Topics in Current Genetics

14

Series Editor: *Stefan Hohmann*

Markus J. Tamás • Enrico Martinoia (Eds.)

Molecular Biology of Metal Homeostasis and Detoxification

From Microbes to Man

With 45 Figures, 2 in Color; and 13 Tables

Springer

Dr. Markus J. Tamás
Department of Cell and Molecular Biology/
Microbiology
Göteborg University
Box 462
405 30 Göteborg
Sweden

Dr. Enrico Martinoia
Institute of Plant Biology
University Zurich
Zollikerstr. 107
8008 Zurich
Switzerland

The cover illustration depicts pseudohyphal filaments of the ascomycete *Saccharomyces cerevisiae* that enable this organism to forage for nutrients. Pseudohyphal filaments were induced here in a wild-type haploid MATa Σ1278b strain by an unknown readily diffusible factor provided by growth in confrontation with an isogenic petite yeast strain in a sealed petri dish for two weeks and photographed at 100X magnification (provided by Xuewen Pan and Joseph Heitman).

ISSN 1610-2096
ISBN-10 3-540-22175-1 Springer Berlin Heidelberg New York
ISBN-13 978-3-540-22175-3

Springer-Verlag is a part of Springer Science + Business Media
springeronline.com

Printed in Germany

Typesetting: Camera ready by editors
Data-conversion: PTP-Berlin, Stefan Sossna e.K.
Cover Design: Design & Production, Heidelberg
Printed on acid-free paper – 39/3152-YK – 5 4 3 2 1 0

Topics in Current Genetics publishes review articles of wide interest in volumes that centre around a specific topic in genetics, genomics as well as cell, molecular and developmental biology. Particular emphasis is placed on the comparison of several model organisms. Volume editors are invited by the series editor for special topics, but further suggestions for volume topics are highly welcomed.

Each volume is edited by one or several acknowledged leaders in the field, who ensure the highest standard of content and presentation. All contributions are peer-reviewed. All papers are published online prior to the print version. Individual DOIs (digital object identifiers) make each article fully citable from the moment of online publication.

Subscribers to the print version of *Topics in Current Genetics* receive free access to the online version. An online-only license is also available.

Editorial office:
Topics in Current Genetics
Series Editor: Stefan Hohmann
Cell and Molecular Biology
Göteborg University
Box 462
40530 Göteborg, Sweden
Phone: +46 733 547297
FAX: +46 31 7732595
E-mail: editor@topics-current-genetics.se
Website: http://www.topics-current-genetics.se

Preface

Markus J. Tamás and Enrico Martinoia

One of the challenges faced by every cell as well as by whole organisms is to maintain appropriate concentrations of essential nutrient metals while excluding nonessential toxic metals. The transition metals iron, copper, zinc, nickel, and manganese are required as micronutrients. Yet, these same metals can produce toxicity when present in excess whereas shortage is detrimental for vital cellular functions. Nonessential metals such as cadmium, arsenic, mercury, and lead have no known beneficial function in the cell and are toxic even at low concentrations. Nevertheless, they are taken up by cells due to overlapping specificities of some transport proteins. Hence, all organisms, ranging from unicellular microorganisms such as bacteria and yeast to multicellular organisms like plants and mammals, have developed mechanisms for metal homeostasis and detoxification to maintain metal levels within physiological limits. These homeostasis and detoxification mechanisms involve metal uptake pathways, chelation and/or trafficking within the cell, delivery of metals into cellular compartments, organelles, and enzymes as well as systems for metal storage and efflux. Metal-specific sensing and regulation mechanisms including signal transduction pathways and transcriptional regulators coordinate these processes depending on metal levels and the metabolic and physiological state of the cell. Various regulatory mechanisms are also in place to control long-distance transport and distribution of metals to various cells and organs in multicellular organism, depending on their specialized functions and developmental stage.

In recent years, the importance of metals in biological systems has received increasing attention with a large and growing number of groups working in this research area. Novel molecular mechanisms of metal homeostasis and detoxification have been elucidated in a range of organisms and also contributed to an increased understanding of the pathophysiology of metal deficiency and overload in relation to disease. Of particular importance is the realization that most transition and heavy metals do not exist as free ions in the cytosol but are sequestered by various types of metal chaperones and/or intracellular carrier proteins. Of the same dignity is the molecular understanding of diseases caused by perturbed metal homeostasis including Menkes and Wilson disease (copper), hereditary hemochromatosis and anemias (iron) and Acrodermatitis enteropathica (zinc). Until recently, the mechanisms of tolerance to various nonessential metals in eukaryotic organisms have remained poorly explored. However, the increasing use of toxic metals in medical therapy, *e.g.*, the use of arsenic for the treatment of certain forms of cancer and of diseases caused by protozoan parasites, as well as the need to develop systems for phytoremediation of contaminated sites, has spurred research in this field and led to a significant progress in understanding metal responses and tolerance acquisition mechanisms.

The volume '*Molecular Biology of Metal Homeostasis and Detoxification; from microbes to man*' covers essential nutrient metals as well as nonessential toxic metals in various eukaryotic model systems including yeasts, plants and mammals focussing on the cellular systems controlling metal transport, intracellular distribution and immobilization as well as on systems regulating metal-dependent transcription. The pathophysiology of metal deficiency and overload in relation to disease as well as environmental aspects including phytoremediation are also covered. The chapters are organised into different sections focusing on the essential nutrient metals copper, zinc and iron in yeast, mammals and plants, respectively (Chapters 2 to 9). Other chapters describe the current knowledge of heavy metal immobilization and phytoremediation (Chapters 10 and 11), the molecular basis of metal dependent transcriptional regulation covering transcriptional regulators from bacteria to mammals (Chapter 12), the molecular mechanisms of nonessential toxic metal tolerance in yeast (Chapter 13) and nonessential metal transport in mammals (Chapter 14). The volume is concluded with a chapter bringing together concepts, visions and developments for the future (Chapter 15).

'*Molecular Biology of Metal Homeostasis and Detoxification; from microbes to man*' is not only a timely publication, but is also unique in its composition bringing together current knowledge of the molecular basis of metal homeostasis and detoxification in various eukaryotic model systems. Hence, our hope is that '*Molecular Biology of Metal Homeostasis and Detoxification; from microbes to man*' will be a milestone in this exciting and rapidly emerging field and be of great interest to a broad readership.

Martinoia, Enrico
Universität Zürich, Institut für Pflanzenbiologie, Zollikerstrasse 107, CH-8008 Zürich, Switzerland
enrico.martinoia@botinst.unizh.ch

Tamás, Markus J.
Department for Cell and Molecular Biology, Göteborg University, Box 462, 40530 Göteborg, Sweden
markus.tamas@gmm.gu.se

Table of contents

Molecular mechanisms of copper homeostasis in yeast 1
Jaekwon Lee, David Adle, Heejeong Kim 1
Abstract 1
1 Introduction 1
2 Cu uptake at the plasma membrane 4
2.1 High affinity Cu transporters 4
2.2 Cu transporters identified from other yeast 6
2.3 Low affinity Cu transporters: Fet4, Smf1, and Pho84 7
2.4 Cu reductases 8
3 Intracellular Cu distribution 9
3.1 Atx1-mediated Cu delivery to the secretory compartment 9
3.2 Ccc2, and other factors necessary for Cu incorporation into Fet3 10
3.3 Interaction between Atx1 and Ccc2 for Cu transfer 12
3.4 CCS, a Cu chaperone for Cu,Zn superoxide dismutase (SOD1) 13
3.5 Cu transport to the mitochondria 14
3.6 How do the cytoplasmic Cu chaperones acquire Cu? 17
3.7 Ctr2-mediated mobilization of intracellular Cu stores 17
3.8 Pca1, a P-type ATPase 18
4 Defense systems to Cu toxicity 19
4.1 Metallothioneins 19
4.2 Cu,Zn superoxide dismutase (SOD1) 20
4.3 CuS biomineralization 20
4.4 P-type ATPase-mediated Cu export 20
4.5 Multi-drug resistance protein 21
4.6 Vacuole and Cu sequestration 21
5 Regulation of Cu metabolism 22
5.1 Ace1/Amt1 23
5.2 Mac1 24
5.3 Cuf1 25
5.4 Post-translational regulation of Cu transporters 26
5.5 Regulation of Cu metabolism by stress and other nutritional factors 26
6 Conclusions 27
Acknowledgements 27
References 27
Abbreviations 37

Zinc in yeast: mechanisms involved in homeostasis 37
Lisa M. Regalla and Thomas J. Lyons 37
Abstract 37
1 Introduction 37

2 Zap1p: The zinc sensor 38
2.1 Regulation of Zap1p activity 38
2.2 The Zap1p regulon 40
3 Zinc transporters 41
3.1 Import into the cytoplasm 41
3.2 Export out of the cytoplasm 43
3.3 Zinc regulation of transporter function 44
4 The zinc proteome 44
4.1 Structural zinc 45
4.2 Catalytic zinc 45
5 Prioritizing zinc 47
5.1 Zinc chaperones 50
5.2 Remodeling 50
6 Zinc storage and detoxification 51
6.1 The vacuole 51
6.2 Metallothionein 52
7 Zinc signals and other regulators of zinc homeostasis 52
8 Conclusions 53
References 54

Iron in yeast: Mechanisms involved in homeostasis 59
Ernest Kwok and Daniel Kosman 59
Abstract 59
1 Introduction 59
2 The plasma membrane and exocytoplasmic milieu 60
2.1 Siderophore-mediated iron uptake 60
2.2 Ferrous iron uptake 63
2.3 Ferroxidase, permease-mediated iron uptake 65
2.4 Heme/hemin uptake 66
3 The cytoplasm 67
4 The vacuole 69
5 Mitochondria 73
5.1 Putative iron transporters 73
5.2 Iron-sulfur cluster and heme biosynthesis 75
5.3 Mitochondrial iron homeostasis with respect to the cell 77
6 The nucleus 79
7 Regulation and integration 79
7.1 Iron regulation *via* GATA factors 80
7.2 Iron regulation *via* Aft1 proteins 83
7.3 Sensing cellular iron status 84
7.4 Achieving iron homeostasis 89
Acknowledgement 90
References 90
Abbreviations 99

Copper in mammals: mechanisms of homeostasis and pathophysiology 101
Michael A. Cater and Julian F.B. Mercer 101
Abstract 101
1 The biochemical properties of copper 101
2 Physiological copper homeostasis 102
2.1 Copper absorption from the diet 103
2.2 Copper in the general circulation 103
2.3 Copper excretion 104
3 Cellular copper homeostasis 104
4 Genetic diseases of copper homeostasis 108
4.1 Menkes disease 108
4.2 Mouse models of Menkes disease 109
4.3 Wilson disease 110
4.4 Rodent models for Wilson disease 112
4.5 Possible genetic copper toxicity conditions 113
5 Mechanistic and cellular aspects of the copper-ATPases (ATP7A and ATP7B) 113
5.1 The P-type ATPase family of proteins and cation translocation 113
5.2 Cell biology of ATP7A and ATP7B 116
5.3 Role of the N-terminal metal binding domains of the copper-ATPases 117
6 Brain copper and neurodegenerative diseases 118
7 Conclusions 120
References 120

Zn^{2+}, a dynamic signaling molecule 131
Michal Hershfinkel 131
Abstract 131
1 Zinc in health and disease 131
2 Mechanisms of cellular zinc homeostasis 134
2.1 Cellular zinc pools 134
2.2 Mammalian zinc transport 135
2.3 Zinc signaling 139
3 Future directions 143
References 144
List of abbreviations: 153

Iron in mammals: pathophysiological mechanisms of overload and deficiency in relation to disease 155
Martha B. Johnson, Caroline A. Enns, and An-Sheng Zhang 155
Abstract 155
1 Overview of iron transport and homeostasis 155
2 Misregulation of iron: hereditary hemochromatosis 159
2.1 Hereditary hemochromatosis type 1 159
2.2 Hereditary hemochromatosis type 2 161

2.3 Hereditary hemochromatosis type 3 165
2.4 Hereditary hemochromatosis type 4 167
2.5 Mechanisms of iron homeostasis misregulated in hereditary hemochromatosis 169
3 Misregulation of iron: anemias 171
3.1 β-thalassemia 172
3.2 Anemia of inflammation 173
3.3 Inherited sideroblastic anemias 175
3.4 Anemia due to mutation of DMT1 178
References 179
Abbreviations 190

Cellular and whole organism aspects of iron transport and storage in plants 193
Jean-François Briat 193
Abstract 193
1 Introduction 193
2 Iron acquisition and trafficking 194
2.1 Iron uptake by the roots and its control 195
2.2 Iron fluxes within the plant 198
2.3 Iron subcellular compartmentation 200
3 Iron storage by plant ferritins 201
3.1 Leaf ferritin 202
3.2 Ferritins in roots and nodules 203
3.3 Ferritins in seeds 204
3.4 Iron-dependent regulation of ferritin gene expression 205
4 Deregulation of iron homeostasis in plants 206
4.1 Alteration of iron accumulation in Fe-signaling plant mutants 206
4.2 Ferritin overexpression in transgenic plants and its consequences 207
5 Conclusions 208
References 208

Functions and homeostasis of zinc, copper, and nickel in plants 215
Ute Krämer and Stephan Clemens 215
Abstract 215
1 Introduction 215
2 Requirement, acquisition, and trafficking of Zn, Cu, and Ni in plants 219
2.1 Requirement of Zn, Cu, and Ni in plants 219
2.2 Homeostasis of Zn, Cu, and Ni in higher plants 229
3 Metal Regulation 243
3.1 Known and likely levels of metal regulation in plants 243
3.2 Long distance signaling in metal homeostasis 244
3.3 Principles of Cu and Zn regulation in model organisms 245
4 Toxicity and tolerance in plants of Cu, Ni, Zn 248
4.1 Toxicity of excess concentrations of Cu, Ni, and Zn 248

4.2 Basal metal tolerance in plants ... 249
4.3 Naturally selected metal hypertolerance and hyperaccumulation ... 250
5 Prospects and developments ... 253
Acknowledgements ... 254
References ... 254

Metal immobilization: where and how? ... 273
Stéphane Mari and Michel Lebrun ... 273
Abstract ... 273
1 Introduction ... 273
2 Immobilization in the rhizosphere ... 275
3 Immobilization in the roots ... 279
3.1 The case of phytochelatins ... 279
3.2 The elusive role of metallothioneins ... 282
3.3 The role of the cell wall ... 284
3.4 The vacuolar storage ... 285
4 Immobilization in the aerial parts ... 286
4.1 Metal accumulation in the trichomes ... 286
4.2 Epidermal cells and/or mesophyll ... 287
4.3 Nicotianamine, a potential new actor in heavy metal sequestration ... 289
5 Concluding remarks ... 290
References ... 291

Phytoremediation and hyperaccumulator plants ... 299
Wendy Ann Peer, Ivan R. Baxter, Elizabeth L. Richards, John L. Freeman, Angus S. Murphy ... 299
Abstract ... 299
1 Introduction to phytoremediation ... 299
1.1 Phytoextraction ... 301
1.2 Phytodegradation ... 301
1.3 Phytovolatilization ... 302
1.4 Rhizosphere degradation ... 302
1.5 Rhizofiltration ... 303
1.6 Phytostabilization ... 303
1.7 Phytorestoration ... 304
2 Definitions of tolerant, indicator, and hyperaccumulator species ... 305
2.1 How do plants take up and transport metal? ... 306
2.2 Strategies for phytoremediation using hyperaccumulators ... 309
3 Common elemental contaminants ... 311
3.1 Arsenic (As) ... 311
3.2 Cadmium (Cd) ... 314
3.3 Chromium (Cr) ... 316
3.4 Copper (Cu) ... 317
3.5 Mercury (Hg) ... 318
3.6 Nickel (Ni) ... 319

3.7 Lead (Pb) 321
3.8 Selenium (Se) 323
3.9 Zinc (Zn) 325
4 Future outlook 326
References 328
List of abbreviations 339

Family matters: gene regulation by metal-dependent transcription factors 341
Harm van Bakel and Cisca Wijmenga 341
Abstract 341
1 Introduction 341
2 Metal-responsive transcription factor families in prokaryotes 344
2.1 MerR family 348
2.2 Fur family 352
2.3 Diphtheria toxin regulator family 356
2.4 ArsR/SmtB family 359
2.5 Additional prokaryotic metal-responsive transcriptional systems 363
3 Eukaryotic metal-responsive transcription regulation 366
3.1 Copper-responsive transcription factors 367
3.2 Iron-mediated gene expression 370
3.3 The zinc uptake regulator Zap1 372
3.4 Metal-responsive transcription factor –1 (MTF-1) 372
4 Interplay of transcriptional systems in determining limits to metal ion levels 373
4.1 Metal ion acquisition and regulator specificity 375
5 Conclusions 376
Acknowledgements 377
References 377
Abbreviations 395

Mechanisms of toxic metal tolerance in yeast 395
Markus J. Tamás, Jean Labarre, Michel B. Toledano, and Robert Wysocki 395
Abstract 395
1 Introduction 395
1.1 Metal abundance, distribution, and usage 396
2 Effects of nonessential metals on biological systems 397
2.1 Effects on human health 398
2.2 Toxic metals cause oxidative stress 399
2.3 Possible molecular mechanisms leading to oxidative stress 402
2.4 Selenium and chromium salts 404
3 Metal uptake pathways 405
3.1 Arsenic and antimony 405
3.2 Cadmium 407
3.3 Mercury 409

3.4 Other metals 409
4 Metal transport and detoxification systems 409
4.1 Efflux-mediated tolerance systems 410
4.2 Vacuolar sequestration of toxic metals 411
4.3 Metal-binding peptides and proteins: metallothioneins and phytochelatins 414
4.4 Arsenate reduction – a pathway leading to tolerance and drug activation 415
5 Sulphur and glutathione metabolism 417
5.1 The sulphur pathway of *S. cerevisiae* 418
5.2 Toxic metals induce the synthesis of glutathione 418
5.3 Sulphur sparing in proteins 419
5.4 Other yeasts 420
5.5 Selenate and chromate interferes with the sulphate assimilation pathway 420
6 Signalling and transcriptional regulation 421
6.1 Yap1 protects cells from a variety of oxidants and metals 421
6.2 Yap8: a specific arsenic resistance factor 428
6.3 Hog1 mediates As(III) tolerance through multiple mechanisms 429
6.4 Met4 432
6.5 Other transcriptional regulators 434
7 Conclusions and future perspectives 434
Acknowledgments 435
References 435

Transport of nonessential metals across mammalian cell membranes 455
Nazzareno Ballatori and Michael S. Madejczyk 455
Abstract 455
1 Introduction 455
2 Metal ion interactions with biological molecules 456
3 Metal-selective transporters, pumps, and channels 457
3.1 The SLC11A family of H^+-coupled metal ion transporters: NRAMP1/SLC11A1 and NRAMP2/SLC11A2 (the latter is also known as DMT1 and DCT1) 458
3.2 MTP1/SLC40A1 and hephaestin in cellular iron export 460
3.3 The ZNT/SLC30 and ZIP/SLC39 families of zinc transporters 461
3.4 The hCTR1/SLC31A1 and hCTR2/SLC31A2 copper uptake transporters 461
3.5 ATP7A and ATP7B, and other ATPases 462
3.6 Channels 462
4 Other ion and organic solute transporters 464
4.1 Amino acid and peptide transporters 464
4.2 Uptake on phosphate or sulfate transporters 465
4.3 MRP/ABCC-mediated excretion of GSH complexes and other organometallic complexes 466
4.4 Organic solute carriers: OATP, OAT, OCT, and NTCP 468

4.5 Organic solute transport pumps: MDR1, MDR2, and BSEP 468
5 Endocytosis and exocytosis 468
5.1 Endocytosis of transferrin and other metal complexes 469
6 Simple diffusion 471
7 Summary 472
Acknowledgements 473
References 473

Metals in biology: past, present, and future 485
Barry P. Rosen 485
Abstract 485
1 Introduction 485
2 Concepts and developments 485
2.1 Chaperones and scaffolds 485
2.2 Transporters for assembled metal complexes 489
2.3 Pathways of metalloid uptake and detoxification 490
3 Visions for the future 496
3.1 Metals and medicine 496
3.2 Metalloid chemotherapy 497
4 Conclusion 499
Acknowledgements 499
References 500

Index 507

List of contributors

Adle, David
Department of Biochemistry and Redox Biology Center, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

Ballatori, Nazzareno
Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine, 575 Elmwood Avenue, Rochester, NY 14642
Ned_Ballatori@urmc.rochester.edu

Baxter, Ivan R.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA

Briat, Jean-François
Biochimie et Physiologie Moléculaire des Plantes. Centre National de la Recherche Scientifique (UMR 5004), Institut National de la Recherche Agronomique, Université Montpellier 2, École Nationale Supérieure d'Agronomie, 2 Place Viala, F-34060 Montpellier cedex 2, France
briat@ensam.inra.fr

Cater, Michael A.
Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, Burwood, Victoria Australia

Clemens, Stephan
Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Saale, Germany

Enns, Caroline A.
Department of Cell & Developmental Biology, L-125, 3181 SW Sam Jackson Park Road, Oregon Health & Science University, Portland, OR 97239 USA

Freeman, John L.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA
Current address: Dept. of Biology, Colorado State University, Fort Collins, CO USA

Hershfinkel, Michal
Department of Morphology and the Zlotowski Center for Neuroscience, Faculty of Health Science, Ben Gurion University of the Negev, Beer-Sheva, Israel
hmichal@bgu.ac.il

Johnson, Martha B.
Department of Cell & Developmental Biology, L-125, 3181 SW Sam Jackson Park Road, Oregon Health & Science University, Portland, OR 97239 USA

Kim, Heejeong
Department of Biochemistry and Redox Biology Center, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

Kosman, Daniel
Department of Biochemistry, The University at Buffalo, Department of Biochemistry, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214, USA
camkos@buffalo.edu

Krämer, Ute
Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Golm, Germany
kraemer@mpimp-golm.mpg.de

Kwok, Ernest
Department of Biochemistry, The University at Buffalo, Department of Biochemistry, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214, USA

Labarre, Jean
Service de Biochimie et de Génétique Moléculaire, DBJC, CEA/Saclay, 91191 Gif-sur-Yvette, France

Lebrun, Michel
Unité Mixte de Recherche, Biochimie et Physiologie Moléculaire des Plantes, INRA, CNRS, Université Montpellier II, Agro-M, Place Viala, 34060 Montpellier.
lebrun@univ-montp2.fr

Lee, Jaekwon
Department of Biochemistry and Redox Biology Center, University of Nebraska-Lincoln, N210 Beadle, Lincoln, NE 68588, USA
jlee7@unlnotes.unl.edu

Lyons, Thomas J.
Department of Chemistry, University of Florida, Gainesville, FL 32611, University of Florida, Department of Chemistry, P.O. Box 117200, Gainesville, FL 32611
lyons@chem.ufl.edu

Madejczyk, Michael S.
Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine, 575 Elmwood Avenue, Rochester, NY 14642

Mari, Stéphane
Unité Mixte de Recherche, Biochimie et Physiologie Moléculaire des Plantes, INRA, CNRS, Université Montpellier II, Agro-M, Place Viala, 34060 Montpellier.

Mercer, Julian F.B.
Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, Burwood, Victoria Australia
jmercer@deakin.edu.au

Murphy, Angus S.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA

Peer, Wendy Ann
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA
peerw@purdue.edu

Regalla, Lisa M.
Department of Chemistry, University of Florida, Gainesville, FL 32611, University of Florida, Department of Chemistry, P.O. Box 117200, Gainesville, FL 32611

Richards, Elizabeth L.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA

Rosen, Barry P.
Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, Detroit, MI 48201 USA
brosen@med.wayne.edu

Tamás, Markus J.
Department of Cell and Molecular Biology/Microbiology, Göteborg University, S-405 30 Göteborg, Sweden
markus.tamas@gmm.gu.se

Toledano, Michel B.
Laboratoire Stress Oxydants et Cancer, Service de Biologie Moléculaire Systemique, DBJC, DSV, CEA-Saclay, 91191 Gif-sur-Yvette, France

van Bakel, Harm
Complex Genetics Group, Dept. of Biomedical Genetics, Stratenum 2.117, University Medical Center Utrecht, PO Box 85060, 3508 AB Utrecht, The Netherlands
H.H.M.J.vanBakel@med.uu.nl

Wijmenga, Cisca
Complex Genetics Group, Dept. of Biomedical Genetics, Stratenum 2.117, University Medical Center Utrecht, PO Box 85060, 3508 AB Utrecht, The Netherlands

Zhang, An-Sheng
Department of Cell & Developmental Biology, L-125, 3181 SW Sam Jackson Park Road, Oregon Health & Science University, Portland, OR 97239 USA
zhanga@ohsu.edu

Molecular mechanisms of copper homeostasis in yeast

Jaekwon Lee, David Adle, Heejeong Kim

Abstract

Copper ions play critical roles as electron transfer intermediates in various redox reactions. The yeast *Saccharomyces cerevisiae* has served as a valuable model to study copper metabolism in eukaryotic cells. The systems for copper homeostasis; including the uptake, cytoplasmic trafficking, and metabolism in intracellular organelles, detoxification, and regulation of these systems have been characterized. Most of the molecular components for copper metabolism identified in yeast are functionally and structurally conserved in mammals. These findings have underscored the importance of evolving delicate mechanisms to utilize copper. Studies on copper metabolism in yeast certainly have opened up interesting and important research avenues that have shed light on the molecular details of copper metabolism and the physiological roles of copper.

1 Introduction

Copper (Cu) is a metal-ion abundantly found in the earth's crust. It easily accepts and donates electrons through redox reactions. Aerobic organisms have taken advantage of the chemical properties of Cu by incorporating it in various biological processes. Thus, organisms have developed mechanisms for acquiring Cu from the environment. Mechanisms for homeostatic Cu metabolism have been uncovered in prokaryotes, fungi, plants, and mammals. Among these organisms, the yeast *Saccharomyces cerevisiae* has served as a model organism to study Cu metabolism in eukaryotes. A number of experimental tools are available to understand the molecular mechanisms of Cu homeostasis. The sequencing of the yeast genome has provided an extremely valuable source of information. Deletion or expression control of yeast genes is much easier than in higher eukaryotes. Growth environments of yeast can be easily manipulated. Furthermore, most of the mechanisms and components in physiological and biochemical processes identified in yeast are conserved in higher eukaryotes.

Cu is required for at least three biological processes in yeast, (i) mitochondrial oxidative phosphorylation, (ii) superoxide anion detoxification, and (iii) iron metabolism. In the mitochondria cytochrome c oxidase subunits 1 and 2 contain Cu as an electron transport intermediate in oxidative phosphorylation (Tsukihara et al. 1995; Iwata et al. 1995). Thus, Cu is an essential micronutrient for yeast under

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_91 / Published online: 26 July 2005

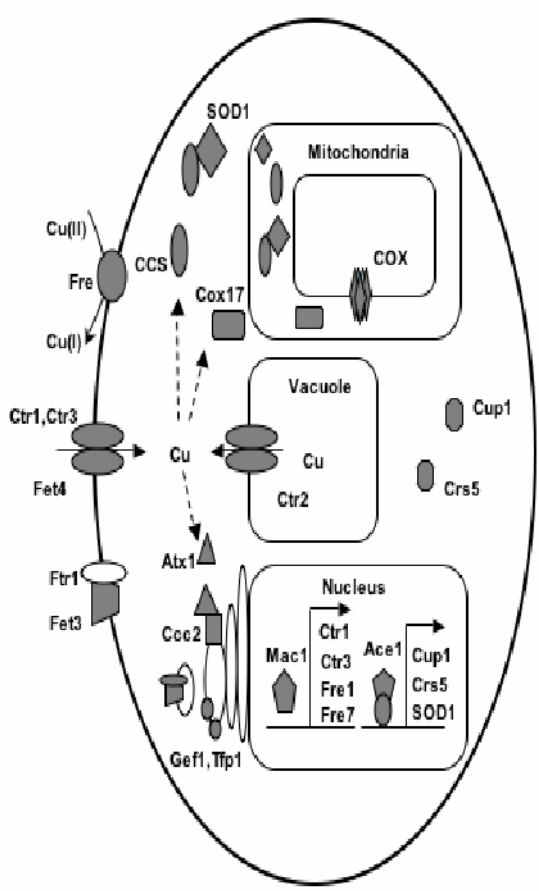


Fig. 1. Copper (Cu) homeostasis in yeast *S. cerevisiae.* Molecular mechanisms of Cu transport, distribution and detoxification have been characterized in yeast. Cu is reduced by cell surface reductases (Fre) prior to uptake by Ctr1 and Ctr3 Cu transporters. Fet4 serves as a low affinity Cu transporter. Ctr2 transports Cu form the vacuole. Cytosolic Cu chaperones Atx1, Cox17 and CCS deliver Cu to the secretory pathway, mitochondria and Cu, Zn superoxide dismutase (SOD1), respectively. At the post-Golgi vesicles Ccc2 accepts Cu from Atx1, followed by incorporation of Cu to Fet3, a multicopper ferroxidase. Gef1 and Tfp1 facilitate the transport and incorporation of Cu into Fet3. Fet3 forms a complex with the iron permease Ftr1 and both proteins are responsible for high affinity iron uptake at the plasma membrane. In mitochondria Cox17 plays essential roles in Cu incorporation into cytochrome c oxidase (COX) subunit 1 (Cox1) and subunit 2 (Cox2). CCS delivers Cu specifically to SOD1 in the cytosol. SOD1 and CCS also localize at the mitochondrial intermembrane space. Two metallothioneins, Cup1 and Crs5, are critical for Cu detoxification. Mac1 and Ace1, Cu-responsive transcription factors, regulate expression of genes involved in Cu metabolism. Mac1 and Ace1 directly bind to the *cis*-acting element of their target genes.

aerobic conditions. Yeast cells that are defective in Cu metabolism are not able to grow on media containing ethanol and glycerol as sole carbon source. These non-fermentable carbons need mitochondrial oxidative phosphorylation to generate energy and Cu serves as an essential cofactor (Keyhani and Keyhani 1975). Cu metabolism is linked to iron (Fe) metabolism, since Cu is a cofactor of Fet3 feroxidase, which plays an essential role in Fe transport at the plasma membrane (Dancis et al. 1994a; Askwith et al. 1994). Cu is also a critical cofactor for Cu,Zn superoxide dismutase (Cu/Zn SOD, SOD1), which detoxifies superoxide anions generated by aerobic biological processes. Thus, yeast cells defective in Cu uptake exhibit sensitivity to superoxide anion stress (Greco et al. 1990). The counterparts of these Cu-containing proteins in higher eukaryotes play the same roles as those in yeast cells (Peña et al. 1999).

Cu is able to catalyze reactions generating reactive oxygen intermediates that are highly toxic to all cellular components (Halliwell and Gutteridge 1984, 1990). The beneficial and detrimental roles of Cu in biological systems demand that cells maintain delicate control of intracellular Cu metabolism. Cu-chelating metallothionein is known as a primary defense system against Cu toxicity (Hamer 1986). Molecular characterization of the metallothionein gene and its transcription regulation has provided an initial picture that reflects the significance of homeostatic Cu metabolism. Further understanding of Cu-metabolism has been taken by cloning the genes encoding components involved in Cu uptake and intracellular distribution in yeast (Fig.1). Phenotypic analyses of yeast cells defective in any of these components led to the identification and characterization of their counterparts in higher eukaryotes.

This chapter will address molecular mechanisms of Cu metabolism in yeast, including baker's yeast, fission yeast and pathogenic yeast, by which uptake, distribution and detoxification of Cu are precisely controlled. The major focus will be in the baker's yeast *S. cerevisiae*. Although this chapter describes the most updated information regarding yeast Cu metabolism in a comprehensive manner, there are excellent review articles focusing on specific topics in Cu metabolism such as transporters (Labbé and Thiele 1999; Puig and Thiele 2002), intracellular distribution (O'Halloran and Culotta 2000; Rosenzweig 2001; Huffman and O'Halloran 2001), mitochondrial Cu metabolism (Carr and Winge 2003), and transcriptional regulation (Thiele 1992; Winge 1998).

2 Cu uptake at the plasma membrane

2.1 High affinity Cu transporters

Initial studies on Cu uptake in yeast demonstrated that Cu transport at the plasma membrane is a saturable and carrier-mediated process (Lin and Kosman 1990). An elegant genetic approach identified the Copper Transporter 1 (Ctr1), which plays critical roles in Cu metabolism (Dancis et al. 1994a). Ctr1 was actually identified from yeast mutants that are unable to transport iron. Subsequent analysis demon-

strated that the Fe-deficiency phenotypes of the Ctr1 mutants were the consequence of Cu deficiency and that Ctr1 is a Cu transporter. Indeed, Cu transported through Ctr1 serves as a cofactor of the Fet3 ferroxidase required for Fe uptake (Askwith et al. 1994). The mammalian Cu-containing ferroxidases, ceruloplasmin and hephaestin, also play an important role in Fe metabolism in mammals (Osaki and Johnson 1969; Vulpe et al. 1999).

Ctr1 appears to be specific for Cu, since Ctr1-mediated Cu uptake is not competed by other metal-ions such as Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, or Zn^{2+} (Dancis et al. 1994a). Expression of Ctr1 is a limiting factor in Cu uptake, since overexpression of Ctr1 increases Cu uptake. Non-functional mutations of the Ctr1 gene result in altered cellular responses to extracellular Cu(I), demonstrating a physiological role for Ctr1 in delivering bio-available Cu. Ctr1-defective cells exhibit deficiency in Cu/Zn SOD activities resulting in growth arrest in Cu-deficient media (Dancis et al. 1994b).

Ctr1 is a 406 amino acid integral membrane protein with three putative transmembrane domains. Ctr1 is heavily glycosylated with *O*-linkages (Dancis et al. 1994b) but it is not known whether glycosylation of Ctr1 is essential for its membrane localization and function. The hydrophilic amino terminal contains eight MXXM or MXM sequence repeats that have been identified as Cu-binding motifs in other Cu-transporting proteins from prokaryotes (Cha and Cooksey 1991; Odermatt et al. 1993). The amino-terminus of Ctr1 localizes at the extra-cellular surface of the plasma membrane (Puig et al. 2002), and those methionine residues appear to play a role in capturing Cu from the environment. Sequence alignment of Ctr family proteins has revealed that a methionine at the extracellular domain and two methionines (MXXXM) at the second transmembrane domain are conserved among these transporters. Site-directed mutagenesis experiments have demonstrated that the conserved methionines are critical for Ctr1 function (Puig et al. 2002). The methionines may serve as Cu ligands in Ctr1-mediated Cu transport. Given that most of the membrane transport proteins possess more than 6 trans-membrane domains, it is reasonable to predict that Ctr1 assembles in a homo or hetero multimer to make a Cu channel at the membrane. This is supported by *in vitro* cross-linking experiments (Dancis et al. 1994b). Interactions of glutamate residues at the third transmembrane domain of each monomer have been implicated in multimer formation (Aller et al. 2004).

Ctr3 was identified in mutant yeast cells defective in Ctr1 (Knight at al. 1996). Ctr3 is a 241 amino acid protein that has 11 cysteine residues of which three pairs are arranged in a potential CXC or CXXC metal binding motif. Ctr3 expression in many laboratory yeast strains is blocked by Ty2, a transposable DNA element. In strains that do not possess a Ty2 transposon, Ctr3 expression is regulated by Cu as is Ctr1. Ctr3 is able to replace Ctr1 function and has the same basic structural features as Ctr1, including three transmembrane domains and a functionally important MXXM motif at the second transmembrane domain. Despite these similarities, Ctr3 bears little sequence identity to Ctr1. The expression of both proteins provides enhanced proficiency in Cu uptake under Cu-limiting conditions. However, it is not clear whether Ctr1 and Ctr3 have any specificity in their roles.

Cu transporters have been identified from other yeast species as well as plants, fruit fly, and mammals due to their functional and structural similarity to yeast Ctr1 or Ctr3 Cu transporters (Puig and Thiele 2002). The structural features observed in yeast Ctr1 are common to the Ctr family of Cu transporters, which suggests that Ctr1 proteins in different organisms function with the same mode of action. However, the actual mechanisms of Ctr1 and Ctr3-mediated Cu transport are poorly understood. In mammalian cells, Fe-containing transferrins bind to their plasma membrane receptor, and Fe is released from transferrin at the endosomes to be transported by a transporter. Similarly, Ctr proteins may simply serve as Cu receptors at the cell membrane. Ctr1 endocytosis may be one mechanism for Cu transport. However, since endocytosis of Ctr3 has not been observed (Peña et al. 2000) as has been for yeast and human Ctr1, endocytosis-mediated intracellular compartmentalization of Cu transporters does not appear to be an absolutely required process in Cu acquisition in yeast. A Cu transport assay of purified Ctr1 protein in a lipid vesicle will be an approach for direct demonstration of Cu transport by Ctr1.

Given that Ctr1 does not possess an obvious ATPase domain, the driving force for Ctr1-mediated Cu transport is not known. Initial characterization of Cu transporting activities in yeast cells suggested a temperature and ATP-dependent high affinity Cu transport process (Lin and Kosman 1990). It is possible that another subunit binding to Ctr1 is an ATPase. Electrochemical measurements have shown that Cu-ion uptake is coupled with K^+ efflux in a 1:2 stoichiometry (De Rome and Gadd 1987), suggesting that Cu transport may take place via a $Cu^+/2K^+$ antiport mechanism. Since elevated extra-cellular K^+ levels enhance Cu uptake in mammalian cells (Lee et al. 2002), there may be a connection between the transport of K^+ and Cu.

An interesting observation is that the Ctr1 Cu transporter determines intracellular accumulation of cisplatin (Ishida et al. 2002). Cisplatin, a platinum-based anticancer drug, is highly active against a wide variety of tumors; however, resistance to this drug upon treatment limits its effectiveness (Loehrer and Einhorn 1984; Giaccone 2000). A genetic screen of yeast loss-of-function mutants for cisplatin resistance has uncovered that deletion of the CTR1 gene results in increased cisplatin resistance and reduced intracellular accumulation of cisplatin. Furthermore, cisplatin regulates Ctr1 stability and trafficking in similar ways as Cu in yeast cells. Cu pre-treatment reduces toxicity of cisplatin. These results suggest that Ctr1 transports cisplatin as well. This has been observed for both yeast and mammalian Cu transporters (Ishida et al. 2002; Lin et al. 2002). The link between Cu transporters and cisplatin may explain some cases of resistance in humans and suggest ways of modulating sensitivity and toxicity to this important anticancer drug.

2.2 Cu transporters identified from other yeast

The fission yeast *S. pombe* is particularly interesting due to its somewhat unique system of Cu uptake. A high affinity Cu uptake protein from *S. pombe*, Ctr4, re-

sembles a chimera between the *S. cerevisiae* Ctr1 and Ctr3 proteins (Labbé et al. 1999; Zhou and Thiele 2001). Ctr4 harbors five MXXMXM repeats in the predicted amino-terminal extracellular region similar to Ctr1, yet transmembrane domains are homologous to Ctr3. A Cu transport assay of Ctr4 in *S. cerevisiae* revealed that Ctr4 fails to complement baker's yeast cells defective in high affinity Cu transport and is not able to localize to the plasma membrane. Selection for *S. pombe* genes, which, when co-expressed with Ctr4, confer high affinity Cu transport to *S. cerevisiae* cells resulted in the identification of Ctr5. Ctr4 forms a complex with Ctr5 to function as a Cu transporter (Zhou and Thiele 2001). Both Ctr4 and Ctr5 are integral membrane proteins, and the physical association between them is interdependent for their secretion to the plasma membrane and for high affinity Cu transport. The specific roles of each subunit in the secretion to plasma membrane and Cu transport have not been defined. It is also interesting to ascertain whether there are homologous subunits in high affinity transport complexes in other eukaryotes.

A Ctr1 Cu transporter has been identified from the pathogenic yeast *C. albicans*. A *C. albicans* Ctr1-null mutant displays phenotypes consistent with the lack of Cu uptake, and its Ctr1 can complement Cu deficiency in *S. cerevisiae* (Marvin et al. 2003). Consequently, *C. albicans* does not appear to have a redundant high affinity Cu transporter similar to Ctr3.

2.3 Low affinity Cu transporters: Fet4, Smf1, and Pho84

The saturable and low-affinity Cu transport activities in Ctr1 and Ctr3 knockout yeast cells are attributed to the Fet4 plasma membrane protein. The Cu transported by Fet4 is available for intracellular Cu-requiring proteins and Cu-responsive transcription factors as is the Cu uptake by Ctr1 and Ctr3 (Hassett et al. 2000). Fet4 has been known as a transporter of divalent metals including Fe, Co, and Cd (Dix et al. 1994), as well as reduced monovalent Cu (Hassett et al. 2000). Mutant Fet4 alleles that are non-functional in Fe transport are also defective in Cu transport. However, since Cu inhibits Fet4-mediated Fe uptake in a non-competitive manner, the Cu and Fe transport by Fet4 is not necessarily through the same mechanism (Hassett et al. 2000).

In addition to Fet4, the Smf1 and Pho84 metal transporter appears to contribute to Cu accumulation in yeast. Smf1 is a plasma membrane transporter for manganese, and transports other metal-ions including Cu (Supek et al. 1996; Liu et al. 1997). Overexpression of Smf1 has been associated with increased accumulation of Cu. Smf1 degradation is controlled by Bsd2-mediated ubiquitination (Liu and Culotta 1999; Hettema et al. 2004). Consequently, a non-functional mutation in Bsd2 upregulates Smf1 expression, resulting in hyper-accumulation of Cu, Cd and Mn (Liu et al. 1997). A phosphate transporter encoded by the PHO84 gene functions as a low affinity transporter of metals including Mn, Zn, Co and Cu (Jensen et al. 2003). This transporter is implicated in metal accumulation when extracellular metals are in excess. However, Smf1 and PHO84-mediated Cu uptake most likely play minimal roles in Cu metabolism. Ctr1, Ctr3, and Fet4 triple knockout

yeast cells do not exhibit saturable Cu transport activities at a wide range of Cu concentration, even though the cells are expressing Smf1 and Pho84 (Hassett et al. 2000). Additionally, Cu transported by Smf1 and Pho84 does not appear to be bioavailable, since cells defective in all other Cu transporters, Ctr1, Ctr2, Ctr3, and Fet4, are not able to acquire Cu for Cu/Zn SOD (Portnoy et al. 2001).

The biological significance of these low affinity Cu transporters is not well understood. The high affinity Cu transport machinery is either down regulated or not expressed to prevent Cu toxicity when yeast are exposed to excess Cu. Under these conditions, the low affinity Cu transporters may play roles in Cu acquisition.

2.4 Cu reductases

Oxygen in the environment oxidizes Cu(I) to Cu(II). Reduction of Cu(II) appears to be an important step for Cu transport into yeast cells. Fre1 and Fre2 metalloreductases initially identified as components of Fe transport also play roles in Cu metabolism. Both FRE1 and FRE2 knockouts have impaired Fe uptake but also Cu transport (Hassett and Kosman 1995; Georgatsou et al. 1997). They are plasma membrane electron transport proteins that mobilize cytoplasmic electrons through the membrane. Five other homologous proteins have been identified from the yeast genome (Martins et al. 1998). Regulation patterns of FRE genes by Cu and Fe further demonstrates that these metalloreductases play roles in Fe and Cu metabolism. FRE1, FRE6, and FRE7 genes are induced when cells are cultured under Cu deficiency. FRE1 to 6 are induced in cells cultured in Fe chelator-treated media as well. The functional specificities among these reductases and subcellular localization have not been fully characterized.

The Fre proteins are homologous to the gp91-*phox* subunit of the human NADPH phagocyte oxidoreductase. These proteins possess two heme-binding motifs, a flavin adenine dinucleotide (FAD) binding site, and two NADPH binding sequences (Roman et al. 1993; Finegold et al. 1996; Lesuisse et al. 1996; Shatwell et al. 1996). Given that gp91-*phox* is a subunit of the NADPH oxidase system (Rotrosen et al. 1992; Chanock et al. 1994), it is feasible to postulate that Cu reductases may need other components for their activities. Secondly, since reduced Cu is easily oxidized in the extracellular aerobic environment, there may be a mechanism that protects reduced Cu during the Cu transport process. Reduced Cu may bind to another molecule, and this complex may be a substrate of Ctr1 or Ctr3-mediated Cu transport. Alternatively, it is possible that Fre proteins may form a complex with Cu transporters passing the reduced Cu to them. Third, given that most other proteins involved in Cu metabolism are conserved between yeast and mammalian cells, yeast Cu reductases may provide critical information for identifying their mammalian counterparts.

3 Intracellular Cu distribution

Once Cu is transported into the cell, a mechanism must be in place to safely distribute Cu to specific intracellular targets. It is known that a class of small proteins referred to as chaperones or metallochaperones are required for this role (Fig.1). There are three known pathways in which Cu is directed in yeast. The Cu chaperone Atx1 directs Cu into the secretory compartments. CCS (copper chaperone for SOD1) incorporates Cu into Cu/Zn SOD (SOD1). The putative Cu chaperone Cox17 distributes Cu to the mitochondria. Chaperones are specific for their targets, since CCS, a Cu chaperon for SOD1, is not required for Cu trafficking to the secretory pathway or the mitochondria. Similarly, overexpression of other Cu chaperones such as Atx1 cannot restore SOD1 activity in cells lacking CCS (Culotta et al. 1997). The physiological necessity of metallochaperones was made apparent by the discovery that the ambient free Cu concentration in yeast cells is exceedingly low (Pufahl et al. 1997). With such a low availability of free Cu in the cell, there must be a mechanism to deliver Cu to the appropriate Cu-dependent enzyme while minimizing its toxic effects.

In addition to the chaperones there are many other downstream components required for intracellular distribution of Cu. These include the Atx1 target, Ccc2, which plays an obligatory role for Cu incorporation into the secretory pathway. There are accessory proteins or co-chaperones, which aid in Cu insertion into specific active sites. Furthermore, a member of the Ctr family has been implicated in mobilizing Cu stores from the vacuole. All together these components act in concert for the precise distribution of intracellular Cu.

3.1 Atx1-mediated Cu delivery to the secretory compartment

The Cu chaperone Atx1 was first identified as a high-copy suppressor of oxidative damage in yeast cells lacking SOD1 (Lin et al. 1995). Atx1 appears to have dual roles as both a scavenger of superoxide anions and a Cu chaperone. The first clue for its role as a Cu chaperone was provided by sequence homology with other metal binding proteins. Another clue came from an observed increase in cellular Cu levels upon overexpression (Lin et al. 1995). Atx1 was found to have conserved homologues in other eukaryotes, including the Hah1(Atox1) in humans (Klomp et al. 1997).

Once Cu is transported into the cytosol, it is captured by Atx1 and shuttled to the trans-golgi network (TGN) for delivery into the secretory pathway. Here, Atx1 transfers its cargo to the Cu translocating ATPase, Ccc2. In an ATP dependent manner, Cu is transported across a vesicular membrane for incorporation into the Fet3 multi-Cu oxidase (Lin et al. 1997). The mature Fet3 with its Cu cofactor forms a complex with the Ftr1 iron permease, which is responsible for iron uptake at the plasma membrane (Steaman et al. 1996). This explains the inextricable link previously observed between iron and Cu metabolism. Consistent with its role in iron metabolism, Atx1 is regulated by the iron sensing transcription factor Aft1 (Lin et al. 1997). However, unlike other Aft1 targets, Atx1 expression is unaf-

fected by a null mutation in AFT1, suggesting regulation by an additional transcription factor. The oxygen sensing transcription factor, Yap1 has been implicated in the regulation of Atx1 as its expression is strongly induced by oxygen (Harshman et al. 1988). The regulation of Atx1 by two different transcription factors is consistent with its dual functionality, Cu and Fe metabolism and oxidative stress defense.

Atx1 is a small cytosolic 8.2 kD polypeptide composed of 73 amino acid residues. The N-terminus of Atx1 contains a single MTCXXC metal binding motif. Electron paramagnetic resonance (EPR) and X-ray absorption spectroscopy (XAS) of Atx1 have supported the binding of a single Cu (I) ion per polypeptide (Pufahl et al. 1997). Extended X-ray absorption fine structure (EXAFS) measurements of Atx1 indicated an all sulfur coordination environment through either a two or three liganded complex. The NMR solution structure indicates that the Atx1 protein has an αβαββαβ fold and places a single Cu ion between Cys15 and Cys18 (Arnesano et al. 2001). Atx1 is comprised of multiple Lys residues generating a positively charged surface (Portnoy et al. 1999). Mutations of conserved lysines crippled Atx1 function *in vivo*. In particular, a mutation of Lys65 to Glu, which introduces a negative charge adjacent to the metal-binding motif, severely abrogates Atx1 function. Interestingly, a basic, neutral or hydrophobic residue at this position was tolerated (Portnoy et al. 1999). The importance of Lys65 has been proposed to partially neutralize the net negative charge resulting from the coordination of Cu(I) by two or more cysteines.

3.2 Ccc2, and other factors necessary for Cu incorporation into Fet3

Several genetic and biochemical experiments support the pathway of Cu(I) delivery from Atx1 to Ccc2 for incorporation into Fet3. Yeast ATX1 null mutants are unable to grow in iron limiting conditions, which can be rescued by Cu supplementation (Lin et al. 1997). *In vivo* ^{64}Cu labeling experiments have confirmed that Cu incorporation into Fet3 is defective in Δatx1 yeast strains (Klomp et al. 1997). Furthermore, overexpression of CCC2 can correct the defect of poor growth on limited iron in Δatx1 mutants corroborating the model in which Atx1 delivers Cu(I) to Ccc2 prior to incorporation into Fet3.

Ccc2 is 110kD transmembrane protein consisting of 1004 amino acid residues which has been localized to the late or post Golgi network (Yuan et al. 1997). It is a P-type ATPase belonging to the P1-type subfamily, which is specific for metal ion transport (Lutsenko and Kaplan 1995). P1-type ATPases contain 8 putative transmembrane domains with a conserved CPC/H motif located in the sixth transmembrane domain which is believed to be critical for metal ion translocation. P1-type ATPases contain 1 to 6 CXXC metal-binding domains at their N-terminus. The N-terminus of Ccc2 contains two of these metal-binding domains. The cytoplasmic domain of Ccc2 contains two loops with the largest containing the catalytic ATP-binding and phosphorylation sites. Ccc2 is a functional and structural homolog of the mammalian WND and MNK, P1-type ATPase Cu transporters, which are impaired in Wilson and Menkes disease, respectively. Both

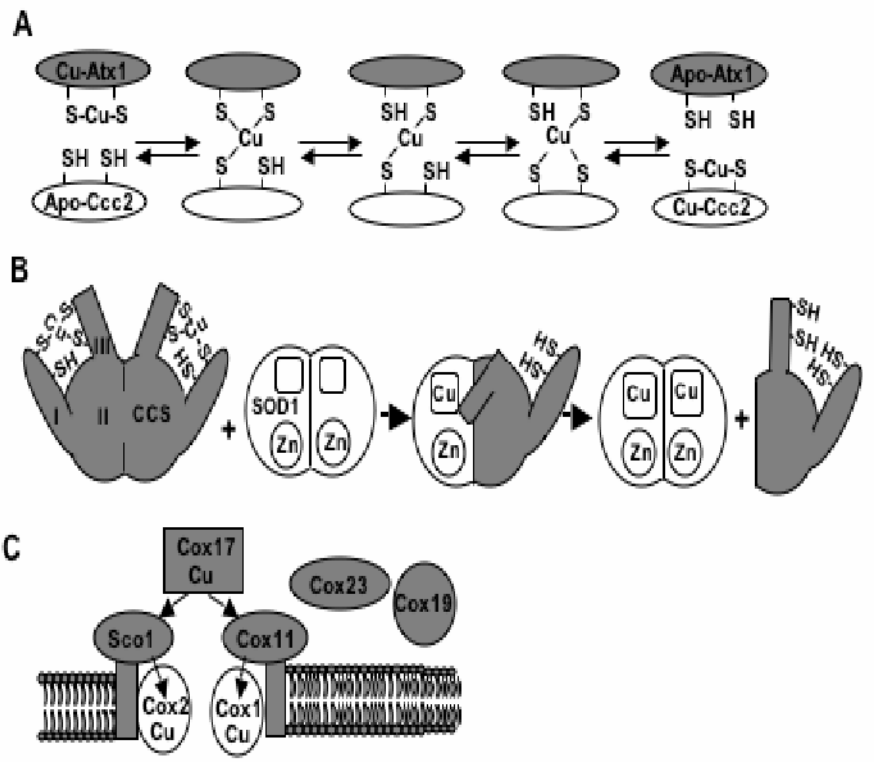


Fig. 2. Schematic illustrating the mechanisms of copper (Cu) transfer between metallochaperones and targets. (A) Atx1 and Ccc2 form a transiently docked complex bringing each metal binding domain into proximity. Cu equilibrates between both metal binding domains involving two and three liganded intermediates for Cu transfer. (B) Dimerization and Cu transfer between CCS and Cu,Zn superoxide dismutase (SOD1). Domain II forms docking interface with SOD1. At low Cu concentrations domain I potentially provides Cu ions to domain III. Domain III swings into position for Cu transfer into the active site of SOD1 (C) Cu incorporation into mitochondrial cytochrome c oxidase (COX) subunits 1 and 2. Cox17 transfers Cu to Sco1 and Cox11 prior to incorporation into the Cox2 Cu_A site and Cox1 Cu_B site, respectively. Cox19 and Cox23 both partially localize to the mitochondrial intermembrane space and are also important for COX assembly. Figures are modified from previous publications, (Fig. 2A: Pufahl et al. 1997; Fig. 2B: Lamb et al. 2001; Fig. 2C: Carr and Winge 2003).

WND and MNK can complement a Ccc2 null mutation in heterologous yeast systems (Hung et al. 1997; Payne and Gitlin 1998).

The Tfp1 subunit of the yeast vacuolar H^+ - ATPase and the yeast chloride channel Gef1 have been implicated in aiding Ccc2 mediated Cu transfer to Fet3 (Gaxiola et al. 1998). Yeast cells lacking Tfp1 or Gef1 are unable to grow on non-fermentable carbon sources; however, they can be rescued by Cu supplementation.

Additionally, Gef1 was also shown to co-localize with Ccc2 (Gaxiola et al. 1998). These observations led to the following model: Tfp1 ensures the maintenance of an acidic environment of the lumen in the late- or post-golgi vesicles required for Cu loading onto Fet3. Acidification and concomitant import of Cu ions lead to an increase of membrane potential further impeding transport of metal cations. The potential can be counter balanced by the influx of Cl^- anions mediated through Gef1 restoring Ccc2 mediated delivery of Cu into the lumen. In addition to the stabilization of the electrochemical potential, Cl^- anions have been proposed to act as an allosteric effector required for the incorporation of Cu into Fet3 (Davis-Kaplan et al. 1998).

3.3 Interaction between Atx1 and Ccc2 for Cu transfer

Structural studies of Atx1 and Ccc2 have shed light onto possible mechanisms of Cu transfer. Yeast two-hybrid experiments have demonstrated a Cu dependent interaction between Atx1 and the N-terminus of Ccc2 (Pufahl et al. 1997). NMR solution structures available for Atx1 (Arnesano et al. 2001) and the soluble N-terminal domain of Ccc2 (Banci et al. 2001) indicated that both have a similar secondary structure. The metal-binding domain of Atx1 is located at a surface-exposed loop which is buried in the metal bound form (Arnesano et al. 2001). Conversely, the Ccc2 N-terminal structure remains relatively invariant upon metal binding (Banci et al. 2001).

It has been demonstrated that a Cu(I) loaded Atx1 can directly transfer Cu to Ccc2 in a reversible manner (Huffman and O'Halloran 2000). Cu(I) rapidly equilibrates between the two proteins suggesting that the thermodynamic gradient is quite shallow. Thus, the vectoral delivery of Cu(I) from Atx1 is not dependent on higher Cu affinity of the Ccc2 target domain. Rather, Atx1 acts as an enzyme by lowering the energy required for transfer specifically for the Ccc2 target site while protecting it from adventitious reactions with non-partner proteins (Huffman and O'Halloran 2000).

A model using current structural information has been proposed for Cu transfer between Atx1 and Ccc2. According to this model, the low activation barrier for metal transfer is achieved from complementary electrostatic forces which orientate the metal binding domains of Cu(I) loaded Atx1 and apo-Ccc2 (Huffman and O'Halloran 2000). The conformational changes observed in the apo-Atx1 to Cu-Atx1 transition are thought to poise Atx1 for direct interaction with Ccc2. Atx1 possesses many positively charged residues on its surface while Ccc2 possesses multiple negatively charged residues at its N-terminus serving as a docking interface (Portnoy et al. 1999). Computer modeling, using the available structural information, shows stable electrostatic interactions amongst specific Atx1 Lys residues and N-terminal Ccc2 Glu and Asp residues (Arnesano et al. 2004). Additionally, an intermolecular hydrogen bond could be placed between the critical Lys65 of Cu-Atx1 and Ccc2. Thus, computer aided modeling has given a structural basis to explain previous mutational data.

It has been proposed that after protein-protein recognition, Cu undergoes a series of rapid associative exchange reactions involving two- and three-coordinate intermediates between the Atx1 and Ccc2 metal-binding motifs (Fig. 2a) (Pufahl et al. 1997). Once Cu is bound to Ccc2, it is believed to be transferred to the intermembrane CXC motif. The binding of Cu to the CXC motif is believed to trigger ATP hydrolysis which drives a conformational change, releasing Cu into a vesicle that is thermodynamically distinct from the cytosol (Huffman and O'Halloran 2000). However, the details of this mechanism still remain poorly understood.

3.4 CCS, a Cu chaperone for Cu,Zn superoxide dismutase (SOD1)

The chaperone which delivers Cu to SOD1 in yeast is CCS (copper chaperone for SOD1). CCS was first identified by mutations in the Lys7 gene, which displayed a phenotype of a non-functional SOD1 (Culotta et al. 1997). A Lys7 null mutation results in the loss of SOD1 function despite normal levels of SOD1 protein. SOD1 function is restored by the supplementation of Cu supporting that its inactivity is the result of inadequate Cu incorporation.

CCS is a 249-amino acid protein, which is much larger in comparison to Atx1. Similar to SOD1, CCS exists as a homodimer (Fig. 2B). There are three functionally distinct domains in CCS (Lamb et. al 1999). The N-terminal domain, domain I, resembles Atx1 and contains the same MXCXXC Cu binding motif; however, is only necessary for function under Cu limited conditions. The central domain, domain II, is homologous to SOD1 and is critical for recognition, but lacks residues important for SOD1 function. Domain III, a short peptide of the carboxyl terminus, bears an invariant CXC Cu binding motif conserved in all members of the CCS family and is crucial for function (Schmidt et. al 1999).

CCS activates SOD1 by directly inserting the Cu cofactor while protecting it from intracellular Cu scavengers. This function was first made apparent as a Cu loaded CCS could activate apo-SOD1 in the presence of Cu chelating agents *in vitro* (Rae et al. 1999). The transfer mechanism must be direct because any Cu ions released into solution would be immediately sequestered by the Cu chelators. Yeast two hybrid assays showed *in vivo* protein-protein interactions between SOD1 and domains II and III of CCS, however, no interaction was observed with the Atx1-like domain I. Instead, this domain most likely captures Cu under limited conditions and transfers it to the CXC motif of Domain III.

It would appear that a similar mechanism of Cu transfer exists for CCS and SOD1 as does for Atx1 and Ccc2. In this case the CCS and SOD1 homodimers would have to disassociate prior to docking with one another. As with Atx1, Cu binding to CCS elicits a conformation suitable for docking with its partner. The Cu transfer is directed by specific protein-protein interactions which guide the Cu binding domains of donor and acceptor within proximity for Cu transfer.

The crystal structure of CCS complexed with SOD1 has provided a structural model for CCS Cu insertion into SOD1 (Lamb et al. 2001). The "frozen" complex was obtained by replacing SOD1 His48 with Phe so that it was unable to bind Cu

yet retain Zn in the active site. The crystal structure shows a heterodimer consisting of a single CCS monomer and a single SOD1 monomer linked together by an intermolecular disulfide bond. Previous biochemical studies have also supported the formation of a heterodimer between CCS and SOD1 which is facilitated by Zn (Lamb et al. 2000). Most contacts are made between SOD1 and the SOD1-like domain II of CCS. Many conformational changes occurred upon the reorganization of CCS and SOD1 homodimers to form a heterodimeric duplex. These conformational changes have given mechanistic clues into how Cu is loaded into the SOD1 active site. CCS has two potential Cu binding sites, one in the MHCXXC sequence motif of the Atx1-like domain I and the other CXC sequence motif in domain III. The structure showed that the MHCXXC motif of domain I is too far away for Cu transfer, however, the cysteines of the CXC motif of domain III lie adjacent to the SOD1 active site. Interestingly, one of these cysteines forms an intermolecular disulfide bond with SOD1 disrupting a previous intramolecular disulfide within SOD1. This displacement appears to open up the active site of SOD1 for Cu incorporation (Lamb et al. 2001).

Recently, the presence of O_2 has been shown to be necessary for the post-translational activation of SOD1 in a CCS dependent manner (Brown et al. 2004). Mutagenesis and biochemical studies have shown that the activity of SOD1 was dependent on an intramolecular disulfide bond which is catalyzed by Cu-CCS in the presence of O_2 (Furukawa et al. 2004). It has been proposed that the oxygen affords an oxidizing environment catalyzing the disulfide-linked heterodimer of CCS and SOD1 which was observed in the crystal structure. Following isomerization the intermolecular disulfide would exchange to form an intramolecular disulfide bond activating SOD1. This would explain how the SOD1 intramolecular disulfide bond is achieved in the reducing environment of the cytosol. This provides for a means of post-translational activation in which a pool of apo-SOD1 could be activated in the presence of oxygen when most needed (Furukawa et al. 2004).

3.5 Cu transport to the mitochondria

Cu is an essential electron carrier for cytochrome c oxidase (COX). COX is the terminal enzyme of the electron transport chain within the mitochondrial inner membrane (IM). COX is composed of many different subunits, three of which (Cox1, Cox2, and Cox3) are encoded and assembled within the mitochondria (Carr and Winge 2003). Two of these subunits, Cox1 and Cox2, utilize Cu as their cofactor. Cox2 contains two Cu ions in the binuclear Cu_A site which functions as the initial electron acceptor. Cox1 contains a single Cu and a heme cofactor forming the heterobimetallic Cu_B site. These mitochondrial encoded subunits are assembled within the mitochondria and are localized to the IM. A mechanism must be in place to deliver Cu ions to the mitochondria, traverse the outer membrane (OM) and incorporate the metal cofactors into the appropriate COX subunits. Since it has already been demonstrated that metallochaperones are responsible for the delivery of Cu to the secretory pathway and to Cu/Zn SOD1, it is likely that Cu delivery to the mitochondria requires metallochaperones as well.

Cox17 has been implicated as the main Cu shuttle, which delivers cytosolic Cu to the mitochondria. Cox17 was first identified by genetic screening of yeast for respiratory deficient mutants (Glerum et al. 1996a). Cells harboring a COX17-1 mutation are respiratory deficient and lack COX activity; however, the phenotypes were reversed by the addition of exogenous Cu suggesting a role of Cox17 in the metalation of COX. Currently, in addition to Cox17, four other proteins (Cox11, Sco1, Cox19, and Cox23) have been implicated in Cu loading of COX (Fig. 2C).

Cox17 is a hydrophilic, cysteine rich polypeptide composed of 69 amino acid residues. It resides partially in the cytosol (40%) and the mitochondrial intermembrane space (IMS) (60%) (Beers et al.1997). This dual localization is consistent for a chaperone that ferries Cu across the mitochondrial OM. Cox17 exists as a dimer or a tetramer where the apo form is predominantly monomeric, suggesting that Cu binding is important for oligimerization and correlated to function (Heaton et al. 2001). Of the seven total cysteines, C23, C24, and C26 are part of a CCXC sequence motif and important for function (Heaton et al. 2000). Mutation of any one cysteine results in loss of function despite retaining the ability to bind Cu and localize to the mitochondria. However, two Cys->Ser mutations of the Cox17 CCXC motif results in the loss of Cu binding yet localization to the mitochondria is retained. Thus, a Cu(I) conformer of Cox17 is not necessary for mitochondrial import as was once previously thought. Recently, the Cox17 NMR solution structure places a single Cu(I) ion coordinated by C23 and C26 of the conserved CCXC motif, which is consistent with previous mutagenesis studies (Abajian et al. 2004). Interestingly, the original COX17-1 mutation turned out to be a C57Y substitution first believed to abrogate Cu binding. It was later found that this mutant retains the ability to bind Cu(I) but no longer localizes to the mitochondria (Heaton et al. 2000). Conversely, a C57S mutant is still functional but only low levels accumulate in the mitochondria suggesting that minimal quantities of Cox17 are required for function (Heaton et al. 2000).

Direct transfer of Cu from Cox17 to both Sco1 and Cox11 has been demonstrated (Horng Y-C et al. 2004). Thus, Cox17 seems to have the unique ability to mediate Cu transfer to two different proteins. Sco1 has been proposed to play a role in Cu delivery to the Cu_A site of Cox2 subunit. Previously, it was shown that overexpression of Sco1 or its close homolog Sco2 could suppress the respiratory deficient phenotype of a Cox17-1 strain (Glerum et al. 1996b). Overexpression of Sco2 only partially restored Cox17-1 respiratory deficiency and only when exogenous Cu was added to the growth medium. In yeast, Δsco1 null mutants are respiratory deficient while Δsco2 null mutants lack an obvious phenotype. Overexpression of Sco2 fails to suppress the respiratory deficiency of Δsco1 null mutants. However, Sco2 overexpression does show partial allele-specific suppression for a Sco1 point mutant. One explanation for the allele-specific suppression observed is that Sco1 and Sco2 physically interact; however, no such interaction was detected. Instead Sco2 may be providing one of the functions lost in the Sco1 mutant suggesting that some redundancy exists between these two proteins (Glerum et al. 1996b). Contrary to human Sco2 which is essential, the functional role of Sco2 in yeast still remains unclear (Lode et al. 2002).

Both Sco1 and Sco2 possess a single transmembrane N-terminal helix and are associated with the mitochondrial IM (Glerum et al. 1996b). Sco1 has been demonstrated to bind Cu through a conserved CXXC sequence, which is critical for *in vivo* function (Nittis et al. 2001; Rentzsch et al. 1999). X- Ray absorption spectroscopy suggested that Cu(I) is coordinated by three ligands provided by the two cysteines of the CXXC motif and a conserved histidine (Nittis et al. 2001). A mutation of any one of these conserved residues of Sco1 abolished function and resulted in a non-functional COX (Rentzsch et al. 1999). These findings established that the function of Sco1 is dependent on Cu(I) binding. Additionally, Sco1 has been demonstrated to interact specifically with the Cox2 subunit, which has further supported its role for the incorporation of Cu(I) into the Cu_A site (Lode et al. 2000).

Cox11 has been proposed to insert Cu into the Cu_B site of the Cox1 subunit. Like the Sco1 and Sco2 proteins, Cox11 contains a single N-terminal transmembrane helix and is localized to the mitochondrial IM (Tzagoloff et al. 1990). Similarly, Cox11 is a Cu(I) binding protein, coordinating Cu(I) by three conserved cysteines (Carr et al. 2003). As with Sco1 any mutation of the Cu(I) coordinating residues resulted in respiratory deficiency due to reduced COX activities (Carr et al. 2003). These findings also correlate Cox11 function with Cu(I) binding. Evidence for specific incorporation of Cu into the Cox1 subunit Cu_B site has been provided by genetic and biochemical experiments. Yeast Δcox11 mutants have lower levels of Cox1 subunit (Tzagoloff et al. 1990). Additionally, COX isolated from *R. sphaeroides* Δcox11 cells lacked Mg^{2+} and a Cu_B center, yet retained all other cofactors including the Cu_A site in Cox2 subunit (Hiser et al. 2000). The combined evidence suggests that Cox11 has a functionally specific role in Cu(I) loading of the Cu_B center in the Cox1 subunit.

Cox19 (Nobrega et al. 2002) and Cox23 (Barros et al. 2004) are important for COX assembly. Like Cox17, Cox19, and Cox23 show a dual localization between the cytosol and the mitochondrial IMS. Cox19 lacks a CCXC Cu (I) binding motif and Δcox19 mutants cannot be rescued by the addition of Cu (Nobrega et al. 2002). It is more likely that Cox19 plays another role in COX assembly other than Cu metalation of COX. However, Cox23 does seem to play a role in mitochondrial Cu homeostasis. Δcox23 unlike Δcox19 mutants can be rescued by exogenous Cu, however, only when transformed with a Cox17 high copy plasmid. Conversely, overexpression of Cox23 does not suppress the Δcox17 respiratory deficient phenotype suggesting that Cox17 functions downstream of Cox23 in the same pathway possibly involving Cox19 (Barros et al. 2004).

One thing that is apparent now is that Cu homeostasis in the mitochondria is much more complicated than once previously thought. Recently, the role of Cox17 as the main mitochondrial Cu shuttle has come into doubt. Cox17, tethered to the mitochondrial IM by fusion with the N-terminal transmembrane domain of Sco2, was still able to compliment the respiratory deficiency of Δcox17 cells (Maxfield et al. 2004). Evidence also exists for a non-proteinaceous pool of Cu in the mitochondrial matrix (Cobine et al. 2004). If Cox17 is not supplying Cu to the mitochondria, then how is it getting there? One possibility is that a Cu transporter exists within the mitochondrial OM; however, such a transporter has remained

elusive. The role of Cox17 seems to be confined to the mitochondrial IMS where it passes Cu(I) to the IM proteins Sco1 and Cox11 for incorporation into the Cu_A and Cu_B sites, respectively (Fig. 2C). The transfer process is likely to be similar to the docking mechanism described between Atx1 and Ccc2 and CCS and Cu/Zn SOD1.

3.6 How do the cytoplasmic Cu chaperones acquire Cu?

Both Cu transporters at the plasma membrane and cytoplasmic Cu chaperones play critical roles in Cu delivery to subcellular targets. However, the mechanisms of Cu acquisition by Cu chaperones are not known. Since Cu transported by Ctr1, Ctr3, Fet4, and Ctr2 is available for intracellular Cu metabolism (Portnoy et al. 2001), it is unlikely that Cu chaperones that are distinct in their structural features interact directly with all these heterogeneous Cu transporters. Furthermore, any common sequence among these Cu transporters that may serve as an interacting motif with cytoplasmic Cu chaperones does not exist either.

A report has addressed the potential direct interaction between yeast Ctr1 and the Atx1 Cu chaperone (Xiao and Wedd 2002). C-terminal sequences (amino acid 280-406) of Ctr1 are hydrophilic and include two Cys-X-Cys motifs. The Ctr1 C-terminus exchanges Cu(I) rapidly with the Atx1 protein *in vitro*. This domain of Ctr1 binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster (Xiao et al. 2004). However, it has not been demonstrated whether other Cu chaperones acquire Cu in the same experimental conditions. Although the metal-binding motifs are not conserved among Cu transporters, three-dimensional structures of the transporters will be much more informative in the investigation of their direct interaction with Cu chaperones. Additionally, since their interactions may be transient, conventional biochemical approaches may not be the best methods for determining their interactions.

3.7 Ctr2-mediated mobilization of intracellular Cu stores

Recent evidence has shown that the yeast *S. cerevisiae* has the ability to mobilize Cu stores from the vacuole and that this mobilization is mediated through the Cu transporter Ctr2 (Portnoy et al. 2001; Rees et al. 2004). Ctr2 was identified along with the *Arabidopsis* COPT1 by sequence homology in an attempt to identify Cu transporters in plants (Kampfenkel et al. 1995). Both proteins were characterized as belonging to the Ctr1 family of integral membrane proteins.

Ctr2 was previously thought to be low affinity plasma membrane Cu transporter. Several early observations led to this misconception. Due to the homology to other Ctr transporters, it seemed logical that Ctr2 would also be a plasma membrane Cu transporter. High levels of Cu could suppress Fe uptake in Ctr1 mutants, which was indicative of the presence of a low affinity Cu transporter at the plasma membrane (Dancis et al.1994). However, disruption of the CTR2 gene did not show any respiratory deficiency when grown on a non-fermentable carbon source

or under iron limiting conditions (Kampfenkel et al. 1995). Overexpression of Ctr2 led to increased sensitivity to high Cu levels while conversely its disruption led to greater resistance. Furthermore, overexpression of Ctr2 did not complement a Ctr1 mutant on a non-fermentable carbon source or under iron limiting conditions suggesting that its contribution for Cu uptake was minimal (Kampfenkel et al. 1995). These findings were all consistent for a low affinity plasma membrane Cu transporter. This role was largely dismissed after Ctr2 was localized to the vacuole (Portnoy et al. 2001; Rees et al. 2004). It is now apparent that Ctr2 plays a role in mobilizing vacuolar Cu stores to readily make Cu available for cellular needs. Evidence for this role was provided by Cu measurements of purified vacuoles. Cu levels in vacuoles from Δctr2 cells were fourfold higher than cells that were constitutively expressing Ctr2 (Rees et al. 2004). Ctr2 has been shown to make Cu available for all known Cu-requiring processes (Portnoy et al. 2001; Rees et al. 2004). Interestingly, Ctr2 does not appear to be regulated as are the high affinity Cu transporters, Ctr1 and Ctr3 (Portnoy et al. 2001).

Sequence homology and predicted topology suggest that Ctr2 utilizes a similar mechanism of transport as Ctr1. This is further supported by genetic and biochemical evidence. It was illustrated that Ctr2 assembles as a homomultimer as does Ctr1 (Rees et al. 2004). Both Ctr1 and Ctr2 possess conserved methionine residues at the N-terminus and possess an MXXXM sequence motif within the second transmembrane domain. Mutations of conserved methionines displayed similar defects in both Ctr1 and Ctr2, which is consistent for a similar mechanism of Cu transport.

If Ctr2 is in fact a Cu transporter which mobilizes Cu from the vacuole, then how does Cu get there in the first place? Since yeast contain an iron and Mn^{2}+ transporter, which stores these metals within the vacuole (Chen et al. 2001), it is quite possible that a similar unidentified Cu transporter is responsible for Cu uptake within the vacuole. Cu transfer mediated through endocytosis or fusion of vesicles loaded with Cu cannot be excluded either.

3.8 Pca1, a P-type ATPase

A second P-type ATPase in *S. cerevisiae* in addition to Ccc2 has been designated as Pca1 (putative P-type cation-transporting ATPase) (Rad et al. 1994). Pca1's role in Cu homeostasis remains obscure. Pca1 is a large 132kD transmembrane protein consisting of 1216 amino acid residues. Pca1 has one N-terminal MTCXXC metal binding motif compared to the two found in Ccc2. Mammals possess two P-type ATPases, which are WND and MNK, and another yeast species *C. albicans* expresses two P-type ATPases that play independent roles, Cu incorporation at the secretory pathway and Cu export to reduce intracellular Cu accumulation. It is curious that *S. cerevisiae* would possess two P-type ATPases that may have distinct roles exampled in mammals and other yeast. Pca1 is believed to play a role in Cu homeostasis and defense against Cu toxicity since Δpca1 strains show sensitivity to high concentrations of Cu (Rad et al. 1994). Interestingly, the single amino acid substitution R970G confers cadmium resistance in yeast

(Shirashi et al. 2000). Microarray data confirmed by quantitative PCR shows that Cu or Fe deficiency regulates Pca1 expression (De Freitas et al. 2004). A respiratory deficient phenotype has been described for Δpca1 null mutants (De Freitas et al. 2004). Thus far, the available evidence suggests some role in Cu and/or Fe homeostasis, however, the details still remain to be studied.

4 Defense systems to Cu toxicity

Cu transporters and cytoplasmic chaperones deliver Cu to Cu-requiring proteins. However, Cu accumulated in excess, like other transition metal ions, is toxic. Consistent to the potential toxicity arising from accumulation or releasing of Cu to its free forms, yeast has equipped defense mechanisms for Cu toxicity. Systems for chelation, sequestration and export of Cu, and scavenging oxygen free radicals generated by Cu-mediated reactions have been characterized in yeast.

4.1 Metallothioneins

Metallothionein (MT) is a 61 amino acid polypeptide, which coordinates seven to eight Cu(I)-ions as a Cu-S polynuclear cluster utilizing 10 of its 12 cysteines (Hamer 1986). Each of the eight Cu(I) ions are bridged trigonally by cysteine residues through Cu(I)-thiolate bonds (Winge et al. 1985; Thrower et al. 1988). Yeast *S. cerevisiae* possesses up to 15 tandem copies of the Cup1 gene encoding metallothionein (Fogel and Welch 1982). The gene copy number of Cup1 is directly correlated to the resistance level to external Cu. Yeast cells lacking Cup1 are hypersensitive to high Cu concentrations (Hamer et al. 1985), which is consistent to the role of MT in Cu detoxification. In addition to Cu chelation, MT partially suppresses the oxygen sensitivity of SOD1-defective yeast cells, since MT carries superoxide dismutase activities (Tamai et al. 1993).

Crs5 encodes a small molecular weight cysteine-rich protein with an amino acid sequence bearing all the features of a eukaryotic metallothionein, yet shares little sequence similarity with Cup1 (Culotta et al. 1994). Given that the Cu(I) ions bound to Crs5 are kinetically more labile, MT plays a dominant role in Cu detoxification (Jensen et al. 1996). The modest effects of Crs5 may imply it may have additional roles that are distinct from Cu chelation.

C. glabrata contains three MT-encoding genes, MTI, MTIIA, and MTIIB, which are highly induced by Cu (Mehra et al. 1989, 1990, 1992). The MTIIA locus contains a tandem array of a gene like Cup1 in *S. cerevisiae*, and Cu tolerance is correlated to the gene copy number (Mehra et al. 1990). The MTIIA and MTIIB genes encode the same polypeptide, but their 5' and 3' non-coding sequences are not identical (Mehra et al. 1992).

4.2 Cu,Zn superoxide dismutase (SOD1)

Cu readily catalyzes reactions that result in the production of hydroxyl radicals through the Fenton and Haber-Weiss reactions ($Cu^{+} + H_2O_2 \rightarrow HO^{\cdot} + HO^{-} + Cu^{2+}$, $O_2^{\cdot -} + Cu^{2+} \rightarrow Cu^{+} + O_2$) (Halliwell and Gutteridge 1984, 1990). Superoxide anion ($O_2^{\cdot -}$) is a critical factor that propagates the reaction generating highly reactive hydroxyl radicals ($HO^{\cdot}$). Thus, superoxide dismutase is obviously linked to a defense mechanism for Cu toxicity. Consistently, excess Cu induces Cu/Zn superoxide dismutase (Cu/Zn SOD, SOD1) expression (Gralla et al. 1991).

SOD1 is primarily located in the cytoplasm, but SOD1 has also been identified in other organelles including mitochondria (Weisiger and Fridovich 1973). It is interesting that a fraction of SOD1 and its Cu chaperone CCS localize to the mitochondrial IMS (Sturtz et al. 2001). Only a very immature form of the SOD1 polypeptide that lacks both Zn and Cu cofactors in its reduced disulfide form efficiently enters the mitochondria (Field et al. 2003), and SOD1 retention in the mitochondria is largely dependent on CCS. When CCS synthesis is repressed, SOD1 levels in the mitochondria are low, and conversely SOD1 levels in the mitochondria are high when CCS is abundant in this organelle. Yeast cells with elevated levels of SOD1 in the mitochondria exhibit pro-longed survival in the stationary phase (Sturtz et al. 2001). Given that death of yeast cells in the stationary phase is linked with mitochondrial reactive oxygen production (Jakubowski et al. 2000; Longo et al. 1999; Ashrafi et al. 1999), SOD1 accumulated in the mitochondria may play a significant role in protection against oxidative stress when cells are in the stationary phase. This study may have an important implication with familial amyotropic lateral sclerosis (ALS), a fatal neurodegenerative disease resulting from gain-of function mutations of SOD1 (Rosen et al. 1993; Gurney et al. 1994). It would be interesting to test whether the SOD1 mutants causing ALS behave abnormally in their retention and mode of actions in the mitochondria.

4.3 CuS biomineralization

The SLF1 gene was identified as a multicopy suppressor of a Cu sensitive mutant (Yu et al. 1996). This protein is important for the physiological Cu sulfide (CuS) mineralization on the cell surface, resulting in a brownish coloration when cells are grown in $CuSO_4$-containing media. Since overexpression of the SLF1 gene confers Cu resistance and disruption of the gene increases Cu sensitivity, CuS biomineralization should be an important mechanism to prevent Cu toxicity.

4.4 P-type ATPase-mediated Cu export

The pathogenic yeast *C. albicans* has a higher resistance to Cu toxicity than baker's yeast. Interestingly, this Cu resistance is mediated by a P-type ATPase (CaCRP1/CRD1) and MT (CaCUP1/CRD2) that have homology to their human counterparts (Weissman et al. 2000; Riggle and Kumamoto 2000). Cu induces

transcription of the CaCRP1/CRD1 gene, while CaCUP1/CRD2 expression is constant. These gene disruptions indicate that the CaCRP1/CRD1 is the major component for Cu resistance. Furthermore, under acidic and anaerobic growth conditions, CaCRP1/CRD1 function becomes essential for survival in the presence of even very low Cu concentration. Cu export mechanisms have been characterized in pathogenic enteric bacteria. Since CaCRP1/CRD1 primarily localizes at the plasma membrane and a knockout of this gene does not affect Fe metabolism, this Cu export pump is a unique example in eukaryotes. The *C. albicans* genome contains another P-type ATPase that may function at the secretory pathway (Weissman et al. 2000). This Cu export mechanism by a P-type ATPase is conserved in mammals, but the P-type ATPases in mammals play dual roles in Cu metabolism, Cu transport at the secretory compartment and Cu export at the plasma membrane (DiDonato and Sarkar 1997; Cox 1999; Schaefer and Gitlin 1999; Llanos and Merser 2002). Given that the baker's yeast *S. cerevisiae* has two P-type ATPases as well, Ccc2 and Pca1 (Yuan et al. 1995; Rad et al. 1994), Pca1 may play the same role as the CaCRP1/CRD1 of *C. albicans*.

4.5 Multi-drug resistance protein

It has been known that yeast cells defective in Pdr13 are more sensitive to Cu toxicity (Kim et al. 2001). Pdr13 activates Pdr1, a transcription factor that upregulates the expression of Pdr5 and Yor1 genes encoding ATP-binding cassette transporters involved in drug efflux (Moye-Rowley 2003). Consistently, a gain of function mutation of Pdr1 transcription factor shows increased resistance to Cu, Fe, and Mn (Tuttle et al. 2003). These results suggest that expression of multidrug resistance genes play a role in Cu resistance by altering their efflux and/or sequestration.

4.6 Vacuole and Cu sequestration

Isolation of yeast mutants sensitive to Cu ion toxicity revealed that genes that play critical roles in vacuolar assembly or acidification, including Pep3, Pep5, and Vma3, are required for normal Cu resistance (Szczypka et al. 1997). Yeast vacuoles serve as a storage organelle for metal-ions including Cu, Fe, and Zn (Bode et al. 1995; Ramsay and Gadd 1997; Paidhungat and Garrett 1998; MacDiarmid et al. 2000). Since baker's yeast Ctr2 protein and Ctr6 of *S. pombe* mobilize stored Cu from the vacuole (Bellemare et al. 2002; Rees et al. 2004), there should be mechanism(s) for Cu sequestration to the lumen of the vacuole.

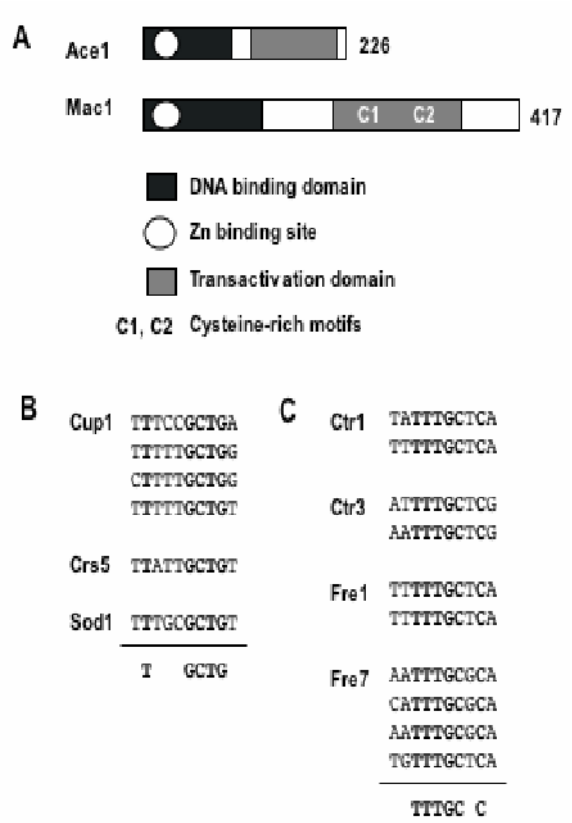


Fig. 3. (A) Ace1 and Mac1 are two Cu-responsive transcription factors in yeast *S. cerevisiae*. They share high sequence similarity in the DNA-binding domain, Cu-binding site and transactivation domain. Carboxyl-terminal cysteine-rich C1 and C2 motifs ($CXCX_4CXCX_2CX_2H$) of the Mac1 are important for its Cu sensing and transcription regulation of its target genes. (B) Sequences of the *cis*-acting elements of Ace1. Ace1 induces expression of *CUP1*, *CRS5* and *SOD1* genes upon binding to the promoter region of theses genes. The bold characters indicate the conserved sequences among the Ace1 DNA-binding sites. (C) Mac1-binding sequences identified from the promoter of *CTR1*, *CTR3*, *FRE1*, and *FRE7* genes.

5 Regulation of Cu metabolism

Copper ion homeostasis in yeast is maintained by different modes of regulation both at the transcriptional and post-translational levels. Cu uptake at the plasma membrane, sequestration of Cu in the cytoplasm, and defense systems against Cu toxicity are all regulated by Cu-activated and Cu-repressed transcription factors.

Turnover of Cu transporters and Cu-responsive transcription factors are also regulated by Cu levels.

5.1 Ace1/Amt1

Studies of the transcriptional regulation of Cup1 metallothionein (MT) gene by the Cu-regulatory transcription factors, Ace1 in *S. cerevisiae* (Thiele 1988; Welch et al. 1989) and Amt1 from *Candida glabrata* (Zhu and Thiele 1991), have provided insights into the mechanisms of Cu sensing and signal transduction. When the extracellular Cu concentration is in excess (10 μM), Ace1 is activated to induce expression of genes including Cup1, Crs5 and Sod1 (Hamer et al. 1988; Grallar et al. 1991; Culotta et al. 1994). Cu(I)-activated gene expression occurs through direct Cu binding to the regulatory domain of the Ace1 transcription factor (Dameron et al. 1991). Cu(I) cluster formation stabilizes a specific conformation of Ace1 that leads to binding with a response element (TXXXGCTG) of its target gene (Fig. 3B) (Buchman et al. 1989; Dobi et al. 1995). Chromosomal footprinting demonstrated that Ace1 binds, as a monomer, to three different regions of the Cup1 promoter comprising four different binding sites (Huibregtse et al. 1989; Evans et al. 1990). The DNA-binding domain of Ace1 was shown to map to the amino-terminal (Fig. 3A) in which 11 cysteine residues (CXXC or CXC) out of 12 are critical for Cu-induced gene expression (Hu et al. 1990). The Cu-regulatory domain binds 4 Cu(I) ions through 8 cysteinyl thiolates forming a polycopper cluster (Furst and Hamer 1989). Ace1 DNA binding is dependent on not only the Cu coordination but also by Ag(I) ions (Dameron et al. 1991; Furst el al. 1988). In addition to a Cu-responsive DNA-binding domain, Ace1 carries two other functional domains. The N-terminal of Ace1 has a Zn(II)-binding module (Farrell et al. 1996). Transcription assays using the fusion protein between a heterologous yeast DNA binding domain and the carboxy-terminal half of Ace1 indicates that this C-terminal region of Ace1 contains a transcription activation domain (Hu et al. 1990).

The trans-acting factor that mediates Cu-induced expression of MT genes in *C. glabrata* is Amt1, which is similar to Ace1 in its structure and mode of action (Zhou and Thiele 1991). Interestingly, unlike Ace1, expression of Amt1 is auto-regulated by Cu (Zhou and Thiele 1993). A homopolymeric (dA·dT) stretch located adjacent to the metal-response element of the Atm1 promoter plays a critical role in rapid transcriptional auto-regulation of the Atm1 gene (Zhu and Thiele1996). This structure fosters binding of Cu-activated Atm1 to the promoter resulting in rapid regulation of Atm1 expression.

The *S. cerevisiae* genome contains Haa1, which is homologous to Ace1. The N-terminal Zn-binding domain and Cu regulatory domain of Haa1 is similar to those of Ace1. Haa1 is indeed a transcription factor, but Cu and other metal-ions do not regulate expression of its target (Keller et al. 2001). The functions of Haa1-regulated genes do not appear to be directly related to Cu metabolism since over-expression of Haa1 does not suppress the Cu sensitivity of Ace1-defective cells (Keller et al. 2001).

5.2 Mac1

Cu-mediated repression of genes involved in Cu uptake in *S. cerevisiae* occurs through Mac1. Mac1 was identified by its similarity with the cysteine-rich copper-binding domain of Ace1 and Amt1 (Jungmann et al. 1993). Cu(I) binding to Mac1 represses an array of genes involved in Cu uptake, including CTR1, CTR3, FRE1, and FRE7, but Cu ion starvation de-represses these genes (Georgatsou et al. 1997; Yamaguchi-Iwai et al. 1997; Labbé et al. 1997; Peña et al. 1998). Deletion of the MAC1 gene results in phenotypes similar to those in cells lacking high affinity Cu transport that can be rescued by the addition of Cu ions.

In the presence of low levels of Cu, Mac1 constantly surveys the intracellular Cu status, and regulates the expression of the Cu transport genes depending on cellular needs. This regulation requires the interaction of Mac1 with the Cu-responsive *cis*-acting elements [CUREs, 5'-TTTGC(T/G)C(A/G)-3'] (Fig. 3C). This sequence is found as tandem or inverted repeats in the promoters of the high affinity Cu transport genes and a reductase encoded by the FRE1 gene (Yamaguchi-Iwai et al. 1997; Labbé et al. 1997; Peña et al. 1998). Consistently, *in vivo* footprinting experiments have shown that the CUREs within the CTR3 promoter are occupied during Cu ion starvation to induce target gene expression (Labbé et al. 1997; Peña et al. 1998). Mac1 appears to bind to these elements as a $(Mac1)_2$/DNA ternary complex with two molecules of Mac1 bound to each CURE element (Joshi et al. 1999). A module in the DNA binding motif interacts with the TTT sequence at the 5' end of the CURE site, and another DNA binding module interacts with the adjacent major groove in the GCTCA sequence (Jamison McDaniels et al. 1999).

Mac1 has several structural features that are consistent with its role as a regulatory protein. Its amino terminal has ~50% similarity to Ace1 and Amt1 (Jungmann et al. 1993). This domain is thought to bind to the minor groove of DNA and to bind Zn(II) (Jensen et al. 1998). The carboxyl terminal of Mac1 has two clusters of Cys-His repeats arranged as Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys-X_2-His (Fig. 3A). These two Cys-rich motifs bind a total of 8 Cu(I) ions (Jensen and Winge 1998). The first cluster appears to play a role in Cu sensing and the second cluster may be involved in transcriptional activation. Cu ions stimulate an intramolecular interaction between the amino terminal DNA binding domain and the cysteine-rich carboxyl terminus of Mac1, resulting in a Cu-dependent stabilization of Mac1 (Jensen and Winge 1998). Such interaction is believed to mask the activation domain of Mac1 resulting in repression of transcription of the Cu transport genes under high Cu conditions. Yeast strains that harbor the dominant allele $MAC1^{up1}$, are hypersensitive to Cu and exhibit high mRNA levels of Cu transport genes where expression is no longer regulated by Cu (Jensen et al. 1998; Hassett and Kosman 1995). The mechanisms of Cu incorporation to Mac1 are not well characterized. Given that intracellular trafficking of Cu to all known targets is mediated by Cu chaperones and the negligible amount of free Cu-ions in cells, there may be a shuttle that transfers Cu to Mac1 when Cu is in excess.

Mac1 has additional modes of regulation. As the intracellular Cu concentration approaches toxic levels (10 μM), Mac1 undergoes proteolytic degradation (Zhu et

al. 1998). This mode of regulation is specific for Cu and does not require the synthesis of new proteins. Conformational changes of Mac1 induced by Cu-binding may expose protease-binding domains. Alternatively, Cu may activate a specific protease that leads to degradation of Mac1. Another level of Mac1 regulation is phosphorylation. Unphosphorylated Mac1 is unable to bind to the Ctr1 promoter (Heredia et al. 2001). The kinase responsible for the phosphorylation of Mac1 is yet to be identified.

Microarray studies have identified two new Mac1-regulated genes in addition to other well-characterized targets (Gross et al. 2000). YFR055W shares homology with a family of trans-sulfuration enzymes involved in cysteine biosynthesis. The other target is YJL217W, which does not share significant sequence similarity to any known proteins. The functions of these genes in Cu metabolism are unclear. Mac1-deleted yeast cells exhibit alterations in expression of a number of genes (De Freitas et al. 2004). Since Cu is a critical cofactor for Fe transport, the responses in Mac1-defective yeast partially reflect Fe-deficiency. Defects in mitochondrial oxidative phosphorylation resulting from reduced activities of mitochondrial Cu-containing cytochrome c oxidase may lead to metabolic reorganization. Genes involved in the metabolism of amino acids, carbohydrates, and lipids are differentially expressed in the Mac1-deficient cells. Other genes that play roles in cell cycle regulation and stress response are also differentially expressed in Mac1-defective yeast. These observations further attest the critical roles of Cu in cellular metabolism.

5.3 Cuf1

The fission yeast *S. pombe* has a Cu-regulated transcription factor Cuf1 that carries homology with Ace1/Atm1 and Mac1 (Labbé et al. 1999). The Cuf1 amino-terminal is more similar to that found in Ace1 and Atm1 than Mac1. The Cys-rich domain near its carboxyl-terminal shares similarity to the domain of Mac1 that is known to play a critical role in Cu ion sensing. Cuf1 activates expression of Cu transporter genes under Cu starvation conditions by binding to a Cu-responsive *cis*-acting element of the Ctr4 Cu transporter (Beaudoin and Labbé 2001). Consistent to sequence similarity of the DNA binding domain between Cuf1 and Ace1, the Cuf1 recognition region bears strong sequence similarity to that of Ace1. A chimeric Cuf1 protein bearing the amino-terminal 63-residue segment of Ace1 functions like Cuf1 (Beaudoin et al. 2003).

Interestingly, Cuf1 activated by Cu starvation represses expression of genes encoding components of the Fe transport machinery (Labbé et al. 1999). This result suggests that, in the absence of sufficient levels of Cu cofactor, *S. pombe* prevents futile synthesis of Fe transporters. It appears that Fet3 in *S. cerevisiae*, which contains Cu as a cofactor is also regulated in a Mac1 activity-dependent manner, but the pattern of regulation is distinct from that of Cuf1. Cu supplementation in wild type cells that leads to Mac1 inactivation induces Fet3 expression, and a hyperactive allele of Mac1 represses Fet3 expression. It would be interesting to test

whether there is a regulatory mechanism of Cu metabolism under Fe starvation to supply sufficient Cu required for Fe metabolism.

5.4 Post-translational regulation of Cu transporters

Cu also triggers the degradation of the *S. cerevisiae* Ctr1 high affinity Cu transporter. Ctr1 is rapidly and specifically degraded at the plasma membrane in the presence of excess extracellular Cu (Ooi et al. 1996). This mechanism of Cu transporter regulation is conserved in its mammalian counterpart (Petris et al. 2003). Regulation of Cu transporters is likely a critical mechanism for preventing excess accumulation of Cu. Studies using mutant yeast strains that have defects in the endocytic pathway, and vacuolar degradation suggest that this process in yeast does not require internalization of Ctr1 or its delivery to the vacuole for proteolytic degradation. A cytosolic metal-binding motif (CX_5CXCX_2H) of Ctr1 appears to play an important role in Ctr1 degradation. Interestingly, the Mac1 transcription factor is also required for Cu-dependent Ctr1 degradation (Yonkovich et al. 2002). Ctr1 is much more stable in the Mac1-deleted cells. It is interesting that a transcription factor controls its target at both transcriptional and post-transcriptional levels. The exact mechanisms and implications of Mac1 in Ctr1 turnover have not been resolved. A simple explanation of this observation is that one or multiple Mac1 target gene(s) play a role in Cu-dependent Ctr1 degradation. Whereas the Ctr3 high affinity Cu transporter has the same functions and similar structural features to those of Ctr1, post-transcriptional regulation of these proteins is distinct. Unlike Ctr1, the Ctr3 transporter is neither regulated at the level of protein degradation nor endocytosis as a function of elevated Cu levels (Peña et al. 2000).

5.5 Regulation of Cu metabolism by stress and other nutritional factors

The Cu transport machinery is not only regulated by extracellular Cu levels but also by growth conditions such as pH and nutrient limitations. First, many other genes relevant to Cu and Fe metabolism such as Fre1, Ctr1, Lys7, and Ccc2 are induced when cultured in an alkaline media (Serrano et al. 2002, 2004). Consistently, yeast cells with deletions in any of these genes are sensitive to alkaline pH, and overexpression of the Ctr1 or Fet4 Cu transporters confers resistance to alkaline pH (Serrano et al. 2004). These results suggest that Cu and Cu-requiring physiological processes are important for yeast growth under such conditions. A possible explanation of this regulation is that Cu uptake is slow under alkaline conditions. Second, it has been shown that Ctr3 is upregulated by carbon-limitations, and Ctr1 and Ctr3 are downregulated under sulfur limitations (Boer et al. 2003). Carbon limitation may force yeast cells to generate energy through mitochondrial oxidative phosphorylation in which Cu and Fe play an essential role in the electron transfer. This may explain why cells induce Cu transport. Downregu-

lation of Ctr1 and Ctr3 in yeast suffering sulfur limitation could be important for preventing Cu toxicity. The underlying mechanisms and biological significance of these regulations remain to be studied.

6 Conclusions

The power of techniques of genetics, molecular biology and chemistry has led to discovery of delicate and fascinating mechanisms of Cu homeostasis. Interestingly, most of the molecular mechanisms of Cu homeostasis are well conserved between yeast and human. Further progress in understanding Cu metabolism will lead to the determination of the roles for Cu homeostasis in biochemical processes and prevention and treatment of Cu-related diseases in human. Advances in understanding Cu metabolism will also lead in to further studies on other physiologically important metal ions.

Acknowledgements

We are grateful to other members in the laboratory, Devis Sinani and Jinyoung Hur, for their comments and helpful suggestions during the preparation of this manuscript. Studies on Cu metabolism in Lee's laboratory are supported by National Institutes of Health grant P20 RR-17675.

References

Abajian C, Yatsunyk LA, Ramirez BE, Rosenzweig AC (2004) Yeast Cox17 solution structure and copper(I) binding. J Biol Chem 279:53584-53592

Aller SG, Eng ET, De Feo CJ, Unger VM (2004) Eukaryotic CTR Copper uptake transporters require two faces of the third transmembrane domain for helix packing, oligomerization, and function. J Biol Chem 279:53435-53441

Arnesano F, Banci L, Bertini I, Huffman DL, O'Halloran TV (2001) Solution structure of the Cu(I) and apo forms of the yeast metallochaperones Atx1. Biochemistry 40:1528-1539

Arnesano F, Banci L, Bertini I, Bonvin A (2004) A docking approach to the study of copper trafficking proteins: Interaction between metallochaperones and soluble domains of copper ATPases. Structure 12:669-676

Ashrafi K, Sinclair D, Gordon JI, Guarente L (1999) Passage through stationary phase advances replicative aging in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 96:9100-9105

Askwith C, Eide D, Van Ho A, Bernard PS, Li L, Davis-Kaplan S, Sipe DM, Kaplan J (1994) The FET3 gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. Cell 76:403-410

Banci L, Bertini I, Ciofi-Baffoni S, Huffman DL, O'Halloran TV (2001) Solution structure of the yeast copper transporter domain Ccc2a in the apo and Cu(I)-loaded states. J Biol Chem 276:8415-8426

Barros MH, Johnson A, Tzagoloff A (2004) COX23, a homologue of COX17, is required for cytochrome oxidase assembly. J Biol Chem 279:31943-31947

Beaudoin J, Labbe S (2001) The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. J Biol Chem 276:15472-15480

Beaudoin J, Mercier A, Langlois R, Labbe S (2003) The *Schizosaccharomyces pombe* Cuf1 is composed of functional modules from two distinct classes of copper metalloregulatory transcription factors. J Biol Chem 278:14565-14577

Beers J, Glerum MD, Tzagoloff A (1997) Purification, characterization, and localization of yeast Cox17p, a mitochondrial copper shuttle. J Biol Chem 272:33191-33196

Bellemare DR, Shaner L, Morano KA, Beaudoin J, Langlois R, Labbe S (2002) Ctr6, a vacuolar membrane copper transporter in *Schizosaccharomyces pombe*. J Biol Chem 277:46676-46686

Bode HP, Dumschat M, Garotti S, Fuhrmann GF (1995) Iron sequestration by the yeast vacuole. A study with vacuolar mutants of *Saccharomyces cerevisiae*. Eur J Biochem 228:337-342

Boer VM, de Winde JH, Pronk JT, Piper MD (2003) The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J Biol Chem 278:3265-3274

Brown NM, Torres AS, Doan PE, O'Halloran TV (2004) Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu,Zn superoxide dismutase. Proc Natl Acad Sci USA 101:5518-5523

Buchman C, Skroch P, Welch J, Fogel S, Karin M (1989) The CUP2 gene product, regulator of yeast metallothionein expression, is a copper-activated DNA-binding protein. Mol Cell Biol 9:4091-4095

Carr HS, Winge DR (2003) Assembly of cytochrome c oxidase within the mitochondrion. Acc Chem Res 36:309-316

Carr HS, George GN, Winge DR (2002) Yeast Cox11, a protein essential for cytochrome c oxidase assembly, is a Cu(I) binding protein. J Biol Chem 277:31237-31242

Cha JS, Cooksey DA (1991) Copper resistance in Pseudomonas syringae mediated by periplasmic and outer membrane proteins. Proc Natl Acad Sci USA 88:8915-8919

Chanock SJ, el Benna J, Smith RM, Babior BM (1994) The respiratory burst oxidase. J Biol Chem 269:24519-24522

Cobine PA, Ojeda LD, Rigby KM, Winge DR (2004) Yeast contain a non-proteinaceous pool of copper in the mitochondrial matrix. J Biol Chem 279:14447-14455

Cox DW (1999) Disorders of copper transport. Br Med Bull 55:544-555

Culotta VC, Howard WR, Liu XF (1994) CRS5 encodes a metallothionein-like protein in *Saccharomyces cerevisiae*. J Biol Chem 269:25295-25302

Culotta VC, Klomp LWJ, Strain J, Casareno RBL, Krems B, Gitlin JD (1997) The copper chaperone for superoxide dismutase. J Biol Chem 272:23469-23472

Dameron CT, Winge DR, George GN, Sansone M, Hu S, Hamer D (1991) A copper-thiolate polynuclear cluster in the ACE1 transcription factor. Proc Natl Acad Sci USA 88:6127-6131

Dancis A, Yuan DS, Haile D, Askwith C, Eide D, Moehle C, Kaplan J, Klausner RD (1994a) Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. Cell 76:393-402

Dancis A, Haile D, Yuan DS, Klausner RD (1994b) The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. J Biol Chem 269:25660-25667

Davis-Kaplan SR, Askwith CC, Bengtzen AC, Radisky D, Kaplan J (1998) Chloride is an allosteric effector of copper assembly for the yeast multicopper oxidase Fet3p: An unexpected role for intracellular chloride channels. Proc Natl Acad Sci USA 95:13641-13645

De Freitas JM, Kim JH, Poynton H, Su T, Wintz H, Fox T, Holman P, Loguinov A, Keles S, Van der Laan M, Vulpe C (2004) Exploratory and confirmatory gene expression profiling of *mac*1Δ. J Biol Chem 279:4450-4458

De Rome L, Gadd GM (1987) Measurement of copper uptake in *Saccharomyces cerevisiae* using a Cu^{2+}-selective electrode. FEMS Microbiol Lett 43:283-287

DiDonato M, Sarkar B (1997) Copper transport and its alterations in Menkes and Wilson diseases. Biochim Biophys Acta 1360:3-16

Dix DR, Bridgham JT, Broderius MA, Byersdorfer CA, Eide DJ (1994) The FET4 gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae*. J Biol Chem 269:26092-260929

Dobi A, Dameron CT, Hu S, Hamer D, Winge DR (1995) Distinct regions of Cu(I) ACE1 contact two spatially resolved DNA major groove sites. J Biol Chem 270:10171-10178

Evans CF, Engelke DR, Thiele DJ (1990) ACE1 transcription factor produced in *Escherichia coli* binds multiple regions within yeast metallothionein upstream activation sequences. Mol Cell Biol 10:426-429

Farrell RA, Thorvaldsen JL, Winge DR (1996) Identification of the Zn(II) site in the copper-responsive yeast transcription factor, AMT1: a conserved Zn module. Biochemistry 35:1571-1580

Field LS, Furukawa Y, O'Halloran TV, Culotta VC (2003) Factors controlling the uptake of yeast copper/zinc superoxide dismutase into mitochondria. J Biol Chem 278:28052-28059

Finegold AA, Shatwell KP, Segal AW, Klausner RD, Dancis A (1996) Intramembrane bisheme motif for transmembrane electron transport conserved in a yeast iron reductase and the human NADPH oxidase. J Biol Chem 271:31021-31024

Fogel S, Welch JW (1982) Tandem gene amplification mediates copper resistance in yeast. Proc Natl Acad Sci USA 79:5342-5346

Furst P, Hamer D (1989) Cooperative activation of a eukaryotic transcription factor: interaction between Cu(I) and yeast ACE1 protein. Proc Natl Acad Sci USA 86:5267-5271

Furst P, Hu S, Hackett R, Hamer D (1988) Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. Cell 55:705-717

Furukawa Y, Torres AS, O'Halloran TV (2004) Oxygen-induced maturation of SOD1: A key role for disulfide formation by the copper chaperone CCS. EMBO J 23:2872-2881

Gaxiola RA, Yuan DS, Klausner RD, Fink GR (1998) The yeast CLC chloride channel functions in cation homeostasis. Proc Natl Acad Sci USA 95:4046-4050

Georgatsou E, Mavrogiannis LA, Fragiadakis GS, Alexandraki D (1997) The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. J Biol Chem 272:13786-13792

Giaccone G (2000) Clinical perspectives on platinum resistance. Drugs 59 Suppl 4:9-17

Glerum MD, Shtanko A, Tzagoloff A (1996a) Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J Biol Chem 271**:**14504-14509

Glerum MD, Shtanko A, Tzagoloff A (1996b) SCO1 and SCO2 act as high copy supressors of mitochondrial copper recruitment defect in *Saccharomyces cerevisiae* J Biol Chem 271:20531-20535

Gralla EB, Thiele DJ, Silar P, Valentine JS (1991) ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. Proc Natl Acad Sci USA 88:8558-8562

Greco MA, Hrab DI, Magner W, Kosman DJ (1990) Cu,Zn superoxide dismutase and copper deprivation and toxicity in *Saccharomyces cerevisiae*. J Bacteriol 172:317-325

Gross C, Kelleher M, Iyer VR, Brown PO, Winge DR (2000) Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. J Biol Chem 275:32310-32316

Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 264:1772-1775

Halliwell B, Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1-114

Halliwell B, Gutteridge JM (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186:1-85

Hamer DH (1986) Metallothionein. Annu Rev Biochem 55:913-951

Hamer DH, Thiele DJ, Lemontt JE (1985) Function and autoregulation of yeast copperthionein. Science 228:685-690

Harshman KD, Moyle-Rowley WS, Parker CS (1988) Transcriptional activation by the SV40 AP-1 recognition element in yeast is mediated by a factor similar to AP-1 that is distinct from GCN4. Cell 53:321-330

Hassett R, Kosman DJ (1995) Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. J Biol Chem 270:128-134

Hassett R, Dix DR, Eide DJ, Kosman DJ (2000) The Fe(II) permease Fet4p functions as a low affinity copper transporter and supports normal copper trafficking in *Saccharomyces cerevisiae*. Biochem J 351:477-484

Heaton D, Nittis T, Srinivasan C, Winge DR (2001) Mutational analysis of the mitochondrial copper metallochaperone Cox17. J Biol Chem 275:37582-37587

Heredia J, Crooks M, Zhu Z (2001) Phosphorylation and Cu^{+} coordination-dependent DNA binding of the transcription factor Mac1p in the regulation of copper transport. J Biol Chem 276:8793-8797

Hettema EH, Valdez-Taubas J, Pelham HR (2004) Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. EMBO J 23:1279-1288

Hiser L, Di Valentin M, Hamer AG, Hosler JP (2000) Cox11p is required for stable formation of the CuB and magnesium centers of cytochrome of cytochrome c oxidase. J Biol Chem 275:619-623

Horng YC, Cobine PA, Maxfield AB, Carr HS, Winge DR (2004) Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome c oxidase. J Biol Chem 34:35334-35340

Hu S, Furst P, Hamer D (1990) The DNA and Cu binding functions of ACE1 are interdigitated within a single domain. New Biol 2:544-555

Huffman DL, O'Halloran TV (2000) Energetics of copper trafficking between the Atx1 metallochaperone and the intracellular copper transporter, Ccc2. J Biol Chem 275:18611-18614

Huffman DL, O'Halloran TV (2001) Function, structure, and mechanism of intracellular copper trafficking proteins. Annu Rev Biochem 70:677-701

Huibregtse JM, Engelke DR, Thiele DJ (1989) Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences. Proc Natl Acad Sci USA 86:65-69

Hung I, Suzuki M, Yamaguchi Y, Yuan DS, Klausner RD, Gitlin JD (1997) Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. J Biol Chem 272:21461-21466

Ishida S, Lee J, Thiele DJ, Herskowitz I (2002) Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci USA 99:14298-14302

Iwata S, Ostermeier C, Ludwig B, Michel H (1995) Structure at 2.8 A resolution of cytochrome c oxidase from *Paracoccus denitrificans*. Nature 376:660-669

Jakubowski W, Bilinski T, Bartosz G (2000) Oxidative stress during aging of stationary cultures of the yeast *Saccharomyces cerevisiae*. Free Radic Biol Med 28:659-664

Jamison McDaniels CP, Jensen LT, Srinivasan C, Winge DR, Tullius TD (1999) The yeast transcription factor Mac1 binds to DNA in a modular fashion. J Biol Chem 274:26962-26967

Jensen LT, Winge DR (1998) Identification of a copper-induced intramolecular interaction in the transcription factor Mac1 from *Saccharomyces cerevisiae*. EMBO J 17:5400-5408

Jensen LT, Howard WR, Strain JJ, Winge DR, Culotta VC (1996) Enhanced effectiveness of copper ion buffering by CUP1 metallothionein compared with CRS5 metallothionein in *Saccharomyces cerevisiae*. J Biol Chem 271:18514-18519

Jensen LT, Posewitz MC, Srinivasan C, Winge DR (1998) Mapping of the DNA binding domain of the copper-responsive transcription factor Mac1 from *Saccharomyces cerevisiae*. J Biol Chem 273:23805-23811

Jensen LT, Ajua-Alemanji M, Culotta VC (2003) The *Saccharomyces cerevisiae* high affinity phosphate transporter encoded by PHO84 also functions in manganese homeostasis. J Biol Chem 278:42036-42040

Joshi A, Serpe M, Kosman DJ (1999) Evidence for (Mac1p) DNA ternary complex formation in Mac1p-dependent transactivation at the CTR1 promoter. J Biol Chem 274:218-226

Jungmann J, Reins HA, Lee J, Romeo A, Hassett R, Kosman D, Jentsch S (1993) MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. EMBO J 12:5051-5056

Kampfenkel K, Kushnir S, Babiychuk E, Inze D, Van Montagu M (1995) Molecular characterization of a putative *Arabidopsis thaliana* copper transporter and its yeast homologue. J Biol Chem 270:28479-28486

Keller G, Ray E, Brown PO, Winge DR (2001) Haa1, a protein homologous to the copper-regulated transcription factor Ace1, is a novel transcriptional activator. J Biol Chem 276:38697-38702

Keyhani E, Keyhani J (1975) Cytochrome c oxidase biosynthesis and assembly in *Candida utilis* yeast cells. Function of copper in the assembly of active cytochrome c oxidase. Arch Biochem Biophys 167:596-602

Kim DY, Song WY, Yang YY, Lee Y (2001) The role of PDR13 in tolerance to high copper stress in budding yeast. FEBS Lett 508:99-102

Klomp LWJ, Lin SJ, Yuan D, Klausner RD, Culotta VC, Gitlin JD (1997) Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis. J Biol Chem 272:9221-9226

Knight SA, Labbe S, Kwon LF, Kosman DJ, Thiele DJ (1996) A widespread transposable element masks expression of a yeast copper transport gene. Genes Dev 10:1917-1929

Labbe S, Pena MM, Fernandes AR, Thiele DJ (1999) A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe.* J Biol Chem 274:36252-36260

Labbe S, Thiele DJ (1999) Pipes and wiring: the regulation of copper uptake and distribution in yeast. Trends Microbiol 7:500-505

Labbe S, Zhu Z, Thiele DJ (1997) Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. J Biol Chem 272:15951-15958

Lamb AL, Torres AS, O'Halloran TV, Rosenzweig AC (2000) Heterodimer formation between superoxide dismutase and its copper chaperone. Biochemistry 39:14720-14727

Lamb AL, Wernimont AK, Pufahl RA, Culottaa VC, O'Halloran TV, Rosenzweig AC (1999) Crystal structure of the copper chaperone for superoxide dismutase. Nat Struct Biol 6:724-729

Lamb AL, Torres AS, O'Halloran TV, Rosenzweig AC (2001) Heterodimeric structure of superoxide dismutase in complex with its metallochaperone. Nat Struct Biol 8:750-755

Lee J, Pena MM, Nose Y, Thiele DJ (2002) Biochemical characterization of the human copper transporter Ctr1. J Biol Chem 277:4380-4387

Lesuisse E, Casteras-Simon M, Labbe P (1996) Evidence for the *Saccharomyces cerevisiae* ferrireductase system being a multicomponent electron transport chain. J Biol Chem 271:13578-13583

Li L, Chen OS, McVey Ward D, Kaplan J (2001) Ccc1 is a transporter that mediates vacuolar iron storage in yeast. J Biol Chem 276:29515-29519

Lin CM, Kosman DJ (1990) Copper uptake in wild type and copper metallothionein-deficient *Saccharomyces cerevisiae*. Kinetics and mechanism. J Biol Chem 265:9194-9200

Lin SJ, Culotta, VC (1995) The ATX1 gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. Proc Natl Acad Sci USA 92:3784-3788

Lin SJ, Pufahl RA, Dancis A, O'Halloran TV, Culotta VC (1997) A Role for the *Saccharomyces cerevisiae* ATX1 gene in copper trafficking and iron transport. J Biol Chem 272:9215-9220

Lin X, Okuda T, Holzer A, Howell SB (2002) The copper transporter CTR1 regulates cisplatin uptake in *Saccharomyces cerevisiae*. Mol Pharmacol 62:1154-1159

Liu XF, Culotta VC (1999) Post-translation control of Nramp metal transport in yeast. Role of metal ions and the BSD2 gene. J Biol Chem 274:4863-4868

Liu XF, Supek F, Nelson N, Culotta VC (1997) Negative control of heavy metal uptake by the *Saccharomyces cerevisiae* BSD2 gene. J Biol Chem 272:11763-11769

Llanos RM, Mercer JF (2002) The molecular basis of copper homeostasis copper-related disorders. DNA Cell Biol 21:259-270

Lode A, Kuschel M, Paret C, Rodel G (2000) Mitochondrial copper metabolism in yeast: Interaction between Sco1p and Cox2p. FEBS lett 448:1-6

Lode A, Paret C, Rodel G (2002) Molecular characterization of *Saccharomyces cerevisiae* Sco2p reveals a high degree of redundancy with Sco1p. Yeast 19:909-922

Loehrer PJ, Einhorn LH (1984) Drugs five years later. Cisplatin. Ann Intern Med 100:704-713

Longo VD, Liou LL, Valentine JS, Gralla EB (1999) Mitochondrial superoxide decreases yeast survival in stationary phase. Arch Biochem Biophys 365:131-142

Lutsenko S, Kaplan JH (1995) Organization of P-type ATPases: significance of structural diversity. Biochemistry 34:15607-15613

MacDiarmid CW, Gaither LA, Eide D (2000) Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*. EMBO J 19:2845-2855

Martins LJ, Jensen LT, Simon JR, Keller GL, Winge DR (1998) Metalloregulation of FRE1 and FRE2 homologs in *Saccharomyces cerevisiae*. J Biol Chem 273:23716-23721

Marvin ME, Williams PH, Cashmore AM (2003) The *Candida albicans* CTR1 gene encodes a functional copper transporter. Microbiology 149:1461-1474

Maxfield AB, Heaton DN, Winge DR (2004) Cox17 is functional when tethered to the mitochondrial inner membrane. J Biol Chem 279:5072-5080

McCord JM, Fridovich I (1969) The utility of superoxide dismutase in studying free radical reactions: Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. J Biol Chem 244:6049-6055

Mehra RK, Garey JR, Butt TR, Gray WR, Winge DR (1989) *Candida glabrata* metallothioneins. Cloning and sequence of the genes and characterization of proteins. J Biol Chem 264:19747-19753

Mehra RK, Garey JR, Winge DR (1990) Selective and tandem amplification of a member of the metallothionein gene family in *Candida glabrata*. J Biol Chem 265:6369-6375

Mehra RK, Thorvaldsen JL, Macreadie IG, Winge DR (1992) Disruption analysis of metallothionein-encoding genes in *Candida glabrata*. Gene 114:75-80

Moye-Rowley WS (2003) Transcriptional control of multidrug resistance in the yeast *Saccharomyces*. Prog Nucleic Acid Res Mol Biol 73:251-279

Nittis T, George GN, Winge DR (2001) Yeast Sco1, a protein essential for cytochrome c oxidase function is a Cu(I)-binding protein. J Biol Chem 276:42520-42526

Nobrega MP, Simone CB, Bandeira, Beers J, Tzagoloff A (2002) Characterization of Cox19, a widely distributed gene required for expression of mitochondrial cytochrome oxidase. J Biol Chem 277:40206-40211

Odermatt A, Suter H, Krapf R, Solioz M (1993) Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. J Biol Chem 268:12775-12779

O'Halloran TV, Culotta VC (2000) Metallochaperones, an intracellular shuttle service for metal ions. J Biol Chem 275:25057-25060

Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD (1996) Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. EMBO J 15:3515-3523

Osaki S, Johnson DA (1969) Mobilization of liver iron by ferroxidase (ceruloplasmin). J Biol Chem 244:5757-5758

Paidhungat M, Garrett S (1998) Cdc1 and the vacuole coordinately regulate Mn^{2+} homeostasis in the yeast *Saccharomyces cerevisiae*. Genetics 148:1787-1798

Payne AS, Gitlin JD (1998) Functional expression of the Menkes disease protein reveals common biochemical mechanisms among the copper-transporting P-type ATPases. J Biol Chem 273:3765-3770

Pena MM, Koch KA, Thiele DJ (1998) Dynamic regulation of copper uptake and detoxification genes in *Saccharomyces cerevisiae*. Mol Cell Biol 18:2514-2523

Peña MM, Lee J, Thiele DJ (1999) A delicate balance: homeostatic control of copper uptake and distribution. J Nutr 129:1251-1260

Pena MM, Puig S, Thiele DJ (2000) Characterization of the *Saccharomyces cerevisiae* high affinity copper transporter Ctr3. J Biol Chem 275:33244-33251

Petris MJ, Smith K, Lee J, Thiele DJ (2003) Copper-stimulated endocytosis and degradation of the human copper transporter, hCtr1. J Biol Chem 278:9639-9646

Portnoy ME, Rosenzweig AC, Rae T, Huffman DL, O'Halloran TV, Culotta VC (1999) Structure-function analyses of the ATX1 metallochaperone. J Biol Chem 274:15041-15045

Portnoy ME, Schmidt PJ, Rogers RS, Culotta VC (2001) Metal transporters that contribute copper to metallochaperones in *Saccharomyces cerevisiae*. Mol Genet Genomics 265:873-882

Pufahl RA, Singer CP, Peariso KL, Lin SJ, Schmidt PJ, Fahrni CJ, Culotta VC Penner-Hahn JE, O'Halloran TV (1997) Metal ion chaperone function of the soluble Cu(I) receptor Atx1. Science 278:853-856

Puig S, Thiele DJ (2002) Molecular mechanisms of copper uptake and distribution. Curr Opin Chem Biol 6:171-180

Puig S, Lee J, Lau M, Thiele DJ (2002) Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. J Biol Chem 277:26021-26030

Rad MR, Kirchrath L, Hollenberg CP (1994) A putative P-type Cu (2+)-transporting ATPase gene on chromosome II of *Saccharomyces cerevisiae*. Yeast 10:1217-1225

Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV (1999) Undetectable intracellular free copper: The requirement of a copper chaperone for superoxide dismutase. Science 284:805-807

Ramsay LM, Gadd GM (1997) Mutants of *Saccharomyces cerevisiae* defective in vacuolar function confirm a role for the vacuole in toxic metal ion detoxification. FEMS Microbiol Lett 152:293-298

Rees EM, Lee J, Theile DJ (2004) Mobilization of intracellular copper stores by the Ctr2 vacuolar copper transporter. J Biol Chem (in press)

Rentzsch A, Krummeck-Weiss G, Hofer A, Bartuschka A, Ostermann K, Rodel G (1999) Mitochondrial copper metabolism in yeast: mutational analysis of Sco1p involved in the biogenesis of cytochrome c oxidase. Curr Genet 35:103-108

Riggle PJ, Kumamoto CA (2000) Role of a *Candida albicans* P1-type ATPase in resistance to copper and silver ion toxicity. J Bacteriol 182:4899-4905

Roman DG, Dancis A, Anderson GJ, Klausner RD (1993) The fission yeast ferric reductase gene frp1+ is required for ferric iron uptake and encodes a protein that is homologous to the gp91-phox subunit of the human NADPH phagocyte oxidoreductase. Mol Cell Biol 13:4342-4350

Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362:59-62

Rosenzweig AC (2001) Copper delivery by metallochaperone proteins. Acc Chem Res 34:119-128

Rotrosen D, Yeung CL, Leto TL, Malech HL, Kwong CH (1992) Cytochrome b558: the flavin-binding component of the phagocyte NADPH oxidase. Science 256:1459-1462

Schaefer M, Gitlin JD (1999) Genetic disorders of membrane transport. IV. Wilson's disease and Menkes disease. Am J Physiol 276:G311-314

Schmidt PJ, Rae TD, Pufahl RA, Hamma T, Strain J, O'Halloran TV, Culotta VC (1999) Multiple protein domains contribute to the action of the copper chaperone for superoxide dismutase. J Biol Chem 274:23719-23725

Schmidt PJ, Kunst C, Culotta VC (2000) Copper activation of superoxide dismutase 1 (SOD1) *in vivo*. J Biol Chem 275:33771-33776

Serrano R, Ruiz A, Bernal D, Chambers JR, Arino J (2002) The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signaling. Mol Microbiol 46:1319-1333

Serrano R, Bernal D, Simon E, Arino J (2004) Copper and iron are the limiting factors for growth of the yeast *Saccharomyces cerevisiae* in an alkaline environment. J Biol Chem 279:19698-19704

Shatwell KP, Dancis A, Cross AR, Klausner RD, Segal AW (1996) The FRE1 ferric reductase of *Saccharomyces cerevisiae* is a cytochrome b similar to that of NADPH oxidase. J Biol Chem 271:14240-14244

Shiraishi E, Masahiro I, Masanori J, Hiroshi T (2000) The cadmium-resistant gene, CAD2, which is a mutated putative copper-transporter gene (PCA1), controls the intracellular cadmium-level in the yeast *S. cerevisiae*. Curr Genet 37:79-86

Srinivasan C, Posewitz MC, George GN, Winge DR (1998) Characterization of the copper chaperone Cox17 of *Saccharomyces cerevisiae.* Biochemistry 37:7572-7577

Steaman R, Yuan D, Yamaguchi-Iwan Y, Klausner RD, Dancis A (1996) A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science 271:1552-1557

Sturtz LA, Diekert K, Jensen LT, Lill R, Culotta VC (2001) A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. J Biol Chem 276:38084-38089

Supek F, Supekova L, Nelson H, Nelson N (1996) A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. Proc Natl Acad Sci USA 93:5105-5110

Szczypka MS, Zhu Z, Silar P, Thiele DJ (1997) *Saccharomyces cerevisiae* mutants altered in vacuole function are defective in copper detoxification and iron-responsive gene transcription. Yeast 13:1423-35

Tamai KT, Gralla EB, Ellerby LM, Valentine JS, Thiele DJ (1993) Yeast and mammalian metallothioneins functionally substitute for yeast copper-zinc superoxide dismutase. Proc Natl Acad Sci USA 90:8013-8017

Thiele DJ (1988) ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. Mol Cell Biol 8:2745-2752

Thiele DJ (1992) Metal-regulated transcription in eukaryotes. Nucleic Acids Res 20:1183-1191

Thrower AR, Byrd J, Tarbet EB, Mehra RK, Hamer DH, Winge DR (1988) Effect of mutation of cysteinyl residues in yeast Cu-metallothionein. J Biol Chem 263:7037-7042

Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S (1995) Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 A. Science 269:1069-1074

Tuttle MS, Radisky D, Li L, Kaplan J (2003) A dominant allele of PDR1 alters transition metal resistance in yeast. J Biol Chem 278:1273-1280

Tzagoloff A, Capitanio N, Nobrega MP, Gatti D (1990) Cytochrome oxidase assembly in yeast requires the product of COX11, a homologue of the *P. denitrificans* protein encoded by ORF3 EMBO J 9:2759-2764

Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat Genet 21:195-199

Weisiger RA, Fridovich I (1973) Mitochondrial superoxide disimutase. Site of synthesis and intramitochondrial localization. J Biol Chem 248:4793-4796

Weissman Z, Berdicevsky I, Cavari BZ, Kornitzer D (2000) The high copper tolerance of *Candida albicans* is mediated by a P-type ATPase. Proc Natl Acad Sci USA 97:3520-3525

Welch J, Fogel S, Buchman C, Karin M (1989) The CUP2 gene product regulates the expression of the CUP1 gene, coding for yeast metallothionein. EMBO J 8:255-260

Winge DR (1999) Copper-regulatory domain involved in gene expression. Adv Exp Med Biol 448:237-246

Winge DR, Nielson KB, Gray WR, Hamer DH (1985) Yeast metallothioneins: Sequence and metal-binding properties. J Biol Chem 260:14464-14470

Xiao Z, Loughlin F, George GN, Howlett GJ, Wedd AG (2004) C-terminal domain of the membrane copper transporter Ctr1 from *Saccharomyces cerevisiae* binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster: sub-femtomolar Cu(I) affinity of three proteins involved in copper trafficking. J Am Chem Soc 126:3081-3090

Xiao Z, Wedd AG (2002) A C-terminal domain of the membrane copper pump Ctr1 exchanges copper(I) with the copper chaperone Atx1. Chem Commun (Camb) 6:588-589

Yamaguchi-Iwai Y, Serpe M, Haile D, Yang W, Kosman DJ, Klausner RD, Dancis A (1997) Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. J Biol Chem 272:17711-17718

Yonkovich J, McKenndry R, Shi X, Zhu Z (2002) Copper ion-sensing transcription factor Mac1p post-translationally controls the degradation of its target gene product Ctr1p. J Biol Chem 277:23981-23984

Yu W, Farrell RA, Stillman DJ, Winge DR (1996) Identification of SLF1 as a new copper homeostasis gene involved in copper sulfide mineralization in *Saccharomyces cerevisiae*. Mol Cell Biol 16:2464-2472

Yuan DS, Stearman R, Dancis A, Dunn T, Beeler T, Klausner RD (1995) The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. Proc Natl Acad Sci USA 92:2632-2636

Yuan DS, Dancis A, Klausner RD (1997) Restriction of copper export in *Saccharomyces cerevisiae* to a late golgi of post-golgi compartment in the secretory pathway. J Biol Chem 272:25787-25793

Zhou PB, Thiele DJ (1991) Isolation of a metal-activated transcription factor gene from *Candida glabrata* by complementation in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 88:6112-6116

Zhou P, Thiele DJ (1993) Rapid transcriptional autoregulation of a yeast metalloregulatory transcription factor is essential for high-level copper detoxification. Genes Dev 7:1824-1835

Zhu Z, Thiele DJ (1996) A specialized nucleosome modulates transcription factor access to a *C. glabrata* metal responsive promoter. Cell 87:459-470

Zhou H, Thiele DJ (2001) Identification of a novel high affinity copper transport complex in the fission yeast *Schizosaccharomyces pombe*. J Biol Chem 276:20529-20535

Zhu Z, Labbe S, Pena MM, Thiele DJ (1998) Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. J Biol Chem 273:1277-1280

Abbreviations

ATP: adenosine triphosphate
COX: cytochrome c oxidase
FAD: flavin adenine dinucleotide
IM: inner membrane
IMS: intermembrane space
kD: kilodalton
OM: outer membrane
MT: metallothionine
MNK: Menkes disease protein
NMR: nuclear magnetic resonance
NADPH: nicotinamide adenine dinucleotide phosphate
Cu,Zn SOD: Cu, Zn Superoxide Dismutase
SOD1: Cu, Zn Superoxide Dismutase
WND: Wilson's disease protein

Adle, David
Department of Biochemistry and Redox Biology Center, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

Kim, Heejeong
Department of Biochemistry and Redox Biology Center, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

Lee, Jaekwon
Department of Biochemistry and Redox Biology Center, University of Nebraska-Lincoln, N210 Beadle, Lincoln, NE 68588, USA
jlee7@unlnotes.unl.edu

Zinc in yeast: mechanisms involved in homeostasis

Lisa M. Regalla and Thomas J. Lyons

Abstract

The first eukaryotic zinc uptake transporter was discovered in the yeast, *Saccharomyces cerevisiae*. Since then, this organism has been an invaluable tool for the discovery of genes involved in zinc homeostasis. Genomic and proteomic studies have revealed an abundance of Zn^{2+}-regulated genes and Zn^{2+}-binding proteins. The large number of essential functions of Zn^{2+} necessitates a complex homeostatic mechanism involving the transport and storage of Zn^{2+} as well as its allocation to essential sites. Studies in yeast have elucidated the opposing roles of the ZIP and CDF Zn^{2+} transporter families and uncovered additional transport systems. The transcription factor, Zap1p, functions as the central Zn^{2+} sensor by regulating genes involved in Zn^{2+} uptake and adaptation to Zn^{2+}-deficiency. The investigation of the role of Zn^{2+} in the regulation of signaling pathways is becoming a primary research direction, and yeast will undoubtedly play a major role in any discoveries in this field as well.

1 Introduction

Cellular organisms are constrained by an absolute requirement for ionic Zn^{2+} (Vallee and Falchuk 1993). The relatively high bioavailability and useful chemical properties of Zn^{2+} allow its extensive use in three general biochemical capacities. Zn^{2+} is primarily used as a structural component of proteins, serving to stabilize a wide variety of architectures. The Lewis acidity of Zn^{2+} also makes it an excellent cofactor for catalysis and many enzymes require Zn^{2+} for full catalytic potential. Finally, Zn^{2+}, like Ca^{2+}, is highly labile and capable of forming transient, yet robust, associations with proteins (Bertini and Luchinat 1994). It is this property that allows zinc to function as a signaling molecule.

The versatility and abundance of Zn^{2+} have made it indispensable. As a consequence, cells must maintain optimal levels of cellular Zn^{2+}, regardless of supply, via a complex process known as homeostasis (Eide 2003). Under conditions of low nutritional Zn^{2+}, cells must ensure that adequate quantities are acquired from the environment. This entails the activation of specific transporters that scavenge Zn^{2+} from the surroundings and transport it across the plasma membrane. Furthermore, the various intracellular uses of Zn^{2+} must be prioritized so that growth can be optimized during periods of limitation. When cells encounter nutritional

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_98 / Published online: 11 August 2005

can be optimized during periods of limitation. When cells encounter nutritional surplus, one general strategy is to exclude excess Zn^{2+} from the interior of the cell by downregulating the plasma membrane transporters. Another strategy involves the continuous acquisition of Zn^{2+} so that it can be stockpiled for leaner times. In the latter case, cells require both a means of storing large quantities of zinc in a manner that does not upset homeostasis and a controlled way to release these stores at the appropriate times.

A proper understanding of Zn^{2+} homeostasis requires the identification of all the players in the game. It is, therefore, beneficial to study an organism for which the most information is known. The yeast, *Saccharomyces cerevisiae*, has proven to be an invaluable model system for this purpose. The genome sequence is complete and decades of research have allowed an in-depth analysis of almost every biochemical system. More importantly, the proteins involved in metal metabolism are remarkably conserved from *S. cerevisiae* to humans. This review will summarize what is known about zinc metabolism in *S. cerevisiae* and the mechanism by which homeostasis is sustained. Appropriate consideration will be given to the discussion of zinc metabolism in other yeast species.

2 Zap1p: The zinc sensor

Any discussion of Zn^{2+} in *S. cerevisiae* should begin with Zap1p (Zinc-regulated Activator Protein). Zap1p is an 880 amino acid transcription factor that functions as the central sensor and regulator of zinc homeostasis (Bird et al. 2003). In response to Zn^{2+}-deficiency, Zap1p becomes active and binds to Zinc Response Elements (ZREs) in the promoters of genes involved in Zn^{2+} uptake. The ZRE is an 11 base pair palindrome that has the consensus sequence ACCTTNAAGGT. Zap1p is comprised of a C-terminal DNA binding domain and two distinct activation domains (AD1 and AD2) that recruit RNA polymerase II to the promoter (Fig. 1). Close homologues of Zap1p are found in fungi alone and only the DNA binding domain is fully conserved. [PSI-BLAST and homology searches were performed on the NCBI website (Altschul et al. 1997) or the Saccharomyces Genome Database (Christie et al. 2004).]

2.1 Regulation of Zap1p activity

Zap1p is constitutively located in the nucleus; therefore, its translocation from the cytosol to the nucleus does not seem to be a primary determinant of its transcriptional activity. In addition, there is no evidence to suggest that Zap1p activity is regulated by any type of posttranslational modification. The current state of understanding is that nuclear localized Zap1p generally binds to ZREs during Zn^{2+}-deficiency, but not during Zn^{2+}-repletion and that a direct interaction with Zn^{2+} is responsible for this phenomenon (Bird et al. 2000).

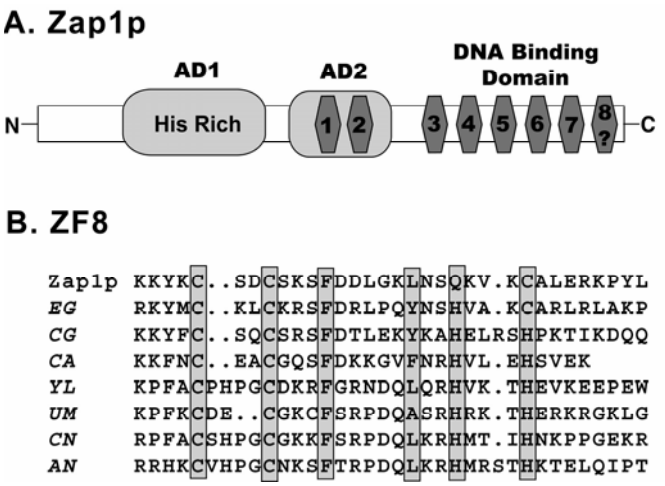


Fig. 1. Structural characteristics of Zap1p. A. The major structural domains of Zap1p. AD = activation domain. Zinc fingers (ZF) are numbered beginning with the most N-terminal finger. B. A multiple sequence alignment of ZF8, including fungal Zap1p homologues in which all eight zinc fingers are conserved. Shaded boxes show conserved residues. *EG, Eremothecium gossypii*; *CG, Candida glabrata*; *CA, Candida albicans*; *YL, Yarrowia lipolytica*; *UM, Ustilago maydis*; *CN, Cryptococcus neoformans*; *AN, Aspergillus nidulans*.

There are at least three direct mechanisms by which Zn^{2+} binding affects the activity of Zap1p. First, the DNA binding domain is Zn^{2+}-regulated (Bird et al. 2003). This domain contains five classical TFIIIA-type zinc fingers (ZF3-ZF7 in Fig. 1A). Each contains one Zn^{2+} ion that facilitates domain folding by coordinating two cysteines and two histidines via their side chain sulfur and nitrogen atoms, respectively. Conserved phenylalanine and leucine residues help form the hydrophobic core of the fingers. Although not strictly conserved in all species, the unequivocal presence of an additional non-canonical zinc finger (ZF8) at the extreme C-terminus of Zap1p is elucidated by alignment with other fungal Zap1p homologues (Fig. 1B). ZF4, ZF5, ZF6, and ZF7 are believed to make direct contact with bases in the ZRE (Evans-Galea et al. 2003). Enigmatically, the structural integrity of these fingers is essential for DNA binding, yet the domain shows decreased DNA binding activity at high Zn^{2+} concentrations. The means by which excess Zn^{2+} decreases the affinity for the ZRE is unknown, however, ZF3 and/or perhaps ZF8 may be involved in this process.

The two other mechanisms of Zap1p regulation involve the repression of activation domain function by Zn^{2+} binding. AD1 is a very large region of the protein rich in histidine residues. This domain is the least conserved across species and almost nothing is known about how Zn^{2+} affects its activity. The working hypothesis is that Zn^{2+} binding to histidine residues alters the conformation of the domain and abrogates its interaction with RNA polymerase. AD2 contains two atypical TFIIIA-like fingers (ZF1 and ZF2) that lack the consensus phenylalanine and leucine residues. In vitro, these fingers have a decreased affinity for Zn^{2+}

when compared with fingers from the DNA binding domain, perhaps due to the loss of the hydrophobic core residues. At higher Zn^{2+} concentrations, the folding of these fingers is postulated to induce a conformational change that results in decreased AD2 activity (Bird et al. 2003).

It is important to note that, on a few promoters, Zap1p remains active even under Zn^{2+}-replete conditions. Two notable examples are the *ZRT2* (Bird et al. 2004) and *ZPS1* (Lamb et al. 2001) genes that encode a low affinity zinc transporter and a metalloprotease-like protein, respectively. Unlike classical Zap1p target genes, *ZRT2* has high Zap1p-dependent expression in Zn^{2+}-replete cells. This elevated expression can be repressed by the addition of excess Zn^{2+}. In the case of *ZPS1*, expression in Zn^{2+}-replete conditions is induced by alkaline pH in a manner that is dependent upon both the Zap1p protein and the pH-responsive transcription factor Rim101p. Zap1p and Rim101p interact in a yeast two-hybrid screen (Uetz et al. 2000), suggesting they may collaborate during the regulation of *ZPS1*. The induction profiles of *ZRT2* and *ZPS1* suggest that the inactivation of Zap1p can be prevented or perhaps shifted to higher Zn^{2+} concentrations by other proteins in the nucleus.

2.2 The Zap1p regulon

Zap1p was first discovered as a positive regulator of both *ZRT1* and *ZRT2,* the genes encoding the high- and low-affinity Zn^{2+} uptake transporters, respectively (Zhao and Eide 1997). Three distinct ZREs can be found in the promoter regions of both of these genes. A ZRE was subsequently identified in the promoter of the *ZAP1* gene as well (Zhao et al. 1998). The autoregulation of *ZAP1* by Zap1p represents a fourth, indirect mechanism by which Zn^{2+} regulates the activity of Zap1p.

DNA microarrays were used to identify all Zap1p-target genes in the yeast genome (Lyons et al. 2000). Global expression changes in response to Zn^{2+}-depletion were monitored in wild type and *zap1*Δ cells. This screen yielded over forty genes whose expression suggested Zap1p-dependent regulation and whose promoter regions contained sequences that resemble the consensus ZRE. There is no reason to believe that the Zap1p regulon defined in these experiments is complete. The inherent errors of DNA microarray analysis notwithstanding, many yeast genes are constitutively repressed under the conditions used for these experiments (i.e. glucose as a carbon source, aerobic culture, etc.) (Courey and Jia 2001). It is possible that, if different culture conditions were used, new Zap1p-target genes would be found. Many of the genes belonging to the Zap1p regulon were either known or expected to be Zap1p targets due to their predicted roles in Zn^{2+} metabolism. The majority of Zap1p target genes, however, encode proteins not directly involved in Zn^{2+} metabolism (Lyons et al. 2000). Some of these genes will be discussed in later sections.

3 Zinc transporters

The main line of defense against ion loss or overaccumulation are membranes. The lipid bilayer presents a formidable barrier to the diffusion of charged molecules. This necessitates the existence of specific transporters that can selectively allow the passage of ions in response to environmental conditions. Several major families of Zn^{2+} transporters have been characterized and extensively reviewed.

3.1 Import into the cytoplasm

The ZIP (Zrt-like, Irt-like Proteins) family of proteins is ubiquitous in biology, indicating a very early origin. ZIP proteins are responsible for transporting Zn^{2+} into the cytoplasm from either outside of the cell or from various internal organelles. The ZIP family is defined by a characteristic topology. Although most members have eight transmembrane domains (TM), some have as few as five. TMs 4 and 5 contain conserved histidines that are predicted to line a channel involved in metal binding and transport. Another conserved region of unknown importance is the cytoplasmic loop between TM3 and TM4 that contains an $(HX)_n$ motif (Fig. 2) (Eide 2004).

S. cerevisiae possess five genes encoding ZIP proteins (Fig. 2). Indeed, the aforementioned Zrt1p and Zrt2p high- and low-affinity plasma membrane Zn^{2+} transporters are the flagship members of this family. The third ZIP protein, Zrt3p, is closely related to the Zn^{2+} uptake transporter, ZupT, from *Escherichia coli* (Grass et al. 2002). Zrt3p is involved in the liberation of Zn^{2+} from vacuolar stores (Section 6) (MacDiarmid et al. 2000). Of the remaining ZIP proteins, only Atx2p has been characterized. Atx2p is thought to reside in the Golgi complex where it may function in the transport of Mn^{2+} from the lumen to the cytoplasm, but its involvement in zinc homeostasis has not been investigated (Lin and Culotta 1996). The last ZIP protein, Yil023cp, although completely uncharacterized, is closely related to the human ZIP4 protein mutated in congenital zinc deficiency (acrodermatitis enteropathica).

Interestingly, a strain lacking both Zrt1p and Zrt2p (*zrt1*Δ*zrt2*Δ) is still viable, indicating that these transporters are not the sole vehicles for Zn^{2+} transport from outside of the cell. Another protein, Fet4p, was found to function as a low affinity Fe^{2+}, Cu^{2+}, and Zn^{2+} uptake transporter (Waters and Eide 2002). Fet4p has an interesting evolutionary history. It is yeast-specific and no closely related proteins can be found in homology searches. More extensive BLAST searches, however, revealed that Fet4p is distantly related to a widely dispersed family of bacterial proteins (COG5478) that have two transmembrane domains. Fet4p is a fusion protein made up of four tandem repeats of the COG5478 motif. The most highly conserved amino acids are two tryptophan residues found within the transmembrane domains (Fig. 2).

Since a *zrt1*Δ*zrt2*Δ*fet4*Δ triple mutant strain is still viable when grown in high Zn^{2+}, other Zn^{2+} uptake mechanisms must exist (Waters and Eide 2002). The current assessment of the phosphate transporter, Pho84p, suggests it too functions as

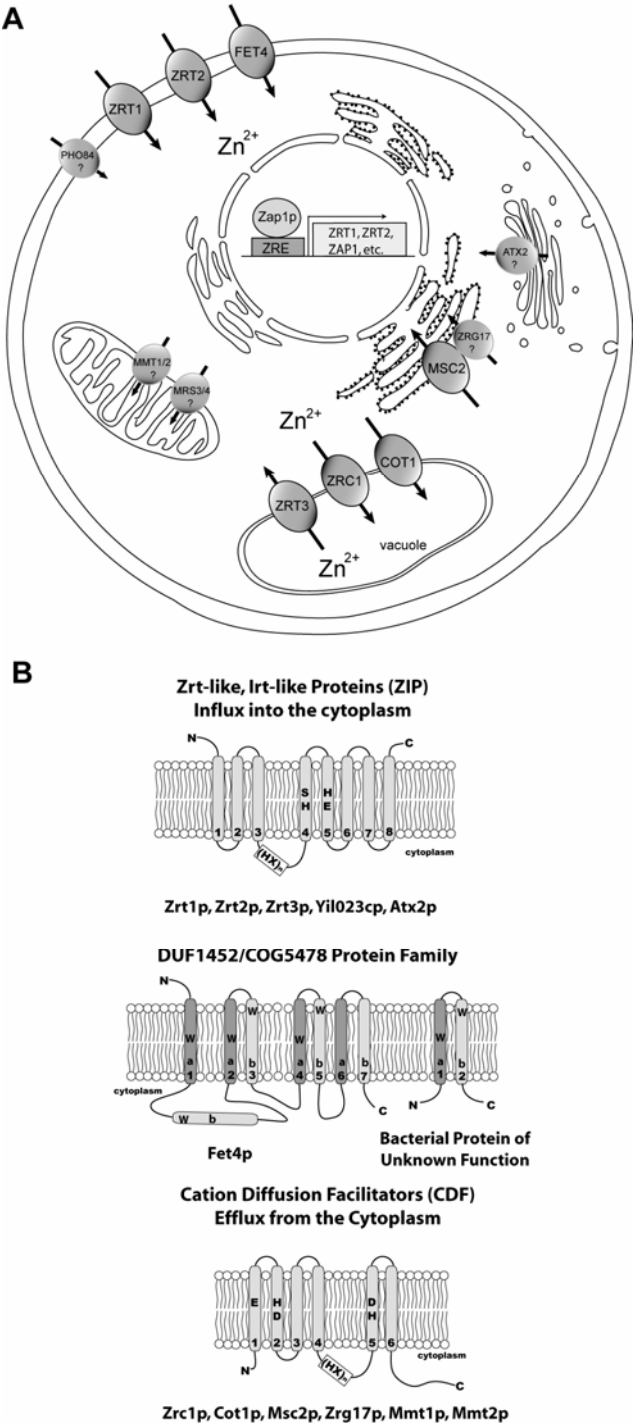

A
FET4
ZRT2
ZRT1
PHO84
?
Zn2+
Zap1p
ZRE
ZRT1, ZRT2,
ZAP1, etc.
ATX2
?
ZRG17
?
MSC2
MMT1/2
?
MRS3/4
?
Zn2+
COT1
ZRC1
ZRT3
vacuole
Zn2+
B
Zrt-like, Irt-like Proteins (ZIP)
Influx into the cytoplasm
N
C
S
H
H
E
1
2
3
4
5
6
7
8
(HX)n
cytoplasm
Zrt1p, Zrt2p, Zrt3p, Yil023cp, Atx2p
DUF1452/COG5478 Protein Family
N
W
a
1
W
a
2
b
3
W
a
4
b
5
a
6
b
7
C
N
C
cytoplasm
W
b
Fet4p
Bacterial Protein of
Unknown Function
Cation Diffusion Facilitators (CDF)
Efflux from the Cytoplasm
E
H
D
D
H
1
2
3
4
5
6
N
(HX)n
cytoplasm
C
Zrc1p, Cot1p, Msc2p, Zrg17p, Mmt1p, Mmt2p

Fig. 2 (overleaf). Zinc transporters in *Saccharomyces cerevisiae*. A. A model of a yeast cell with the predicted locations and directionality of Zn^{2+} transport for known and putative transporters. B. Predicted topology and important structural features of the ZIP, Fet4p, and CDF proteins.

a low affinity transporter of Zn^{2+} and other divalent cations, presumably via metal-phosphate complexes (Jensen et al. 2003).

3.2 Export out of the cytoplasm

It is clear that yeast possess many systems for the transport of Zn^{2+} into the cytoplasm. Once Zn^{2+} builds up in the cytoplasm, however, it must be trafficked across the membranes of various internal organelles to maintain homeostasis. The CDF (Cation Diffusion Facilitator) family of proteins assumes this responsibility. Like the ZIP family, the CDF proteins are ubiquitous. Their predicted topology generally consists of proteins with six transmembrane domains, although some CDF proteins have twelve. As with the ZIP proteins, a long loop region between TM4 and TM5 contains an $(HX)_n$ motif. The highly amphipathic nature of TM1, TM2, TM5, and TM6, along with a preserved intermembrane aspartate residue, suggest a cation transporting channel (Fig. 2) (Palmiter and Huang 2004).

In *S. cerevisiae*, Zrc1p and Cot1p are the best characterized members of the CDF family. Both confer resistance to metals when overexpressed and sensitivity when deleted (Kamizono et al. 1989; Conklin et al. 1992; MacDiarmid et al. 2000). Zrc1p and Cot1p are found on the vacuolar membrane and function to transport Zn^{2+} into this compartment (Section 6) (MacDiarmid et al. 2000). Msc2p is believed to transport Zn^{2+} into the lumen of the endoplasmic reticulum and perhaps an additional organelle involved in the secretory pathway. Protein folding in the ER is impaired in an *msc2*Δ strain, a phenotype which can be rescued by addition of excess Zn^{2+} (Ellis et al. 2004). Msc2p has been shown to physically interact with a fourth CDF protein, Zrg17p. These two proteins function as a complex to transport Zn^{2+} into the secretory pathway (Ellis 2005). The final two members of the CDF family in *S. cerevisiae*, Mmt1p and Mmt2p, are thought to participate in the transport of iron into the mitochondrion (Li and Kaplan 1997). No evidence to date links them to the metabolism of Zn^{2+}.

Mrs3p and Mrs4p comprise another pair of homologous proteins, unrelated to the CDF family, that are thought to transport iron into the mitochondrion. Two lines of evidence suggest these two proteins also play a role in mitochondrial Zn^{2+} uptake. First, *MRS3* expression seems to be regulated by Zap1p, either directly or indirectly (Lyons et al. 2000). Second, mitochondrial Zn^{2+} concentrations in iron-deficient yeast are highest in strains overexpressing Mrs3p or Mrs4p and lowest in strains that lack these transporters (Muhlenhoff et al. 2003).

3.3 Zinc regulation of transporter function

Due to their role in scavenging extracellular or stored Zn^{2+}, it is not surprising that the primary transcriptional regulator of *ZRT1, ZRT2, ZRT3,* and *FET4* is Zap1p (Lyons et al. 2000). The *ZRT2* gene, however, presents an interesting case. Not only does *ZRT2* retain elevated expression in high Zn^{2+} (Section 2.1), at very low Zn^{2+} concentrations, its expression is repressed by Zap1p. This phenomenon is due to the binding of Zap1p to a weak ZRE adjacent to the TATA box, thereby, preventing the recruitment of RNA polymerase. These findings reflect the function of Zrt2p as a low affinity transporter. Since Zrt2p does not function at extremely low Zn^{2+} concentrations, its expression is not needed. During Zn^{2+}-repletion, it may play a role in constitutive zinc uptake (Bird et al. 2004).

In mammalian cells, excess Zn^{2+} is sensed by the MTF-1 transcription factor that induces the expression of proteins which expel Zn^{2+} from the cytoplasm (Andrews 2001). This mechanism is absent in *S. cerevisiae*. The only CDF gene induced by Zn^{2+}-excess is *COT1* and this effect is not direct (Section 7) (Lyons et al. 2004). On the contrary, *ZRC1* and *ZRG17* are induced by Zn^{2+}-deficiency via Zap1p (Lyons et al. 2000). It is possible that essential proteins in the vacuole and ER require Zn^{2+} for function and the upregulation of *ZRC1* and *ZRG17* indicates an increased need for Zn^{2+} transport to these sites. While this may be the case for *ZRG17*, the induction of *ZRC1* during Zn^{2+}-deficiency is more complex and will be discussed in Section 6.

The shift from a nutrient-limiting to nutrient-replete environment is problematic because many nutrients are toxic at high concentrations. Zn^{2+} is no exception (Dineley et al. 2003). Transcriptional changes are unlikely to occur quickly enough for cells to adapt to rapid environmental changes. Therefore, yeast respond to these extreme changes via posttranslational control of Zn^{2+} transporters, particularly Zrt1p. Under conditions of Zn^{2+} limitation, Zrt1p is a stable protein. Upon exposure to high levels of Zn^{2+}, Zrt1p is internalized via ubiquitin-dependent endocytosis. Although, the exact trigger for ubiquitination is poorly understood, the modification is known to occur on lysine 195. After endocytosis, Zrt1p traffics to the vacuole where it is degraded, thereby preventing additional Zn^{2+} uptake (Gitan et al. 1998, 2003). To date, this is the only known posttranslational regulatory mechanism of Zn^{2+} transporters in yeast.

4 The zinc proteome

To gain a complete understanding of zinc homeostasis in yeast, one must first identify all of the genes and gene products involved in the process. Several papers have attempted to define the "zinc proteome" in both *E. coli* and yeast using 2D gel electrophoresis and mass spectrometry (Obata et al. 1996; Zhu et al. 2002). Many new Zn^{2+}-containing proteins have since been identified. There is also a wealth of genomic data that can be mined to identify putative Zn^{2+} proteins using known Zn^{2+}-binding motifs.

4.1 Structural zinc

Since the characterization of the classical TFIIIA-type zinc finger motif, a plethora of distinct structural Zn^{2+}-binding motifs have been discovered and characterized by x-ray crystallographic and nuclear magnetic resonance techniques. In general, structural Zn^{2+} sites have only four tetrahedrally coordinated protein side chain atoms. With rare exceptions, these coordinating atoms are cysteine sulfurs and histidine nitrogens. The most common ligand sets are four cysteines (C_4), three cysteines and one histidine (C_3H) and two cysteines and two histidines (C_2H_2). The characteristics of zinc finger and related domains have been extensively reviewed elsewhere (Grishin 2001; Laity et al. 2001; Matthews and Sunde 2002; Krishna et al. 2003).

The entire yeast proteome can be scanned for proteins that contain known Zn^{2+} binding motifs. A summary of such a search, including Zn^{2+}-binding proteins discovered by other means, is shown in Table 1. Proteins are listed by structural motif or functional classification. As can be seen, there are hundreds of proteins with known Zn^{2+}-binding motifs. Due to the prevalence of CxxC motifs (where x = any amino acid) in structural Zn^{2+} sites, proteins that have closely spaced CxxC pairs (leading to a C_4 ligand set) are also predicted to bind Zn^{2+}. If these putative Zn^{2+}-binding proteins were added to Table 1, they would place the number of proteins that require structural Zn^{2+} at approximately four hundred. Since glutamate and aspartate can occasionally replace cysteines and histidines in Zn^{2+}-binding motifs, it is likely that the proteome search performed for this review missed many bona fide Zn^{2+}-binding proteins. Moreover, novel Zn^{2+}-binding motifs are being discovered at a regular pace and it is likely that Table 1 is far from complete. Approximately 6-7% of the yeast proteome (depending on varying estimates for its size) require zinc for structural integrity. Based on this fact, zinc can be thought of as an essential building block for proteins.

4.2 Catalytic zinc

Several excellent reviews have considered the role of Zn^{2+} as a catalytic cofactor (Coleman 1992, 1998; Parkin 2004). While hundreds of enzymes are known to utilize Zn^{2+}, most can be categorized into two basic groups, both of which use the positive charge of zinc to stabilize negative charges on substrates. The first class of enzymes uses Zn^{2+} to coordinate the oxygen or sulfur atoms in water molecules, alcohols or thiols. Coordination to Zn^{2+} polarizes the O-H or S-H bond, making the proton more acidic and allowing its abstraction by a basic amino acid side chain. The hydroxide, alkoxide or thiolate generated can then act as a nucleophile in catalysis. The second class of enzymes utilizes Zn^{2+} as an electron-withdrawing group to polarize carbonyls. This makes the carbon atom more electrophilic, thereby, stabilizing enolates or making carbonyls more amenable to nucleophilic attack. Still other enzymes, such as α-1,2-mannosidase, are thought to use zinc for substrate recognition (van den Elsen et al. 2001). Table 2 lists all of the proteins in yeast that are known or suspected to contain tightly bound catalytic Zn^{2+}.

Table 1. Compendium of yeast proteins with structural zinc sites

Structural Class/Function	Proteins
Single zinc binding domains	
Classical TFIIIA tandem zinc fingers (C_2H_2)	Ace2, Adr1, Azf1, Crz1, Fzf1, Gis1, Lpz12, Lpz14, Nrg1, Nrg2, Map1, Met31, Met32, Mig1, Mig2, Mig3, Mot3, Msn2, Msn4, Mub1, Pzf1, Rgm1, Rim101, Rme1, Rph1, Rpn4, Set5, Sfp1, Stp1, Stp2, Stp3, Stp4, Swi5, Usv1, Zap1, Zms1, Yer130c, Ygr067c, Yml081c, Ypr022c
U1-like zinc fingers (C_2H_2)	Bud20, Dbf4, Jjj1, Luc7, Prp9, Prp6, Prp11, Reh1, Rei1, Rts2, Sad1, Snu23, Spt10, Yhc1, Yod1, Ydr049w
TFIIIA-like (C_2H_2)	Abf1, Eco1, Luc7, Pcf11, Sas2, Sas3
GATA-type zinc finger (C_4)	Ash1, Dal80, Gat1, Gat2, Gat3, Gat4, Gln3, Gzf3, Rad16, Srd1, Srd2
ACE1 structural zinc (C_3H)	Ace1, Haa1, Mac1
Viral-type zinc knuckle (C_2HC)	Air1, Air2, Atg14, Bik1, Gis2, Itt1, Mpe1, Msl5, Slu7, Ykr017c, Yol029c
TIS11 RNA binding finger (C_3H)	Cth1, Dus3, Lee1, Nab2, Tis11, Yth1, Yor091w
Rad50 zinc hook (C_4)	Rad50
PKC1-like fold/ARF GAP (C_4)	Age1, Age2, Gcs1, Glo3, Gts1, Sps18
MOB1 four helix bundle (C_2H_2)	Mob1
NEW1/DHHC (predicted)	Akr1, Akr2, Erf2, Swf1, Ydr459c, Ynl155w, Ynl326c, Yol003c
Class II histone deacetylase(C_4)	Hst1, Hst2, Hst3, Hst4, Sir2
DnaJ/CSL zinc finger (C_4)	Apj1, Dph3, Hua1, Jjj1, Mdj1, Nob1, Scj1, Xdj1, Ydj1
Ubiquitin interacting zinc fingers	
HIT znf-UBP (C_4)	Bcd1, Hit1, Plb1, Plb2, Plb3, Spo1, Ubp8, Ubp14, Vps71, Yhl010c
RBZ/NFZ (C_4)	Npl4, Nrp1, Ubp14, Vps36
E1 protein zinc finger (C_4)	Atg7, Uba2, Uba3, Uba4
Deubiquitinase finger(C_4)	Ubp1, Ubp4, Ubp7, Ubp8, Ubp9, Ubp10, Ubp11, Ubp13, Ubp14, Ubp16
Sec23/24 zinc finger (C_4)	Sec23, Sec24, Sfb2, Sfb3, Yhr035w
ZPR1 finger	Zpr1
tRNA binding proteins (C_4)	Ism1p, Mes1p, Nam2p, Trm1
DNA replication machinery (C_4)	Mcm2, Mcm6, Mcm7, Mcm10, Pol1, Pol2, Pol3, Rev3, Rfa1
RNA polymerase complex (at least 8 zinc/complex)	Brf1, Dst1, Rpa9, Rpa135, Rpa190, Rpb1, Rpb2, Rpb3, Rpb9, Rpb10, Rpc2, Rpc10, Rpc11, Rpo31, Spt4, Sua7, Tfa1, Tfb4
Ribosome associated proteins	Mrpl32, Rps26, Rps27, Rps29, Rpl34, Rpl37, Rpl43, Tif5, Sui3
TIM22 complex	Tim8, Tim9, Tim10, Tim12, Tim13

Multinuclear zinc binding domains	
PKC1	Pkc1
FYVE	Fab1, Pep7, Pib1, Pib2, Vps27
ZZ domain	Ada2, Rsc8
RING finger and related motifs	Asr1, Apc11, Asi1, Asi3, Asr1, Bre1, Cst9, Cwc24, Dma1, Dma2, Far1, Hex3, Hrd1, Hrt1, Hul4, Itt1, Mag2, Nfi1, Pep3, Pep5, Pex2, Pex10, Pex12, Pib1, Psh1, Rad5, Rad16, Rad18, Ris1, San1, Sig1, Siz1, Slx1, Slx8, Ssm4, Ste5, Tfb3, Tul1, Ubr1, Ubr2, Vps8, Ybr062c, Ydr128w, Ydr266c, Yhl010c, Ykr017c, Ylr247c, Ymr187c, Ymr247c, Yol138c
PHD	Asr1, Bye1, Cti6, Ecm5, Hop1, Ioc2, Nse1, Nto1, Pho23, Rco1, Rds3, Set2, Set3, Set4, Snt2, Spp1, Yng1, Yng2, Yer051w, Yjr119c
LIM domain/RHO GAP	Lrg1, Pxl1, Rga1, Rga2
Binuclear zinc clusters (Zn_2Cys_6)	Arg80, Aro81, Cat8, Cep3, Cha4, Dal81, Ecm22, Eds1, Gal4, Hal9, Hap1, Leu3, Lys14, Mal13/2/23/33/83, Oaf1, Pdr1, Pdr3, Pdr8, Pip2, Ppr1, Put3, Rdr1, Rds1, Rds2, Rgt1, Rsc3, Rsc30, Sef1, Sip4, Stb4, Stb5, Sut1, Sut2, Tbs1, Tea1, Thi2, Uga3, Ume6, Upc2, War1, Yrm1, Yrr1, Ybr239c, Ydr520c, Yer184c, Yfl052w, Yil130w, Yjl103c, Yjl206c, Ykl222c, Ykr064w, Yll054c, Ylr278c, Ynr063w
Multinuclear metalloenzymes with known and potential structural zinc sites	
PPP family phosphatases	Cmp2, Cna1, Glc7, Ppg1, Pph3, Pph21, Pph22, Ppq1, Ppt1, Ppz1, Ppz2, Sit4
Pseudouridine synthase	Deg1, Pus1, Pus2
Alcohol dehydrogenases	Adh1, Adh2, Adh3, Adh5, Adh6, Adh7, Bdh1, Sfa1, Yalo61w
Miscellaneous	Car1, Cox4, Sod1

Many more enzymes can be activated by Zn^{2+} in vitro, however, the functional cation in vivo is not known. Such enzymes, known as Zn^{2+}-activated enzymes, may be far more numerous than is currently recognized. One example of this type of enzyme is enolase, which is highly active in the presence of Zn^{2+} in vitro. The pI of enolase has been shown to change when cells are grown under Zn^{2+}-deficient conditions, suggesting that Zn^{2+} is the functional cofactor in vivo as well. Based on Table 2, it is estimated that at least 100 enzymes, or 1-2% of the yeast proteome, require or can utilize Zn^{2+} for catalysis.

5 Prioritizing zinc

Estimates for the concentration of Zn^{2+} inside eukaryotic cells are remarkably consistent from species to species. In yeast, this value is approximately 180 μM (Lyons and Eide, unpublished data). Back-of-the-envelope calculations based on several different studies suggest that a yeast cell grown in normal media (Zn^{2+}-

Table 2. Compendium of yeast proteins that use zinc in a catalytic capacity

Functional Class	Proteins
Nucleophile stabilization by -X-H bond polarization	**Zn^{2+} + X-H ------> Zn^{2+}-X^- + H^+**
Alcohol dehydrogenases (R-O-H)	Adh1, Adh2, Adh3, Adh5, Adh6, Adh7, Bdh1, Sfa1, Sor1, Sor2, Xyl2, Yal061w
Prokaryotic-type carbonic anhydrase (H-O-H)	Nce103
Hydrolases (H-O-H)	
AlkP (alkaline phosphatase) superfamily: bi-nuclear metallohydrolase	Gpi13, Las21, Mcd4, Pho8, Ycr026c, Yel016c
Type I cyclic nucleotide phosphodiesterase	Pde2
Trinuclear zinc phosphodiesterase	Apn1
HIT family diadenosine polyphosphatase hydrolases	Apa1, Apa2, Hnt1, Hnt2
β-lactamase fold: binuclear zinc site	
Phosphodiesterases	Pde1, Pso2, Trz1, Ysh1
Glyoxalases I and II	Glo2, Glo4
Class I histone deacetylases	Hda1, Hos1, Hos2, Hos3, Rpd3
Cytosine deaminase fold	
Nucleic acid/riboflavin deaminase	Amd1, Cdd1, Dcd1, Fcy1, Gud1, Rib2, Tad1, Tad2, Tad3
Cyclic imidohydrolases/dihydropyrimidase family	Dal1, Ura4, Yjl213w
Jab1/MPN proteasomal metalloprotease	Ron8, Rpn11, Rri1
MH clan binuclear zinc metalloproteases ($HxDx_nDx_nEEx_nDx_nH$)	Ape3, Cps1, Lap4, Vps70, Ybr074w, Ybr281c, Ydr415c, Yfr044c, Yhr113w, Yol153c
MA clan zinc metalloproteases ($HExxHx_nE$)	Aap1, Afg3, Ape2, Lta1, Oct1, Prd1, Rca1, Yme1, Zps1, Yil137c, Ynr020c
MC clan metalloprotease ($HxxEx_nHx_nE$)	Ecm14
ME clan metalloprotease ($HxxEHx_nEx_nE$)	Axl1, Cym1, Mas1, Mas2, Ste23, Yol098c
MG clan binuclear metalloprotease ($Dx_nDx_nHx_nEx_nE$)	Map1, Map2
Integral membrane proteases (HExxH)	Oma1, Ste24
Thiol Activation (R-S-H)	
Methionine synthases	Met6, Mht1, Sam4
Prenyltransferases	Bet4, Ram2
Methionine sulfoxide reductase (potential)	MsrB
Disulfide isomerases (potential)	Eug1, Mpd1, Mpd2, Pdi1
Electrophile stabilization by R=O bond polarization	**Zn^{2+} + O=C-R ------> Zn^{2+}-$^{\partial-}$O-$C^{\partial+}$-R**
Alcohol dehydrogenase (reverse rxn, carbonyl activation)	See above
Aldol cleavage/condensation (enolate stabilization)	
Type II aldolase	Fba1
DAHP synthase	Aro3, Aro4
5-aminolevulinate dehydratase/PBGS	Hem2

HMGL fold	
Homocitrate synthase	Lys20, Lys21
Isopropylmalate synthase	Leu4, Yor108w
Pyruvate carboxylase	Pyc1, Pyc2
Phosphomannose isomerase (enediolate stabilization)	Pmi40
Substrate recognition	
Alpha-1,2-mannosidase	Ams1

replete) contains roughly 6-8 million Zn^{2+} atoms (Korhola and Edelmann 1986; Obata et al. 1996; Lyons and Eide, unpublished data). When grown in normal media, however, yeast stop growing due to glucose-depletion before Zn^{2+} becomes limiting. Therefore, much of the Zn^{2+} content may result from the continuous uptake and storage of Zn^{2+} by cells that have entered stationary phase. In support of this hypothesis, the Zn^{2+} content in yeast significantly drops when Zn^{2+} is the limiting nutrient. Under these conditions, estimates for intracellular Zn^{2+} range from 600,000 to 3 million atoms per cell (Obata et al. 1996; Lyons and Eide, unpublished data). This value, albeit crude, can be thought of as the minimum cellular Zn^{2+} requirement for cells that have undergone growth arrest due to lack of Zn^{2+}.

Although Zn^{2+} is clearly abundant inside of cells, Table 1 and 2 show that the proteins requiring this metal for proper function are as well. It is therefore important for yeast to prioritize the uses of Zn^{2+} so the most important functions are retained during Zn^{2+}-limitation. To complicate matters, most intracellular Zn^{2+} seems to be tightly bound to a variety of intracellular ligands. 'Free Zn^{2+}', or the amount of Zn^{2+} that remains unchelated inside of a cell is predicted to be quite low (Finney and O'Halloran 2003). Work done in *E. coli* estimates the amount of 'free Zn^{2+}' to be less than one atom per cell (Outten and O'Halloran 2001).

How then does the cell distribute the infinitesimal amount of 'free Zn^{2+}' to the appropriate sites? The answer may lie in the lability of Zn^{2+}. The relatively fast ligand exchange rate of Zn^{2+} makes it likely to associate and dissociate quickly from solvent accessible sites. Thus, when an ample supply exists, Zn^{2+} may diffuse rapidly throughout the cell without ever being 'free' for very long. Small molecules such as glutathione may also mediate the fast exchange of Zn^{2+} from site to site (Mason et al. 2004).

As Zn^{2+} is depleted from the cytoplasm, those sites with the lowest binding affinity or that exchange the fastest are likely to lose Zn^{2+} more rapidly. It is possible that natural selection has tuned the K_d and solvent accessibility of the numerous Zn^{2+}-binding sites so that the dispensable functions of Zn^{2+} are lost first. Since structural Zn^{2+} sites have generally high Zn^{2+}-binding affinities (Cox and McLendon 2000) and low solvent accessibilities (Auld 2001), they are probably the last to lose Zn^{2+}. The fact that the DNA binding zinc fingers of Zap1p retain their function even when cells have stopped growing due to Zn^{2+}-limitation supports this conclusion (Lyons et al. 2000). The likelihood is minimal that cells would continue to grow if Zn^{2+}-deficiency had advanced to the point of depleting structural Zn^{2+} sites.

5.1 Zinc chaperones

Another possibility for the distribution of Zn^{2+} involves the existence of specific proteins that escort Zn^{2+} to essential sites, regardless of their physical properties. In the case of copper homeostasis, proteins known as copper chaperones are responsible for directional transcytoplasmic trafficking. For example, the yeast copper chaperone, Ccs1p, is specifically required for the delivery of copper to superoxide dismutase (Elam et al. 2002).

It is important to note that, with the exception of Atx1p, which delivers copper to the entire secretory pathway, all known copper chaperones have specific copper protein targets. This is not surprising since the copper proteome is quite small, consisting of no more than a handful of proteins. It is not a burden for cells to carry genes for both the copper protein and the accessory copper chaperone. On the other hand, the enormity of the zinc proteome makes it unlikely that each zinc protein possesses a cognate zinc chaperone. Although, it is possible that classes of zinc proteins, such as the Zn^{2+}-dependent alcohol dehydrogenases, may have chaperones that serve all the members of the class. To date, however, no zinc chaperone has been identified in eukaryotes.

5.2 Remodeling

The yeast cell is a complex mixture of proteins competing for limited supplies of Zn^{2+}. In the absence of zinc chaperones or some other type of active partitioning, kinetics and thermodynamics determine the fate of Zn^{2+} as nutritional supplies dwindle. Therefore, it may become necessary for the cell to remodel the cellular protein profile to ensure the reallocation of Zn^{2+} to essential sites. If a Zn^{2+}-containing protein is abundant and dispensable, cells may downregulate its expression in response to Zn^{2+}-deficiency, thus, releasing much needed Zn^{2+} for other, more important, uses.

An example of this phenomenon can be seen with the major isoform of Zn^{2+}-containing alcohol dehydrogenase (Adh1p). Based on crude estimates of approximately 250,000 monomers per cell, Adh1p can be considered to be very abundant in yeast cells (Racker 1950). Since each monomer contains two Zn^{2+} ions, Adh1p would consume an enormous percentage of the cellular supply if expressed under conditions of Zn^{2+}-limitation. Part of this problem is solved by thermodynamics, since Adh1p purified from Zn^{2+}-limited yeast is both less active and Zn^{2+}-deficient (Dickenson and Dickinson 1976). Clearly, Adh1p is unable to compete with the myriad of other Zn^{2+}-chelators. Yeast also address this problem by placing the expression of the iron-dependent alcohol dehydrogenase isozyme, Adh4p, under the control of Zap1p, thereby, eliminating the need for Zn^{2+} to perform the dehydrogenase function (Lyons et al. 2000). Lastly, it appears that Zap1p, either directly or indirectly, represses the expression of the *ADH1* gene (Lyons et al. 2000). This remodeling allows yeast to conserve important Zn^{2+}-dependent functions at the expense of Adh1p activity.

Other potential examples of this type of remodeling have recently come to light. In *E. coli* and *B. subtilis*, several non-Zn^{2+}-dependent ribosomal proteins are specifically induced by Zn^{2+}-deficiency, ostensibly to replace Zn^{2+}-binding subunits that can no longer function due to loss of Zn^{2+} (Panina et al. 2003; Nanamiya et al. 2004). An alternative interpretation is that the replacement of ribosomal proteins that require Zn^{2+} with ones that do not is a matter of economy. Ribosomes are numerous. Ergo, ribosomes that require less Zn^{2+} are beneficial during Zn^{2+}-deficiency because they consume less of a limiting resource.

In yeast, a similar situation may exist. Yeast ribosomes are predicted to contain at least six proteins with predicted structural Zn^{2+}-binding sites (Rivlin et al. 1999). Since a vegetative yeast cell is estimated to contain 200,000 ribosomes (Warner 1999), they probably represent the largest pool of Zn^{2+} in the cytoplasm. Zn^{2+}-deficient yeast show repressed expression of over a hundred genes encoding ribosomal proteins. This is not surprising since the repression of ribosomal genes in yeast seems to be a generalized response to stress. Unexpectedly, ribosomal genes showed lower expression in wild type cells than in *zap1*Δ cells when both were grown under Zn^{2+}-deficiency, suggesting a role for Zap1p in the repression of ribosomal gene expression (Lyons et al. 2000).

6 Zinc storage and detoxification

Part of homeostasis is the evolution of mechanisms by which excess nutrients are managed. If accumulated in the wrong location, Zn^{2+} is an effective cellular poison. Although the exact mechanism(s) by which Zn^{2+} exerts its toxic effect(s) are not known, Zn^{2+} may replace other cations in non-Zn^{2+}-dependent enzymes, thereby inactivating them. For example, Zn^{2+} is known to compete with iron for insertion into porphyrin by ferrochelatase (Labbe et al. 1999). Zn^{2+} is also capable of acting as an inhibitor by binding to adventitious sites on enzymes, a mechanism believed to explain its inhibition of mitochondrial function (Link and von Jagow 1995). Whatever the mechanism of toxicity, Zn^{2+} cannot be allowed to hyperaccumulate in the cytoplasm.

6.1 The vacuole

With respect to zinc metabolism, the primary function of the yeast vacuole is storage and detoxification. When Zn^{2+} is plentiful, Zrc1p transports Zn^{2+} from the cytoplasm into the vacuolar compartment. Transport is thought to proceed via secondary active transport driven by the proton gradient (MacDiarmid et al. 2002). Zn^{2+} may also traffic to the vacuole by other indirect pathways, perhaps from the ER or Golgi via secretory vesicles or from the plasma membrane via endosomes. Yeast grown in excess zinc are capable of accumulating large quantities of vacuolar Zn^{2+}, over 80 million atoms/cell (Obata et al. 1996). The speciation of stored Zn^{2+} inside the vacuole is unknown, although it is predicted to form a complex

with polyphosphate. When external supplies diminish, these stores are released primarily by Zrt3p (MacDiarmid et al. 2000).

Although its sole purpose is Zn^{2+} detoxification, Zrc1p is highly expressed in Zn^{2+}-limited cells via Zap1p. On the surface, it makes little sense why a protein involved in Zn^{2+}-detoxification would be turned on by low Zn^{2+}-bioavailability. The answer to this mystery lies in the cell's inherent proactive defense against zinc shock, a condition brought about by the induction of Zrt1p during Zn^{2+}-deficiency. If cells expressing Zrt1p are exposed to large quantities of extracellular Zn^{2+}, Zrt1p is endocytosed and inactivated. However, the endocytic process is not fast enough to prevent the rapid influx and temporary cytoplasmic accumulation of Zn^{2+}. Therefore, Zrc1p is induced as a preventative measure, thus, allowing the vacuole to absorb the excess Zn^{2+} before it can exert its toxic effects in the cytoplasm. *zrc1*Δ cells are exceptionally sensitive to a shift from Zn^{2+}-depleted conditions to media containing even small amounts of Zn^{2+}. This phenotype can be rescued by the concomitant deletion of the *ZRT1* gene (MacDiarmid et al. 2003).

6.2 Metallothionein

Many organisms express small cysteine-rich proteins, called metallothioneins, that function as cytoplasmic stores for Zn^{2+}. Mammalian metallothioneins are induced by the MTF-1 transcription factor in response to elevated Zn^{2+} (Andrews 2001). Similar systems can be found in lower eukaryotes and cyanobacteria (Robinson et al. 2001). *Saccharomyces cerevisiae* does have genes encoding metallothioneins, however, their gene products are involved in the detoxification of copper and are not Zn^{2+}-regulated (Pena et al. 1998). The fact that *S. cerevisiae* has co-opted metallothionein function for copper homeostasis may reflect its unique evolutionary history resulting from domestication. The distantly related fission yeast, *Schizosaccharomyces pombe*, does have a Zn^{2+}-inducible system of metal tolerance that includes a Zn^{2+}-binding metallothionein, Zym1 (Borrelly et al. 2002). Taking this into account, *S. pombe* may represent a much better model system for understanding eukaryotic zinc homeostasis than *S. cerevisiae*.

7 Zinc signals and other regulators of zinc homeostasis

There exists the possibility that Zn^{2+} is not the only direct regulator of Zap1p, as is demonstrated by the influence of Rim101p in Zn^{2+}-replete conditions (Lamb et al. 2001). In addition, Zap1p is not the only transcription factor that regulates the expression of Zn^{2+} transporters. For example, *ZRT1* is also regulated by the cell cycle (Cho et al. 1998), nitrogen metabolism (Cox et al. 1999) and the Rpd3p histone deacetylase (Bernstein et al. 2000). It is exciting to speculate that the regulation of proteins and genes involved in zinc metabolism by other biochemical systems points toward a larger role for Zn^{2+} as a signaling molecule.

The prospect that Zn^{2+}, like Ca^{2+}, acts as a second messenger has intrigued researchers in the field for quite some time. Like Ca^{2+}, Zn^{2+} is highly labile, redox inert and fairly promiscuous regarding the ligand sets and geometries it will accept (Vallee and Falchuk 1993; Bertini and Luchinat 1994). These properties allow Zn^{2+} to transmit signals inside cells either by modulating the activity of Zn^{2+}-activated proteins or by binding to structural Zn^{2+} sites. Indeed, Zn^{2+} has many pharmacological effects in eukaryotic cells, including the ability to alter signaling pathways (Korichneva et al. 2002; Min et al. 2003).

Some mammalian tissues accumulate high levels of Zn^{2+} in secretory vesicles (Palmiter et al. 1996). Ample evidence suggests that, in some cases, this pool of Zn^{2+} functions in signaling (Cuajungco and Lees 1997). Localization of Zn^{2+} with fluorescent dyes has also indicated the existence of distinct vesicular compartments, called 'zincosomes', filled with 'labile Zn^{2+}' (Beyersmann and Haase 2001). It remains to be seen whether or not these zincosomes are real and function in zinc homeostasis or are merely artifactual. Zincosomes that transiently accumulate Zn^{2+} in response to influx have been visualized in yeast (Devirgiliis et al. 2004). Since this pool of Zn^{2+} is considered 'labile', it is tempting to postulate a role in zinc signals, although this hypothesis remains to be tested.

Whether or not Zn^{2+} acts as a direct messenger, it is clear that the expression of hundreds of genes show altered expression in response to perturbations in zinc homeostasis. Zn^{2+}-deficiency, in particular, has a profound effect on transcription (Lyons et al. 2000). The majority of transcriptional alterations caused by deficiency are part of a generalized environmental stress response. Other changes, such as the induction of the Unfolded Protein Response, are specifically caused by the loss of Zn^{2+} in critical sites (Ellis et al. 2004). The putative Zap1p regulon, however, includes a variety of proteins that have no apparent role in zinc homeostasis. *NRG2*, for example, encodes a transcriptional repressor involved in the regulation of glucose metabolism. *DPP1* encodes diacylglycerol pyrophosphate (DAGPP) phosphatase, an enzyme involved in the generation of lipid molecules that may act as second messengers (Han et al. 2001).

By comparison, the perturbation of zinc homeostasis by excess Zn^{2+} has far fewer transcriptional consequences (Lyons et al. 2004). DNA microarrays revealed that Zn^{2+}-toxicity resulted in the induction of only two interconnected transcriptional regulons: the Aft1p iron-responsive regulon and the Mga2p hypoxia-responsive regulon. The CDF protein, Cot1p, is slightly induced under these conditions as part of the Aft1p regulon. It is likely that Zn^{2+} affects mitochondrial iron metabolism which, in turn, affects the hypoxia regulon.

8 Conclusions

Saccharomyces cerevisiae affords the unique opportunity to characterize the process of zinc homeostasis in its entirety. Much is known, yet much more remains to be discovered. Several ZIP and CDF proteins remain uncharacterized. In addition, improved structural data is needed to elucidate the chemical mechanisms by which

Zn^{2+} is transported or sensed. New avenues of research will include the complete characterization of the zinc proteome and further studies on the transcriptional effects of imbalances in zinc homeostasis. New Zn^{2+}-specific fluorescent probes will be invaluable in defining the role of Zn^{2+} as a signaling molecule. When appropriate, extrapolation of the lessons learned from yeast will yield a better understanding of zinc metabolism in humans.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402

Andrews GK (2001) Cellular zinc sensors: MTF-1 regulation of gene expression. Biometals 14:223-237

Auld DS (2001) Zinc sites in metalloenzymes and related proteins. In: I. Bertini, A. Sigel and H. Sigel, Editors, *Handbook on Metalloproteins*, Marcell Dekker, New York, pp. 881–959

Bernstein BE, Tong JK, Schreiber SL (2000) Genomewide studies of histone deacetylase function in yeast. Proc Natl Acad Sci USA 97:13708-13713

Bertini I, Luchinat C (1994) The reaction pathways of zinc enzymes and related biological catalysts. In: Bertini I, Gray H, Lippard S, Valentine JS (eds) Bioinorganic Chemistry. University Science Books, Mill Valley, CA, pp 37-106

Beyersmann D, Haase H (2001) Functions of zinc in signaling, proliferation and differentiation of mammalian cells. Biometals 14:331-341

Bird AJ, Blankman E, Stillman DJ, Eide DJ, Winge DR (2004) The Zap1 transcriptional activator also acts as a repressor by binding downstream of the TATA box in ZRT2. EMBO J 23:1123-1132

Bird AJ, McCall K, Kramer M, Blankman E, Winge DR, Eide DJ (2003) Zinc fingers can act as Zn2+ sensors to regulate transcriptional activation domain function. EMBO J 22:5137-5146

Bird AJ, Zhao H, Luo H, Jensen LT, Srinivasan C, Evans-Galea M, Winge DR, Eide DJ (2000) A dual role for zinc fingers in both DNA binding and zinc sensing by the Zap1 transcriptional activator. EMBO J 19:3704-3713

Borrelly GP, Harrison MD, Robinson AK, Cox SG, Robinson NJ, Whitehall SK (2002) Surplus zinc is handled by Zym1 metallothionein and Zhf endoplasmic reticulum transporter in *Schizosaccharomyces pombe*. J Biol Chem 277:30394-30400

Cho RJ, Campbell MJ, Winzeler EA, Steinmetz L, Conway A, Wodicka L, Wolfsberg TG, Gabrielian AE, Landsman D, Lockhart DJ, Davis RW (1998) A genome-wide transcriptional analysis of the mitotic cell cycle. Mol Cell 2:65-73

Christie KR, Weng S, Balakrishnan R, Costanzo MC, Dolinski K, Dwight SS, Engel SR, Feierbach B, Fisk DG, Hirschman JE, Hong EL, Issel-Tarver L, Nash R, Sethuraman A, Starr B, Theesfeld CL, Andrada R, Binkley G, Dong Q, Lane C, Schroeder M, Botstein D, Cherry JM (2004) Saccharomyces Genome Database (SGD) provides tools to identify and analyze sequences from *Saccharomyces cerevisiae* and related sequences from other organisms. Nucleic Acids Res 32 Database issue:D311-314

Coleman JE (1992) Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu Rev Biochem 61:897-946

Coleman JE (1998) Zinc enzymes. Curr Opin Chem Biol 2:222-234

Conklin DS, McMaster JA, Culbertson MR, Kung C (1992) COT1, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. Mol Cell Biol 12:3678-3688

Courey AJ, Jia S (2001) Transcriptional repression: the long and the short of it. Genes Dev 15:2786-2796

Cox EH, McLendon GL (2000) Zinc-dependent protein folding. Curr Opin Chem Biol 4(2):162-5

Cox KH, Pinchak AB, Cooper TG (1999) Genome-wide transcriptional analysis in *S. cerevisiae* by mini-array membrane hybridization. Yeast 15:703-713

Cuajungco MP, Lees GJ (1997) Zinc metabolism in the brain: relevance to human neurodegenerative disorders. Neurobiol Dis 4:137-169

Devirgiliis C, Murgia C, Danscher G, Perozzi G (2004) Exchangeable zinc ions transiently accumulate in a vesicular compartment in the yeast *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 323:58-64

Dickenson CJ, Dickinson FM (1976) Some properties of an alcohol dehydrogenase partially purified from baker's yeast grown without added zinc. Biochem J 153:309-319

Dineley KE, Votyakova TV, Reynolds IJ (2003) Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. J Neurochem 85:563-570

Eide DJ (2003) Multiple regulatory mechanisms maintain zinc homeostasis in *Saccharomyces cerevisiae*. J Nutr 133:1532S-1535S

Eide DJ (2004) The SLC39 family of metal ion transporters. Pflugers Arch 447:796-800

Elam JS, Thomas ST, Holloway SP, Taylor AB, Hart PJ (2002) Copper chaperones. Adv Protein Chem 60:151-219

Ellis CD, Wang F, MacDiarmid CW, Clark S, Lyons T, Eide DJ (2004) Zinc and the Msc2 zinc transporter protein are required for endoplasmic reticulum function. J Cell Biol 166:325-335

Ellis CD, MacDiarmid CW and Eide DJ (2005) Heteromeric protein complexes mediate zinc transport into the secretory pathway of eukaryotic cells. J Biol Chem, in press

Evans-Galea MV, Blankman E, Myszka DG, Bird AJ, Eide DJ, Winge DR (2003) Two of the five zinc fingers in the Zap1 transcription factor DNA binding domain dominate site-specific DNA binding. Biochemistry 42:1053-1061

Finney LA, O'Halloran TV (2003) Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300:931-936

Gitan RS, Luo H, Rodgers J, Broderius M, Eide D (1998) Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. J Biol Chem 273:28617-28624

Gitan RS, Shababi M, Kramer M, Eide DJ (2003) A cytosolic domain of the yeast Zrt1 zinc transporter is required for its post-translational inactivation in response to zinc and cadmium. J Biol Chem 278:39558-39564

Grass G, Wong MD, Rosen BP, Smith RL, Rensing C (2002) ZupT is a Zn(II) uptake system in *Escherichia coli*. J Bacteriol 184:864-866

Grishin NV (2001) Treble clef finger--a functionally diverse zinc-binding structural motif. Nucleic Acids Res 29:1703-1714

Han GS, Johnston CN, Chen X, Athenstaedt K, Daum G, Carman GM (2001) Regulation of the *Saccharomyces cerevisiae* DPP1-encoded diacylglycerol pyrophosphate phosphatase by zinc. J Biol Chem 276:10126-10133

Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc Natl Acad Sci USA 98:4569-4574

Jensen LT, Ajua-Alemanji M, Culotta VC (2003) The *Saccharomyces cerevisiae* high affinity phosphate transporter encoded by PHO84 also functions in manganese homeostasis. J Biol Chem 278:42036-42040

Kamizono A, Nishizawa M, Teranishi Y, Murata K, Kimura A (1989) Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. Mol Gen Genet 219:161-167

Korhola M, Edelmann K (1986) Yeast preparations enriched with trace elements. Acta Pharmacol Toxicol (Copenh) 59 Suppl 7:148-151

Korichneva I, Hoyos B, Chua R, Levi E, Hammerling U (2002) Zinc release from protein kinase C as the common event during activation by lipid second messenger or reactive oxygen. J Biol Chem 277:44327-44331

Krishna SS, Majumdar I, Grishin NV (2003) Structural classification of zinc fingers: survey and summary. Nucleic Acids Res 31:532-550

Labbe RF, Vreman HJ, Stevenson DK (1999) Zinc protoporphyrin: A metabolite with a mission. Clin Chem 45:2060-2072

Laity JH, Lee BM, Wright PE (2001) Zinc finger proteins: new insights into structural and functional diversity. Curr Opin Struct Biol 11:39-46

Lamb TM, Xu W, Diamond A, Mitchell AP (2001) Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the RIM101 pathway. J Biol Chem 276:1850-1856

Li L, Kaplan J (1997) Characterization of two homologous yeast genes that encode mitochondrial iron transporters. J Biol Chem 272:28485-28493

Lin SJ, Culotta VC (1996) Suppression of oxidative damage by *Saccharomyces cerevisiae* ATX2, which encodes a manganese-trafficking protein that localizes to Golgi-like vesicles. Mol Cell Biol 16:6303-6312

Link TA, von Jagow G (1995) Zinc ions inhibit the QP center of bovine heart mitochondrial bc1 complex by blocking a protonatable group. J Biol Chem 270:25001-25006

Lyons TJ, Gasch AP, Gaither LA, Botstein D, Brown PO, Eide DJ (2000) Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast. Proc Natl Acad Sci USA 97:7957-7962

Lyons TJ, Villa NY, Regalla LM, Kupchak BR, Vagstad A, Eide DJ (2004) Metalloregulation of yeast membrane steroid receptor homologs. Proc Natl Acad Sci USA 101:5506-5511

MacDiarmid CW, Gaither LA, Eide D (2000) Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*. EMBO J 19:2845-2855

MacDiarmid CW, Milanick MA, Eide DJ (2002) Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. J Biol Chem 277:39187-39194

MacDiarmid CW, Milanick MA, Eide DJ (2003) Induction of the ZRC1 metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. J Biol Chem 278:15065-15072

Mason AZ, Perico N, Moeller R, Thrippleton K, Potter T, Lloyd D (2004) Metal donation and apo-metalloenzyme activation by stable isotopically labeled metallothionein. Mar Environ Res 58:371-375

Matthews JM, Sunde M (2002) Zinc fingers--folds for many occasions. IUBMB Life 54:351-355

Min YK, Park JH, Chong SA, Kim YS, Ahn YS, Seo JT, Bae YS, Chung KC (2003) Pyrrolidine dithiocarbamate-induced neuronal cell death is mediated by Akt, casein kinase 2, c-Jun N-terminal kinase, and IkappaB kinase in embryonic hippocampal progenitor cells. J Neurosci Res 71:689-700

Muhlenhoff U, Stadler JA, Richhardt N, Seubert A, Eickhorst T, Schweyen RJ, Lill R, Wiesenberger G (2003) A specific role of the yeast mitochondrial carriers MRS3/4p in mitochondrial iron acquisition under iron-limiting conditions. J Biol Chem 278:40612-40620

Nanamiya H, Akanuma G, Natori Y, Murayama R, Kosono S, Kudo T, Kobayashi K, Ogasawara N, Park SM, Ochi K, Kawamura F (2004) Zinc is a key factor in controlling alternation of two types of L31 protein in the *Bacillus subtilis* ribosome. Mol Microbiol 52:273-283

Obata H, Hayashi A, Toda T, Umebayashi M (1996) Effects of zinc deficiency on the growth, proteins and other constituents of yeast, *Saccharomyces cerevisiae*, cells. Soil Sci Plant Nutr 42:147-154

Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488-2492

Palmiter RD, Cole TB, Quaife CJ, Findley SD (1996) ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci USA 93:14934-14939

Palmiter RD, Huang L (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch 447:744-751

Panina EM, Mironov AA, Gelfand MS (2003) Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc Natl Acad Sci USA 100:9912-9917

Parkin G (2004) Synthetic analogues relevant to the structure and function of zinc enzymes. Chem Rev 104:699-767

Pena MM, Koch KA, Thiele DJ (1998) Dynamic regulation of copper uptake and detoxification genes in *Saccharomyces cerevisiae*. Mol Cell Biol 18:2514-2523

Racker E (1950) Crystalline alcohol dehydrogenase from baker's yeast. J Biol Chem 184:313-319

Rivlin AA, Chan YL, Wool IG (1999) The contribution of a zinc finger motif to the function of yeast ribosomal protein YL37a. J Mol Biol 294:909-919

Robinson NJ, Whitehall SK, Cavet JS (2001) Microbial metallothioneins. Adv Microb Physiol 44:183-213

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM (2000) A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature 403:623-627

Vallee BL, Falchuk KH (1993) The biochemical basis of zinc physiology. Physiol Rev 73:79-118

van den Elsen JM, Kuntz DA, Rose DR (2001) Structure of Golgi alpha-mannosidase II: a target for inhibition of growth and metastasis of cancer cells. EMBO J 20(12):3008-17

Warner JR (1999) The economics of ribosome biosynthesis in yeast. Trends Biochem Sci 24:437-440

Waters BM, Eide DJ (2002) Combinatorial control of yeast FET4 gene expression by iron, zinc, and oxygen. J Biol Chem 277:33749-33757

Zhao H, Butler E, Rodgers J, Spizzo T, Duesterhoeft S, Eide D (1998) Regulation of zinc homeostasis in yeast by binding of the ZAP1 transcriptional activator to zinc-responsive promoter elements. J Biol Chem 273:28713-28720

Zhao H, Eide DJ (1997) Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*. Mol Cell Biol 17:5044-5052

Zhu H, Pan S, Gu S, Bradbury EM, Chen X (2002) Amino acid residue specific stable isotope labeling for quantitative proteomics. Rapid Commun Mass Spectrom 16:2115-2123

Lyons, Thomas J.
Department of Chemistry, University of Florida, Gainesville, FL 32611, University of Florida, Department of Chemistry, P.O. Box 117200, Gainesville, FL 32611
lyons@chem.ufl.edu

Regalla, Lisa M.
Department of Chemistry, University of Florida, Gainesville, FL 32611, University of Florida, Department of Chemistry, P.O. Box 117200, Gainesville, FL 32611

Iron in yeast: Mechanisms involved in homeostasis

Ernest Kwok and Daniel Kosman

Abstract

Iron homeostasis results from matching iron uptake to cell growth and division in the context of the overall cell requirement for iron. Fungi achieve this balance by transcriptional regulation of the genes that encode iron uptake activities; post-transcriptional regulation of the synthesis of proteins that use iron; and storage and recycling of iron to meet short-term needs in times of iron deprivation. In the Fungal Kingdom, both repression and activation mechanisms of transcriptional regulation have been elucidated; both mechanisms rely on transcription factors that directly or indirectly are regulated by cell iron status. Among fungi, however, one or the other transcriptional regulatory mechanism is used by a given organism but not both. In contrast, of those fungi examined in detail, all employ at least two of the four iron uptake mechanisms characterized in fungi in general: siderophore iron uptake; direct ferrous iron permeation; coupled ferroxidase/permease uptake; and heme/hemin uptake. All of these pathways rely on the activity of a metalloreductase enzyme at some point. The yeast vacuole serves as iron store while the mitochondrion, as the site of heme and Fe-S cluster biosynthesis, is the primary end-user of cell iron. The recycling of iron from both organelles plays a role in the maintenance of homeostasis both in terms of iron utilization and regulation of iron uptake.

1 Introduction

There are six cellular compartments that are known to be involved in iron homeostasis in yeast: plasma membrane, cytoplasm, vacuole, mitochondria, nucleus, and lastly, the exocytoplasmic milieu. Indeed, in free-living organisms like yeasts and other fungi, the iron status of the exocytoplasmic milieu *determines* the mechanisms adopted to maintain cellular iron homeostasis. This review describes the protein components and iron metabolic events that occur in each of these compartments and then summarizes current knowledge as to how these events are integrated so as to maintain the cell's iron balance. Several other excellent reviews that cover somewhat earlier literature and/or various aspects of fungal iron metabolism in more depth are recommended complements to this one (Winkelmann 2002; Van Ho et al. 2002; Schroder et al. 2003; Nelson 1999; Kosman 2003; Kap-

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_92 / Published online: 26 July 2005

lan 2002; Howard 2004; De Luca and Wood 2000; Boukhalfa and Crumbliss 2002).

2 The plasma membrane and exocytoplasmic milieu

The iron metabolic process that dominates these two compartments is iron uptake. Yeasts and fungi exhibit three primary mechanisms of iron accumulation: 1) siderophore-mediated; 2) ferrous iron transporter-mediated; and 3) ferroxidase, permease complex-mediated (Van Ho et al. 2002; Kosman 2003; Howard 2004). All three mechanisms are metalloreductase-dependent with the Fe^{3+}/Fe^{2+} redox reaction catalyzed by this enzyme activity required either at the end of the accumulation process (siderophore-mediated uptake) or at the beginning (the transporter and permease pathways). De Luca and Wood have nicely contrasted the mechanisms of iron accumulation *via* these two reductive pathways (De Luca and Wood 2000). Hemin/heme also is a source of iron for some pathogenic fungi including *Candida albicans* (Santos et al. 2003; Weissman and Kornitzer 2004) and *Histoplasma capsulatum* (Foster 2002). The iron could be released from the organic matrix in either case by ferrireduction in the exocytoplasmic space with the Fe^{2+} produced as substrate for subsequent uptake *via* any one of the three mechanisms above (with an autooxidation to Fe^{3+} preceding siderophore binding). Timmerman and Woods have characterized a glutathione-dependent extracellular reductase activity produced by *H. capsulatum* that they suggest supports this particular mechanism (Timmerman and Woods 1999, 2001). On the other hand, cell-surface heme-binding proteins have been identified in *C. albicans* indicating that like siderophore iron, Fe^{3+} in heme/hemin also can be mobilized intracellularly by metalloreduction following endocytic internalization (Santos et al. 2003). Following, we briefly review the three most broadly used uptake mechanisms; these are illustrated in Fig. 1 using as paradigm the yeast, *S. cerevisiae*.

2.1 Siderophore-mediated iron uptake

Although few yeasts (as distinct from fungi; see below) produce their own siderophore(s), all are likely to express siderophore receptors and the means to release the iron exocytoplasmically and to process the siderophore-iron complex once internalized. The *S. cerevisiae* genome encodes receptors that recognize members of several classes of siderophores, *e.g.,* FOB (*SIT1*) (Lesuisse et al. 1998); TAF (*TAF1*) (Heymann et al. 1999; Lesuisse et al. 2001); ferricrocin (*ARN1*) (Heymann et al. 2000b); and FC (*ARN1*, *TAF1*). A fourth locus – *ENB1* – encodes a facilitator that exhibits relative specificity for enterobactin (Heymann et al. 2000a), but, as this brief summary indicates, one is better served to consider all of these receptors to have, at best, limited specificity. Orthologs to some of these four *S. cerevisiae* genes (also known generically as *ARN* loci since all are upregulated by the iron-responsive transcription factor, Aft1p) (Yun et al. 2000) have

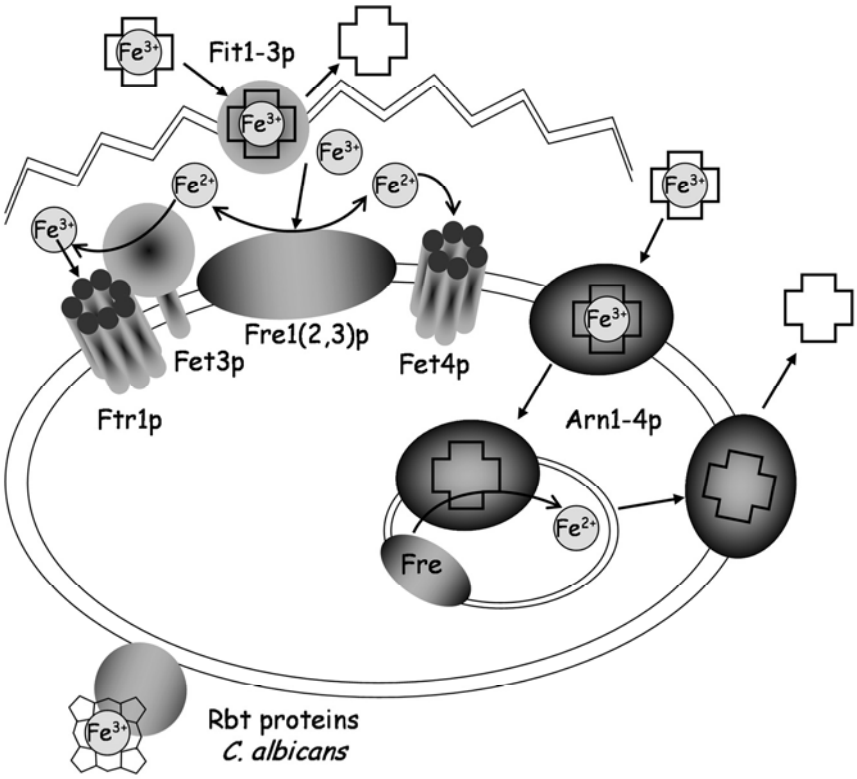


Fig. 1. The three dominant iron uptake pathways in yeasts and fungi. Metalloreductase activity supplied by the Fre proteins is essential to all three pathways. In *S. cerevisiae*, there are seven of these Fre proteins, Fre1-7p. All are membrane proteins; Fre1-3p have been localized directly or indirectly to the PM (plasma membrane). These proteins reductively labilize Fe^{3+} from exocytoplasmic ferric complexes including siderophores. The Fe^{2+} released can enter the cell directly *via* the ferrous iron transporter, Fet4p, or *via* the multicopper ferroxidase (Fet3p), iron permease (Ftr1p) complex. Uptake by Ftr1p is directly coupled to the oxidation (ferroxidation) by Fet3p of the Fe^{2+} produced by the action of the Fre proteins. Fet3p is a multicopper oxidase and thus the activity of the Fet3p/Ftr1p pathway is strongly dependent on copper repletion of the cell. The Fit proteins (Fit1-3p) are bound to the yeast cell wall *via* glycophosphatidylinositol anchors; they appear to bind siderophores thus increasing their effective concentration as substrate for the Fre proteins. The Fet4p and Fet3p/Ftr1p uptake systems also work directly on added Fe^{2+} or that produced by adventitious reduction of exocytoplasmic Fe^{3+}, *e.g.*, by ascorbic acid or any reductant secreted by the yeast cell. The Rbt heme/hemin binding proteins in *C. albicans* illustrate a fourth iron uptake mechanism that may be characteristic of pathogenic fungi.

been characterized in *S. pombe* (Pelletier et al. 2003), *C. albicans* (Heymann et al. 2002; Lesuisse et al. 2002; Ardon et al. 2001; Hu et al. 2002), *A. nidulans* (Haas et al. 2003), and *N. crassa* (Haas et al. 1999; Huschka et al. 1985). The latter two

fungi differ from the yeasts (*e.g. S. cerevisiae*, *S. pombe*, *C. albicans*) in that they are siderophore-producers indicating that for these fungi, at the least, siderophore-mediated Fe-accumulation is a major and not an adventitious means of achieving Fe-homeostasis (Winkelmann 2002; Philpott et al. 2002; Haas 2003).

The iron accumulation *via* the siderophore secretion and uptake pathway characteristic of filamentous fungi and its control are best illustrated by the work on *A. nidulans* (Haas et al. 2003, 1999; Eisendle et al. 2004; Oberegger et al. 2001; Oberegger et al. 2002a; Oberegger et al. 2002b). This fungus encodes three siderophore receptors designated MIRA through MIRC (Haas et al. 2003). MIRA, like the *S. cerevisiae* Enb1p (Arn4p), exhibits specificity towards the heterologous siderophore enterobactin while MIRB transports the native siderophore fusarinine C; it is thus functionally orthologous to Taf1p (Arn2p). MIRC has not yet been characterized in this fashion. *Via* a GATA-like transcription factor, the co-repressor SREA (Haas et al. 1999; Oberegger et al. 2001, 2002b), iron regulates the expression of a gene family whose members encode proteins involved in the biosynthesis of siderophores (*sidA-C*), siderophore uptake (the *mir* loci) and a potential ABC transporter that could play a role in mitochondrial iron handling in *A. nidulans* (*atrH*). In addition, a reductase-encoding gene, *freA*, is upregulated in iron deficiency in an SREA-independent manner (Oberegger et al. 2002a). Regulatory circuits like these will be discussed more fully in the concluding section.

Siderophores specifically bind Fe^{3+} (Boukhalfa and Crumbliss 2002; Neilands 1995); comparable to the reductive mobilization of Fe from transferrin in the mammalian endosome, metalloreductase activity is required for yeasts to utilize siderophore iron at the cell surface or from siderophores brought into the cell *via* endocytosis (De Luca and Wood 2000; Haas 2003; Yun et al. 2001). *S. cerevisiae* produces at least seven reductase proteins encoded by the *FRE1-7* genes (Martins et al. 1998). Two of these reductases (Fre1p and Fre2p) play essential roles in non-siderophore iron uptake while at least two of the others (Fre3p and Fre4p) appear involved in cell accumulation of siderophore iron (Yun et al. 2001). Fre1p and Fre2p, nonetheless, exhibit reactivity towards siderophores of both the hydroxamate and catecholate types whereas Fre3p, which also localizes to the PM, reduces only members of the hydroxamate class (*e.g.* FOB, FC, TAF). Siderophore Fe can be mobilized for cell utilization either at the PM or following endocytosis; both mechanisms appear to obtain in fungi (Lesuisse et al. 2001; Philpott et al. 2002; Yun et al. 2001; Kim et al. 2002; Ardon et al. 1998). In regards to the first of these mechanisms, the *S. cerevisiae* cell wall contains three Fit proteins (facilitator of iron transport) that appear to bind siderophores and thus functionally increase their effective concentration as substrates for the PM reductases, Fre1/2/3p (Protchenko et al. 2001). In this mechanism, the Fe^{2+} released within the exocytoplasmic space from the bound siderophore is substrate for one of the ferrous iron uptake systems described below, an uptake process independent of the siderophore uptake receptor/facilitators noted above (Philpott et al. 2002). Fitp homologs are strikingly absent in any of the archived fungal genomes with the exception of one entry in *Kluyveromyces lactis*, a member also of the family *Saccharomycetaceae*.

The *A. nidulans* genome also encodes a protein that exhibits 24% identity to the Fre2 protein from *S. cerevisiae*; the corresponding ORF has been designated *freA*.

Although the role of FREA in iron accumulation in *A. nidulans* has not been delineated, as noted the expression of *freA* does increase under conditions of iron limitation much as is the case for several of the *FRE* loci in *S. cerevisiae* (Oberegger et al. 2002a). The *C. albicans* genome contains several ORFs with sequence homology to the Fre proteins from *S. cerevisiae*; the proteins encoded by *CFL1* and *CFL95* do support iron uptake in a *S. cerevisiae fre1*Δ strain demonstrating that both are functional reductases. *S. pombe* also produces a functional reductase, frp1, required for iron uptake (Roman Dragos et al. 1993). Reductases in *N. crassa* have not been characterized genetically or biochemically; there are several candidates encoded in the *Neurospora* genome, however. Suffice to say that reductase-dependent iron uptake is a feature of all fungi, indeed, of all organisms living under air.

In general, there is a less detailed understanding of the mechanism of siderophore iron uptake and mobilization in fungi than in bacteria. A number of *E. coli* proteins involved in siderophore accumulation have been crystallographically characterized including FhuA (Ferguson et al. 1998) and FepA (Buchanan et al. 1999) (both of which are siderophore receptors) and FhuD, a periplasmic siderophore binding protein (Clarke et al. 2002). Other well-characterized proteins include another receptor, FhuE (Sauer et al. 1990), and a cytoplasmic membrane-associated reductase, FhuF, that likely contributes to the reductive release of Fe, as Fe^{2+}, from the siderophore complexes, coprogen and ferrichrome (Matzanke et al. 2004). Many of the structure-function details of the interaction of siderophore with these receptors have been reported in bacteria and, based on this work and the coordination chemistry relevant to siderophores in general, much can be deduced about the mechanism of iron release and utilization (Boukhalfa and Crumbliss 2002). These insights provide an excellent starting point for future studies on siderophore iron utilization by fungi.

2.2 Ferrous iron uptake

In contrast to siderophore uptake, direct Fe^{2+} transport function appears limited to a relatively few yeast and fungi with Fet4p from *Saccharomyces cerevisiae* the only characterized member of the small group of ferrous iron transporters (Kosman 2003). Homologous proteins by sequence have been noted in the archived genomes of *Schizosaccharomyces pombe* (NP595134.1), *Candida glabrata* (XP_445982.1), and *Kluyveromyces lactis* (XP_454604.1) but none of these putative ORFs has been further investigated. With such a limited gene cohort to consider, it is speculative to consider whether this function is one lost by most yeast and fungi but retained by baker's yeast, for example; or, conversely, that this ferrous iron uptake process is a specialized function adapted to some environmental condition peculiar to or, in fact, due to the selection process that has resulted in the "wild type" yeast in laboratory use today.

In any event, ferrous iron uptake mediated by Fet4p plays only a limited role in iron homeostasis, at least in *S. cerevisiae*. This is due to the relatively large value for the K_M for Fe^{2+} in the transport process of 35 μM (Dix et al. 1997, 1994). This

kinetic value was determined using $^{59/55}$Fe ferrous iron as substrate (either added directly or derived from Fe^{3+} in the presence of a large stoichiometric excess of dihydroascorbic acid); under any reasonable physiologic condition under air there is little likelihood that Fe^{2+} would accumulate at the exocytoplasmic surface of the yeast PM to this concentration. Therefore, the efficiency of this transporter would be very low; at the more likely [Fe^{2+}] = 1 μM or less, the transporter would be functioning at ~3% maximum efficiency or less. Nonetheless, Fet4p can supply yeast with sufficient iron for normal respiratory growth in the absence of either of the other two uptake functions (siderophore and ferroxidase/permease) in the presence of >50 μM medium iron.

The Fe^{2+} for Fet4p uptake is supplied by the PM-localized reductase Fre1p with a lesser contribution due to the homologous Fre2p (Georgatsou and Alexandraki 1994; Hassett and Kosman 1995). In most yeast, the former reductase accounts for >90% PM Fe^{2+} generation; indeed, introduction of a *fre1Δ* allele alone is sufficient to suppress reductase-dependent iron uptake in *S. cerevisiae* (Georgatsou and Alexandraki 1994; Hassett and Kosman 1995; Dancis et al. 1992). Fre1p and, most likely, all of its paralogs in *S. cerevisiae* and orthologs in other yeasts and fungi, exhibit a broad specificity for one-electron acceptors. These include both Fe^{3+} and Cu^{2+} as well as a number of organic oxidants often used as vital stains (*e.g.* for respiratory activity) (Hassett and Kosman 1995). Based on sequence homology and some biochemical analyses of Fre1p, the fungal Fre proteins are probable flavoheme proteins; the flavin moiety couples the two-electron oxidation of NAD(P)H to the one-electron reduction of the substrate, *e.g.*, Fe^{3+}, *via* the heme prosthetic group. The Fre proteins are predicted to have 7-9 transmembrane domains but none of them have been localized, *e.g.*, by indirect immunofluorescence or visualization of fluorescent fusions in living cells (*cf.* the GFP-fusion results archived by the SGD). All of the Fre proteins possess a recognizable signal sequence that directs their synthesis into the ER membrane, but none carry any additional signaling or sorting motifs, *e.g.*, characteristic of ER retention. Only Fre1p contains the NPF sequence motif that has been correlated with internalization of PM proteins from the cell surface and/or membrane trafficking (Tan et al. 1996). This comment pertains to the role that reductases must play in the release of Fe from siderophore complexes; to the ferrichrome-induced endosome to PM cycling of Arn1p in *S. cerevisiae* (Kim et al. 2002); and to the fact that internalization of siderophore prior to Fe-release has been demonstrated in the fungus, *Ustilago maydis*, the causal agent of corn smut (Ardon et al. 1998). These diverse observations support the implication above that Fre1p, which appears to have the broadest substrate specificity of the Fre proteins, contributes to mobilization of Fe from both hydroxamate and catecholate siderophore complexes either at the PM or within an endosome during cytoplasm/PM cycling (Philpott et al. 2002; Kim et al. 2002). Note, however, that much the same reduction activity is exhibited by Fre2p and Fre3p and, thus, these two PM reductases provide, at the least, redundancy in regards to this function (Philpott et al. 2002; Yun et al. 2001).

2.3 Ferroxidase, permease-mediated iron uptake

Under most laboratory growth conditions, the dominant mechanism by which most yeasts and fungi accumulate iron is *via* the ferroxidase, permease protein complex that resides in the plasma membrane. This high-affinity uptake system is composed at the least of a multicopper oxidase that catalyzes the oxidation of Fe^{2+} to Fe^{3+} (the ferroxidase reaction) and an iron permease that traffics the ferroxidase-generated Fe^{3+} across the PM. The best characterized members of these two protein families are the Fet3p and Ftr1p proteins from *S. cerevisiae* (Askwith et al. 1994; de Silva et al. 1995; Hassett et al. 1998b; Stearman et al. 1996; Severance et al. 2004; Wang et al. 2003). However, this uptake system has been firmly established in at least two other yeasts - *S. pombe* (Askwith and Kaplan 1997) and *C. albicans* (Eck et al. 1999; Knight et al. 2002; Ramanan and Wang 2000) - as well as in plants (*e.g. Chlamydomonas reinhardtii*, (La Fontaine et al. 2002) and paralogs exist in all archived fungal genomes.

The Fet3p, Ftr1p uptake system is high affinity in absolute terms; with a K_M for Fe^{2+} of 0.2 μM, this complex can be expected to efficiently accumulate iron at normonutrient levels of environmental iron (Dancis et al. 1992). This system is high affinity in relative terms, also; this K_M value is 10-100 times more favorable kinetically than either of the other two uptake processes (*i.e.* dependent on either Fet4p or most siderophore receptors) (Philpott et al. 2002; Dix et al. 1997). Its central role in physiologic iron homeostasis is also indicated by the fact that the expression of *FET3* and *FTR1* is tightly and coordinately linked to the iron status of the cell (Van Ho et al. 2002; Rutherford and Bird 2004). While all iron handling activities in *S. cerevisiae* are transcriptionally regulated, the regulation of this high-affinity system is far more acute and quantitatively robust than is the regulation of the other two iron accumulation pathway components. Furthermore, yeasts in general (excluding many fungi), can't rely on siderophore-iron since its availability depends in turn on the presence of a co-habitant that is a siderophore-producer. An interesting question of adaptation is whether yeasts out-sourced the production of siderophores to fungi and other microorganisms or acquired the appropriate receptors so that they could become scavengers of the iron being sequestered in siderophores produced by others.

Iron homeostasis in eukaryotes cannot be discussed without some comment on its strict dependence on copper metabolism (discussed in detail in Chapter 2). This is due to the essential role played by multicopper ferroxidases in either iron uptake (as in fungi); in iron export (as in the intestinal epithelium); or in intracellular iron trafficking (as in the fungal vacuole) (Askwith and Kaplan 1998). Thus, defects in either copper nutritional status or copper handling will in most cases lead to a secondary dysfunction in iron homeostasis due to the failure to supply copper to the *apo*-form of one or more of these ferroxidase enzymes. Those cell activities upstream from the copper binding to the multicopper oxidase are: 1) the high affinity, plasma membrane Cu^{1+} transporter, Ctr1p (and the metalloreductase that supplies the Cu^{1+}); 2) the copper chaperone, Atx1p in yeast (Atox1/HAH1 in humans); and 3) the Cu-ATPase, Ccc2p in yeast (ATP7A or ATP7B in humans). *In vitro* studies suggest that Cu^{1+} translocated by Ctr1p exchanges into Axt1p at

the cytoplasmic face of the plasma membrane (Xiao and Wedd 2002; Xiao et al. 2004). This Atx1p copper subsequently exchanges into the Ccc2p Cu-ATPase that is located in the membrane of a Golgi- or post-Golgi compartment; this Cu-pump concentrates the copper in the lumen of this compartment where it is available for the metallation of *apo*-Fet3p, for example (Huffman and O'Halloran 2001). Additionally, Cl^- appears to synergize this vesicular Cu-uptake and/or metallation as indicated by the failure to activate *apo*-Fet3p in a *gef1*Δ strain (Davis-Kaplan et al. 1998; Gaxiola et al. 1998). *GEF1* in *S. cerevisiae* encodes a homolog of the human CLC proteins that mediate the vectorial transport of Cl^- (Schwappach et al. 1998). Chloride ion is a positive effector of the Cu^{1+} activation of *apo*-Fet3p *in vitro* (Davis-Kaplan et al. 1998). In summary, there are numerous cell activities upon which the accumulation and trafficking of iron *via* the ferroxidase-permease pathways depend.

2.4 Heme/hemin uptake

Pathogenic bacteria appear to prefer heme as iron source (Rouault 2004); there is some evidence that the same might be true for some opportunistic yeasts and fungi. For example, *Histoplasma capsulatum*, of the phylum *Ascomycota*, can cause severe respiratory infections in immunocompromised individuals (Foster 2002). In culture, growth of this fungus is modulated by the presence of heme (added as hemin or heme, or hemoglobin) and not by reductase-accessible iron. This uptake of heme iron was linked to the binding of heme to a protease-sensitive site on the cell surface. This result suggests that *H. capsulatum* expresses a heme receptor and that this organism's virulence will be dependent on this iron uptake pathway.

Heme utilization by *C. albicans* has been characterized in more detail, although its relationship to the virulence of this yeast-like fungi has not been delineated. One should note, however, that siderophore uptake is required for epithelial invasion by this fungus (Heymann et al. 2002). The *C. albicans RBT5* gene is highly induced by iron starvation and encodes a protein essential for robust fungal growth on hemin or hemoglobin (Weissman and Kornitzer 2004). Immunologic screens and sequence homology searches indicate that this cell-surface activity is conserved in other members of the genus *Candida* suggesting that heme-utilization is a relatively widely disseminated means of iron acquisition in fungi. Indeed, there are possibly five *RBT5* paralogs in *C. albicans*, including *RBT51*. Of interest is the fact that although *S. cerevisiae* is unable to utilize heme as a sole iron source, heterologous expression of *RBT51* rescues the iron deficiency of an iron uptake *S. cerevisiae* mutant in the presence of hemoglobin as the sole source of iron (Weissman and Kornitzer 2004). This indicates that except for the putative cell surface receptor for heme/hemoglobin encoded by *RBT51* (and *RBT5*), *S. cerevisiae* produces the other activities required for the subsequent mobilization and trafficking essential to the utilization of any source of iron. One of these activities is likely to be heme oxygenase. Santos et al. demonstrated that a *CaHMX1* mutant was unable to grow on hemin as the sole source of iron although hemin uptake *per*

se was not impaired (Santos et al. 2003). This result is fully consistent with the well-established role of heme oxygenase in heme breakdown that, in the end, results in the recycling of the iron.

3 The cytoplasm

Remarkably little is known about the state of iron in the cytoplasm of yeasts and fungi. Unlike other organisms from both the Bacterial and Eukaryotic Superkingdoms, fungi do not encode a ferritin-like protein nor do they encode an iron response element binding protein that regulates the translation of cytoplasmic messages. This lack of knowledge also contrasts with the well-studied copper chaperone pathways in yeast and higher eukaryotes that serve to keep the "free" [Cu] in the yeast cell diminishingly small (Rae et al. 1999). Copper chaperones bind Cu^{1+} and transfer this cargo to acceptor proteins *via* an associative mechanism that precludes the equilibration of the bound metal ion with bulk solvent (Huffman and O'Halloran 2001; Luk et al. 2003). There are no data that support either the conclusion or inference that fungi (especially yeasts) contain protein (genome-encoded) iron chaperones. Thus, the yeast cytoplasm is most fairly represented as a blank box in a cartoon depicting the six compartments involved in yeast iron homeostasis.

On the other hand, there are data that indicate the yeast cell contains what has been referred to generally as a "labile iron pool" or LIP. This fraction of cell iron is so-called because it is accessible to membrane permeant iron chelating agents; those most useful in this regard are ones that upon Fe-binding exhibit a spectral change of some sort such that they report on the iron status of the LIP. The most widely reported such agent is calcein whose native fluorescence is quenched upon metal binding; in regards to metal binding and fluorescence quenching calcein is relatively specific in reporting the cell level of chelatable iron in comparison to other di- and trivalent metal ions (Ali et al. 2003; Thomas et al. 1999; Epsztejn et al. 1997). There is some controversy as to whether calcein binds Fe^{2+} in preference to Fe^{3+} (Thomas et al. 1999) but this question is not highly relevant to the objectives of this review.

Another useful agent, one that is specific for Fe^{2+}, is 2, 2'-bipyridyl (BIP); the $(BIP)_3Fe(II)$ complex absorbs strongly at 520 nm (ε = 11,000 M^{-1} cm^{-1}) and so can also quantify the accessible Fe^{2+} (Hassett et al. 1998a). In as much as BIP is highly membrane permeant, it has full access to all cell compartments; therefore, the value of 10 μM of accessible Fe^{2+} in the yeast cell includes iron throughout the cell, not just the cytoplasm. Also, given the fact that the cell contains redox buffers that can support the Fe^{2+}/Fe^{3+} equilibrium, BIP in any of these compartments will shift this redox equilibrium towards the lower valent species due to the significantly greater stability of the BIP•Fe^{2+} complex in comparison to the BIP•Fe^{3+} one. Because of these two caveats (which apply to much the same extent for any chelating metal indicator) the precise cell locale and redox state of the iron quantified by BIP cannot be specified. Indeed, a ferric iron EPR signal has been ob-

served in yeast cells treated with desferrioxamine (DFO), presumably of the Fe^{3+}•DFO complex (Srinivasan et al. 2000). Quantification of this DFO-chelatable iron indicated that it accounted for ~5% of total cell iron, or 10 μM. This concentration was the same as that estimated for the BIP-chelatable iron (Hassett et al. 1998a) suggesting, but not proving, that the Fe^{2+} (BIP) and Fe^{3+} (DFO) chelators were accessing the same – and redox labile – iron pool.

Hassett and co-workers quantified the kinetics of transcriptional activation of the *FET3* and *FTR1* genes upon challenge of the yeast cell with the membrane-impermeant Fe^{2+} chelator, bathophenanthroline disulfonic acid (BPS) in comparison to the treatment with BIP (Hassett et al. 1998a). In the first 30 minutes there was only weak expression of these two loci with BPS compared to a >20-fold increase in transcript abundance in those cells treated with BIP. Shifting the cells from an anaerobic to an aerobic environment elicited a similar transcriptional upregulation. This pattern suggests that a BIP-accessible, intracellular pool of Fe^{2+} is what directly or indirectly downregulates Aft1p, the primary transcriptional activator of the expression of the so-called ARN (Aft1p regulon) loci that include *FET3* and *FTR1* (Yamaguchi-Iwai et al. 1995, 1996). As noted above, these results do not demonstrate that this BIP- and oxygen-accessible iron pool is in any specific compartment since both reagents have full access to all within the cell. Also, they do not prove that it is Fe (regardless of valence state) that is sensed by Aft1p; Fe^{2+} chelation by BIP could trigger some downstream metabolic shift that itself was the trigger for Aft1p activation. Other data (below) have suggested that Fe-S clusters and/or heme might be involved in Aft1p regulation; the downstream effect of reducing the cellular pool of Fe^{2+} by chelation or oxidation could be on the biosynthesis of these two iron-containing prosthetic groups.

The eukaryotic cytoplasm and the ER membrane do contain a family of enzymes characterized by having a diiron cluster as an essential redox center (Broadwater et al. 1998). In yeasts and fungi these enzymes include the soluble ribonucleotide reductase (the catalytic, diiron-containing subunit is encoded by the *RNR2* locus) (Ge et al. 2001) and acyl- and sterol desaturases; the ER membrane C-4 methyl sterol oxidase (encoded by the *ERG25* locus) is an example of one of the latter enzymes (Bard et al. 1996). The diiron clusters in these proteins must be assembled *in situ* since their iron coordination spheres are composed of essentially all protein ligands. Therefore, they must be presented with iron in some ligand-exchangeable form, *e.g.*, not in heme, an Fe-S cluster nor an essentially exchange inert oxo-iron species as is likely to be found in the yeast vacuole (see below). Assembly and activation of the R2 subunit of ribonucleotide reductase *in vitro* requires Fe(III), reducing equivalents and molecular oxygen (Stubbe and Riggs-Gelasco 1998). In all likelihood reduced Fe is also needed to activate Erg25p (Broadwater et al. 1998). All of this suggests that there is a pool of exchangeable/chelatable iron (most likely ferrous) in both the cytoplasm (and, for small solutes, in its continuous cell compartments like the nucleus) and the ER and the downstream vesicular trafficking pathway. The mechanism(s) by which these proteins obtain the iron essential to the assembly of their catalytic prosthetic groups remains uncharacterized.

This is not to say that there are no experimental data that at least define the issue. *S. cerevisiae* expresses two genes that encode a diiron-like subunit of ribonucleotide reductase, *RNR2* and *RNR4*. Although the precise function of Rnr4p is not known, it is strongly homologous to Rnr2p (Huang and Elledge 1997); it forms a tight and specific complex with Rnr2p (Voegtli et al. 2001); and it is required for the wild type assembly of the diiron cluster in Rnr2p (Nguyen et al. 1999). While Rnr4p does contain some of the Fe-binding motifs characteristic of Rnr2p and other similar diiron enzymes, it lacks three of them and no data indicate that it binds iron. Therefore, whether it serves solely as a protein chaperone (for Rnr2p), an iron chaperone or both remains undetermined. As for the membrane-bound, ER diiron enzymes like Erg25p that catalyze fatty acyl and sterol desaturation (Broadwater et al. 1998) there is an additional issue to consider and that is the topology and orientation of the protein and the resulting orientation of the diiron cluster in the active enzyme. For Erg25p and its homologs there is no experimental answer to this question, but work on a comparable bacterial enzyme, an acyl desaturase from *Bacillus subtilis*, indicates that the His residues involved in Fe-coordination are in the cytoplasm and at the cytoplasmic face of the ER membrane (Diaz et al. 2002). It is reasonable to conclude that the diiron cluster in Erg25p, which is where the substrate sterol binds and is oxidized (desaturated), would be either in or adjacent to the membrane in which the substrate is partitioned. This locale doesn't preclude a simple activation mechanism in which the *apo*-protein scavenges labile Fe^{2+} from the cytoplasm, but given the increasing number of instances of co-factor chaperoning – including the Rnr4p story above - we shouldn't discount the probability that Erg25p activation benefits from some assistance also.

4 The vacuole

The vacuole is a critical part of cellular iron homeostasis in yeast. This is indicated by three broad facts: 1) the vacuolar membrane contains multiple activities that genetically and functionally have been linked to iron homeostasis; 2) newly arrived cell iron does accumulate in the vacuole; and 3) vacuolar iron appears to be recycled to be used to support essential metabolic activities. The protein activities in the yeast vacuole important to iron homeostasis are of three types: 1) iron transporters/permeases; 2) proton-pumps, most predominantly, the vacuolar V-type ATPase that serves to make the vacuole the yeast's proton buffer; and 3) metalloreductase(s) activity required for the reductive labilization of the Fe_xO_y species that likely accumulate in the relatively oxidizing, acidic environment of the vacuolar lumen.

Iron does accumulate in the yeast vacuole. On a per milligram protein basis, iron is enriched in the yeast vacuolar fraction by as much as 12-fold, and, unlike the cytosol, for example, the vacuole concentrates iron under iron-replete growth conditions (Raguzzi et al. 1988). This vacuolar accumulation depends on the Ccc1p protein (Li et al. 2001; Li and Kaplan 2004), at the least, as well as on the correct targeting of vacuolar proteins (Bode et al. 1995). Ablation of either activity

results in a decline in the steady-state level of iron in the yeast vacuole in growth on standard laboratory media (synthetic or rich). Vacuolar acidification (*vma*) mutants also exhibit defects in iron metabolism, *e.g.* poor or no respiratory growth, and poor growth in low iron conditions (Greene et al. 1993). One may interpret this to indicate that vacuolar iron handling is dependent on acidification, however, these phenotypes are just as or even more likely the result of the failure to acidify the Golgi- or post-Golgi compartments in which Fet3p is activated (*cf.* 1.3 above). *apo*-Fet3p is found on the surface of yeast cells mutant in *VMA* loci, or in a locus (*VPS41*) required for vacuolar protein trafficking (Radisky et al. 1997); such mutants commonly exhibit weak or absent Fet3p/Ftr1p-dependent iron uptake which alone can explain their iron-dependent growth and respiratory defects.

Importantly, however, vacuolar iron does appear to be metabolically active. That is, in yeast switched from glucose (fermentable) to ethanol (respiratory) as carbon source, vacuolar iron declines to 30% of its abundance at the time of the change in carbon source (Raguzzi et al. 1988). This decline correlates to the 30-fold increase in cytochrome oxidase activity induced by this nutrient shift. Respiratory capacity is thus an easily scored phenotype that reflects the continued heme and iron-sulfur cluster biosynthesis required by a yeast deriving its energy from the oxidation of a non-fermentable carbon source. That vacuolar iron supports this iron homeostasis is indicated by the observation that a yeast strain that lacks a putative vacuolar iron export complex lags behind WT in growth adaptation to a non-fermentable carbon source (Urbanowski and Piper 1999).

Four iron-trafficking activities have been localized to the yeast vacuole (Fig. 2). These include (in *S. cerevisiae*) the export complex noted above composed of the Fet3p, Ftr1p orthologs, Fet5p and Fth1p; Ccc1p; and Smf3p. The orientation of Fet5p/Fth1p is identical to that of Fet3p/Ftr1p; thus, the ferroxidase domain of Fet5p and a principal iron trafficking motif of Fth1p (Severance et al. 2004) are located within the vacuolar lumen. In this orientation, the Fet5p/Fth1p ferroxidase/permease complex would function as an exporter of Fe from the vacuole as was indicated by the growth experiments referenced above (Urbanowski and Piper 1999). Ccc1p appears to support vacuolar iron uptake whereas Smf3p appears to function as an exporter of vacuolar iron. For example, vacuolar iron in a *ccc1*Δ mutant is ~30% that of WT while overexpression of *CCC1* leads to a fourfold increase (Le et al. 2001). Smf3p is one of a family of three Smf proteins in *S. cerevisiae* that are homologs of the mammalian Nramp divalent metal ion transporters (Portnoy et al. 2000). Smf3p localizes to the vacuole and appears to specifically support the cell's utilization of iron. This is in contrast to its paralogs, Smf1p and Smf2p, that are required for the accumulation of manganese (Portnoy et al. 2000).

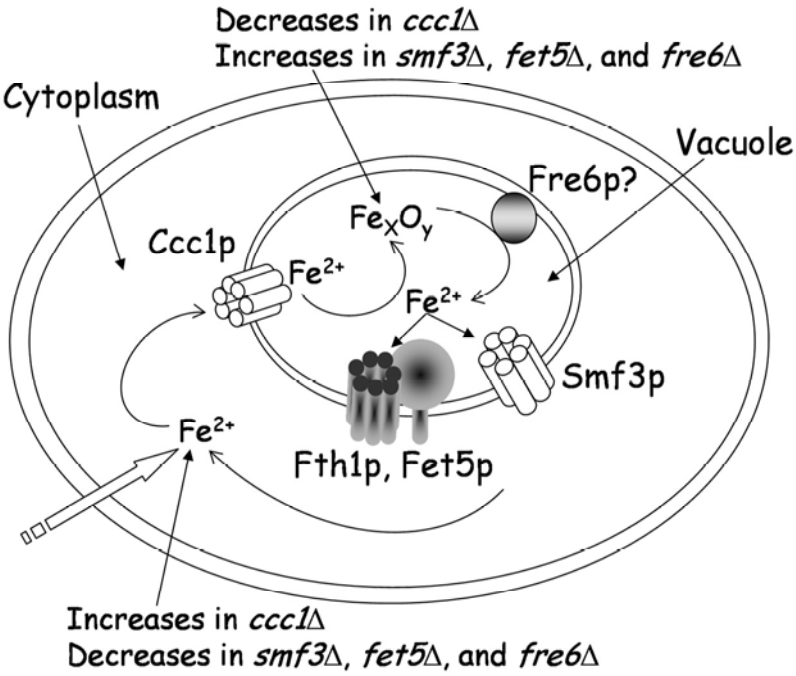


Fig. 2. Vacuolar buffering of cytoplasmic iron. Iron is transported into the cell as Fe^{2+}. This iron is transported into the vacuole *via* Ccc1p and is likely stored there as a ferric hydroxide (phosphate?) species. Recycling likely involves reductive labilization; Fre6p may be the vacuolar reductase responsible for this step (Singh and Kosman, unpublished). The Fe^{2+} released is effluxed from the vacuole *via* Smf3p and the Fet5p/Fth1p complex. Smf3p is a divalent metal iron transporter while the latter complex is paralogous to the PM permease complex described in Fig. 1. The effect of deletion of the corresponding gene loci on the partitioning of iron between cytoplasm and vacuole is indicated.

The role that Smf3p – and Ccc1p – plays in cell iron homeostasis has been evaluated by use of a reporter gene (*lac*Z) under control of an iron-responsive promoter. As discussed in more detail below, a dominant regulator of iron metabolism in *S. cerevisiae* is the Aft1 protein, a transcription activator that is repressed in wild type cells at hyper-nutrient levels of iron (Yamaguchi-Iwai et al. 1995, 1996). The ARE (Aft1p response element) from an Aft1p-regulated locus (*e.g. FET3*, *FTR1*) thus becomes a sensor of the iron status of the cell; to the extent that the vacuole modulates this status the activity of an ARE thus reports on the effectiveness of this modulation. Thus, in a *ccc1*Δ strain in which Fe is *not* being sequestered in the vacuole, Aft1p will be repressed as will the expression from the ARE; in effect, the cell will "act" as if it is more iron replete than WT under the same nutrient condition. In contrast, an *smf3*Δ strain will appear more iron deficient in as much as iron that is pumped into the vacuole by Ccc1p is, in effect, permanently removed from the iron-regulatory circuit; thus, the ARE will sense a functional iron-deficiency not seen in WT under the same condition. Indeed, this

Table 1. List of proteins involved in iron homeostasis of mitochondria in *S. cerevisiae* identified by SGD accession number. Homologs in other yeasts and fungi are identified by NCBI Protein Accession numbers except for *S. pombe* members that are given by their systematic ORF identifiers. Similarity searches were conducted with mature protein sequences derived from experimental data or predictions using MitoProt (Claros and Vincens 1996).

Protein	Location	Activity	Homologs in other species[a]
Mmt1/YMR177W Mmt2/YPL224C	Inner membrane	Fe importer	An(EAA60584) Ca(EAK91620) Nc(EAA33576) Sp(SPCC1020.03)
Mrs3/YJL133W Mrs4/YKR052C	Inner membrane	Fe importer	An(EAA59898) Ca(EAK97010) Nc(EAA30369) Sp(SPAC8C9.12C)
Atm1/YMR301C	Inner membrane	Fe-S exporter	An(EAA62688) Ca(EAK92894) Nc(EAA27101) Sp(SPAC15A10.01)
Yhm1/Ggc1/Shm1/ YDL198C	Inner membrane	GTP/GDP exchanger	An(EAA62313) Ca(EAK94758) Nc(EAA26888) Sp(SPCC1682.09C)
Erv1/YGR029W	Intermembrane space and Cytosol	Sulfhydryl oxidase	An(EAA63598) Ca(EAL02588) Nc(EAA30480) Sp(SPAC3G6.08)
Nfs1/YCL017C	Matrix and Nucleus	Cysteine desulfurase	An(EAA63993) Ca(EAL01528) Nc(EAA29943) Sp(SPBC21D10.11C)
Yfh1/YDL120W	Matrix	Fe chaperone	An(EAA63887) Ca(EAK92316) Nc(EAA28958) Sp(SPCC1183.03C)
Isu1/YPL135W Isu2/YOR226C	Matrix	Fe-S scaffold	An(EAA60457) Ca(EAL02584) Nc(EAA30802) Sp(SPAC227.13C)
Yah1/YPL252C	Matrix	Ferredoxin	An() Ca(EAL01312) Nc(EAA28849) Sp(SPAC22E12.10C)
Arh1/TDR376W	Matrix (membrane associated)	Ferredoxin reductase	An(EAA58656) Ca(EAK92719) Nc(EAA33439) Sp(SPBC3B8.01C)
Ssq1/YLR369W	Matrix	Hsp70 chaperone	An(EAA57651) Ca(EAK96197) Nc() Sp(SPAC664.11)
Jac1 /YGL018C	Matrix	J-type chaperone	An(EAA64599) Ca(EAL00799) Nc(EAA28897) Sp(SPAC144.08)
Grx5/YPL059W	Matrix	Glutaredoxin	An(EAA60465) Ca(EAK92524) Nc(EAA31624) Sp(SPAPB2B4.02)
Hem15/YOR176W	Matrix (membrane associated)	Final step of heme synthesis	An(EAA61540) Ca(EAK94066) Nc(EAA33268) Sp(SPCC320.09)

[a] Species abbreviations used: An, *Aspergillus nidulans*; Ca, *Candida albicans*; Nc, *Neurospora crassa*; Sp, *Schizosaccharomyces pombe.*

transcriptional reporter assay using *FET3*p fused to *lacZ* has demonstrated just these behaviors (Li et al. 2001; Portnoy et al. 2000). These phenotypes are portrayed in the cartoon shown in Fig. 2.

5 Mitochondria

Mitochondria play a special role in iron homeostasis because they are the site of the majority of the cell's biosynthesis of iron-sulfur (Fe-S) clusters and the final step of heme synthesis, the incorporation of ferrous iron into protoporphyrin IX (Dailey 2002). While the demand for iron by mitochondria is high, free iron in the mitochondrial matrix must be limited because the generation of free oxygen species by the electron transport chain makes the organelles sensitive to oxidative stress (Halliwell and Gutteridge 1992). Mitochondria therefore require stringent controls on their iron status. The critical nature of this control is manifested in a variety of human diseases that correlate with dysfunction in mitochondrial iron handling, *e.g.*, Friedrich's ataxia and X-linked sideroblastic anemia with ataxia (Napier et al. 2004). Genetic analysis of mitochondrial iron status in yeasts has led to the identification of numerous proteins involved in iron homeostasis that are conserved across kingdoms (Table 1). These proteins can be broken into two categories: iron transporters and proteins involved in Fe-S cluster or heme biosynthesis.

5.1 Putative iron transporters

Studies with isolated mitochondria indicate that heme synthesis is dependent on import of ferrous iron and requires a membrane potential, but not ATP (Muhlenhoff et al. 2002). To date, however, no proteins have been convincingly demonstrated to be mitochondrial iron importers or exporters. The *S. cerevisiae* genome does contain a number of genes whose products are predicted to encode transporters in the mitochondrial inner membrane. The majority of these proteins are members of conserved families of transporters and as such, similar proteins have been identified in a variety of fungal species.

The inner membrane proteins Mmt1p and Mmt2p (Fig. 3) show a high degree of sequence similarity to each other and to transition metal transporters (Li and Kaplan 1997). A number of experimental findings suggest Mmt1/2p are mitochondrial iron importers. *mmt1/2*Δ strains grow slowly on low iron media (Foury and Roganti 2002). Strains with upregulated *MMT1/2* survive longer than WT after transfer from iron-replete to iron-free media (Foury and Roganti 2002), most likely because the mutants store high levels of iron in their mitochondria (Li and Kaplan 2004). However, the biochemical activities of Mmt1p and Mmt2p have never been demonstrated, and indeed, there are some conflicting data regarding their function. Analysis of Mmt1/2p activity in the *yfh1Δ* mutant (see below) suggests the transporters may actually serve as exporters of mitochondrial iron. For example, deletion of both *MMT1* and *MMT2* appears to exacerbate the high mitochondrial iron phenotype of *yfh1*Δ mutants leading to oxidative stress and loss of mitochondrial DNA in the triple mutant (Foury and Roganti 2002). Also, overproduction of Mmt2p in a *yfh1*Δ mutant lowers mitochondrial iron and suppresses iron sensitivity of the mutants (Li and Kaplan 2004). Both of these results would

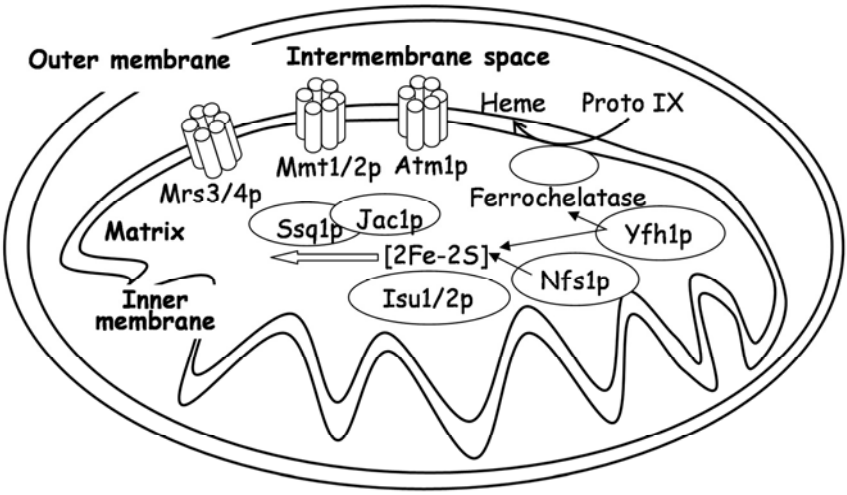


Fig. 3. Proteins involved in iron trafficking and utilization in *S. cerevisiae* mitochondria. A simplified model of the initial steps in iron-sulfur cluster biosynthesis in the matrix is diagramed. Note the role of the frataxin homolog, Yfh1p, in both heme and Fe-S cluster biosynthesis. In the latter pathway, Yfh1p supplies the Fe while Nfs1p supplies the S; assembly takes place on a scaffold provided by Isu1/2p. The Fe-S chaperones, Ssq1p and Jac1p, transfer the newly assembled Fe-S clusters to recipient proteins. The exact functions of the putative Fe-transporters indicated in the inner membrane are not known.

be expected of proteins whose function was export of iron from mitochondria, rather than import.

Mrs3p and Mrs4p are a pair of solute carrier-like proteins that reside in the inner membrane (Muhlenhoff et al. 2003b). Several lines of evidence suggest Mrs3/4p function as iron importers. When grown on low iron media, *mrs3/4*Δ mutants have low mitochondrial iron (Foury and Roganti 2002) which likely leads to the reduced Fe-S and heme protein activity observed in the mutants (Muhlenhoff et al. 2003b). Conversely, overexpression of *MRS3/4* results in high mitochondrial iron and promotes abnormally high Fe-S and heme protein activity (Muhlenhoff et al. 2003b). When *mrs3/4*Δ mutants are grown on high iron media, cells show no Fe-S or heme protein defects, suggesting a separate, low affinity import system also exists (Foury and Roganti 2002).

Yhm1p (also referred to as Ggc1p) is another member of the mitochondrial carrier protein group (Lesuisse et al. 2004). A deletion mutant at this locus has upregulated cellular iron uptake and low cytosolic but high mitochondrial iron levels (Lesuisse et al. 2004). The *yhm1*Δ mutant is similar to the Fe-S cluster synthesis mutants (see below) in having low mitochondrial Fe-S and heme protein activity (Lesuisse et al. 2004). Reconstitution of the protein in liposomes revealed it to be a GTP/GDP anti-porter capable of importing GTP into the mitochondrial matrix (Vozza et al. 2004). Whether this nucleotide exchange activity is necessary

for iron homeostasis or whether the iron phenotypes observed in the deletion strain are secondary effects of the loss of GTP entry into the matrix is unknown.

Atm1p is an ATP Binding Cassette (ABC) transporter located in the inner mitochondrial membrane (Reviewed in Lill and Kispal 2001). In other organisms, ABC transporters import glutathione-metal complexes into the vacuole. The amino acid sequence of *S. cerevisiae* Atm1p indicates its ATPase domain resides in the matrix space, suggesting the protein is an exporter. The nature of the exported molecule is as yet undefined. *atm1*Δ mutants accumulate high mitochondrial iron (Kispal et al. 1997) although the rate of iron uptake across the plasma membrane is unchanged in these mutants (Kispal et al. 1999). When *ATM1* expression is blocked, mutants exhibit reduced Fe-S enzyme activity in the cytosol, but not in the mitochondria, suggesting that the protein exports factors required specifically for Fe-S protein maturation in the cytosol (Kispal et al. 1999).

A close phenotypic relative of *atm1Δ* mutants are strains carrying deletions in *ERV1*. Erv1p is a sulfhydryl oxidase that resides in the mitochondrial intermembrane space (Lee et al. 2000). At the restrictive temperature, $erv1^{ts}$ mutants have reduced cytosolic Fe-S protein activity and high mitochondrial iron (Lange et al. 2001). On the other hand, heme and cytochrome levels and mitochondrial Fe-S protein activities are WT under these conditions. The precise function of Erv1p in cytosolic Fe-S protein maturation remains uncharacterized.

5.2 Iron-sulfur cluster and heme biosynthesis

Mutants defective in mitochondrial iron homeostasis have also been found that are associated with iron-sulfur cluster biosynthesis. The study of Fe-S cluster biosynthesis in the mitochondrial matrix of yeast has been guided by knowledge derived from prokaryotes (Frazzon et al. 2002). A simple model for Fe-S cluster biosynthesis is illustrated in Fig. 3. Nfs1p removes sulfur from cysteine and ligates it to iron that is probably chaperoned by and transferred from Yfh1p. The nascent Fe-S clusters are formed on the scaffold proteins Isu1/2p in a process requiring at the least the action of a redox pair consisting of mitochondrial ferredoxin, Yah1p, and ferredoxin reductase, Arh1p. The transfer of Fe-S clusters from the Isu1/2p scaffolds to apo-Fe-S proteins is thought to require the chaperone pair Ssq1p and Jac1p and the glutaredoxin, Grx5p. Mutation of the gene encoding any one of these proteins results in a suite of common phenotypes that includes: high mitochondrial iron, high plasma membrane iron uptake, and reduced Fe-S and heme protein activity in the mitochondria and cytosol. Of particular interest to this discussion are the proteins involved in the initial formation of Fe-S clusters.

Yfh1p is the yeast homolog of human frataxin, a mitochondrial matrix protein. Recent experiments have revealed Yfh1p to play a central role in iron handling within mitochondria. In humans, Fe-S cluster synthesis is initiated by the transfer of iron from frataxin to the human homolog of Isu1/2p (Yoon and Cowan 2003). *S. cerevisiae* Yfh1p appears to play a similar role, based on its interaction with both Isu1p and Nfs1p (Ramazzotti et al. 2004; Gerber et al. 2003). *yfh1*Δ mutants do retain some Fe-S protein activity suggesting the presence of a second Fe-S

cluster biosynthesis system. One candidate for this activity is Nfs1p, which can catalyze Fe-S cluster formation *in vitro* (Muhlenhoff et al. 2004). Yfh1p also interacts directly with ferrochelatase, at a site overlapping Yfh1p's iron-binding domain, suggesting a role for Yfh1 in heme synthesis (Lesuisse et al. 2003; He et al. 2004). This theory is supported by the observation that *yfh1Δ* mutants have reduced ferrochelatase activity (Lesuisse et al. 2003). Finally, Yfh1p interacts directly with aconitase (a mitochondrial Fe-S protein) in the presence of citrate and activates aconitase by donating Fe^{+2} to aconitase's [4Fe-4S] cluster indicating that Yfh1p can modulate the activity of mature Fe-S proteins (Bulteau et al. 2004). Thus, the yeast homolog of frataxin is central to the formation of heme and the formation and maintenance of Fe-S cluster proteins.

Other prominent players in Fe-S cluster biosynthesis include the scaffold proteins Isu1/2p and the chaperone system, Jac1p/Ssq1p. Isu1p and Isu2p are a pair of similar proteins that serve as scaffolds for the synthesis of [2Fe-2S] clusters. These proteins bind iron *in vitro* in the presence of Nfs1p and sulfur (Muhlenhoff et al. 2003a). Isu1/2p also interact in an iron-dependent manner with Yfh1p and Nfs1p (Gerber et al. 2003). Mitochondrial Isu1/2p are required for both mitochondrial and cytosolic Fe-S protein activity, indicating they are necessary for generation of all cellular Fe-S proteins (Gerber et al. 2004). Isu1/2p interact with both Jac1p and Ssq1p independently; binding of Jac1p to Isu1/2p is believed to target the Isu1/2-Jac1p complex to Ssq1p (Dutkiewicz et al. 2004). Ssq1p and Jac1p are a chaperone pair in the mitochondrial matrix thought to mediate the transfer of Fe-S clusters from Isu1/2p to Fe-S apo-proteins. Ssq1p shows similarity to Hsp70 (Knight et al. 1998), while Jac1p is similar to J-type chaperones (Voisine et al. 2001). Deleting either protein results in the accumulation of Fe-S clusters on Isu1/2p and the loss of Fe-S protein activity in mitochondria and the cytosol (Muhlenhoff et al. 2003a). These data fit a model in which Ssq1p and Jac1p promote the transfer of Fe-S clusters from Isu1/2p to either mitochondrial Fe-S apoproteins or to a molecule that is then exported for activation of cytosolic Fe-S proteins.

Heme biosynthesis is the other mitochondrial activity that consumes iron. The final step of heme biosynthesis is catalyzed by ferrochelatase (product of the *HEM15* gene), which is associated with the matrix side of the mitochondrial inner membrane (Labbe-Bois 1990; Dailey et al. 2000). Ferrous iron from the matrix is inserted into protoporphyrin IX that has been formed in the intermembrane space; the heme produced is likely released into the inner membrane (*cf.* Fig. 3). *In vitro* work suggests that only iron that arrives *via* the intermembrane space is competent for incorporation into heme. That is, iron preloaded into the matrix by incubating mitochondria in ferrous iron followed by removal of exomitochondrial iron by EDTA is not incorporated into heme. This indicates that catalysis of iron insertion by ferrochelatase is probably tied to transport across the inner membrane (Lange et al. 1999). Deletion of *MMT1/2* does not affect heme synthesis, so iron for heme synthesis is likely imported *via* some other route. Surface plasmon resonance experiments indicate Yfh1p binds to ferrochelatase (Lesuisse et al. 2003). Hence, Yfh1p, with roles in both Fe-S cluster biogenesis and heme synthesis may act as a regulatory point between the two major iron-utilizing processes. In support of this

inference, iron has been observed to be unavailable for use by ferrochelatase in *yfh1*Δ mutants (Lesuisse et al. 2003). In contrast, in another study, *yfh1*Δ mutants showed no change in ferrochelatase activity (Lange et al. 2004). Human frataxin has a higher binding affinity for ferrochelatase than for the human Isu1/2p homolog, so heme is probably made at the expense of Fe-S clusters when iron becomes limiting (Yoon and Cowan 2004). In yeast, however, when Fe-S cluster synthesis is limited, an inhibition of ferrochelatase activity is observed as well (Lange et al. 2004). Mutations that depress activation of cytosolic Fe-S proteins do not also correlate with this inhibition of ferrochelatase activity suggesting that this latter activity (heme synthesis) is dependent on mitochondrial Fe-S status alone. Animal ferrochelatases are distinguished from their bacterial homologs by the presence of a carboxy-terminus extension that binds a [2Fe-2S] cluster (Dailey et al. 2000). *S. pombe* ferrochelatase contains an Fe-S cluster, while the *S. cerevisiae* enzyme contains a carboxy-terminal extension, but no Fe-S cluster (Dailey et al. 1994). The [2Fe-2S] cluster is not essential for catalytic activity (Medlock and Dailey 2000). Nevertheless, a role for Fe-S clusters in ferrochelatase activity would explain the inhibition of this activity observed in mitiochondrial Fe-S cluster synthesis mutants; by inference, *S. cerevisiae* ferrochelatase activity may be modulated by a molecule associated with Fe-S cluster biosynthesis (Lange et al. 2004).

5.3 Mitochondrial iron homeostasis with respect to the cell

Mitochondria are expected to exert special influence on cellular iron homeostasis because they are the primary users of cellular iron. Research has shown strong connections between mitochondrial iron status and the activity of both the plasma membrane and vacuolar iron uptake systems. Of primary interest is the relationship of iron-associated mitochondrial proteins and the Aft1/2p system (Table 2).

Expression of the putative mitochondrial iron transporters Mmt2p and Mrs4p is upregulated by Aft1/2p as part of the iron regulon (Foury and Roganti 2002). This co-induction of plasma membrane and mitochondrial uptake systems ensures that mitochondria have access to iron under iron-limiting conditions, especially in light of the fact that Aft1p also induces vacuolar iron-export via Fet5p/Fth1p and Smf3p. Hence Aft1/2p upregulation appears to provide iron for mitochondrial import from both the environment and vacuolar stores. *ISU1* has also been reported to be upregulated by Aft2p (Rutherford et al. 2003). This suggests that Aft1/2p also enhances the Fe-S cluster synthesis activity of mitochondria in low-iron conditions. Similarly, deletion of any one of a number of Fe-S cluster synthesis genes results in upregulation of the iron regulon *via* the activation of Aft1/2p reinforcing the connection between plasma membrane iron uptake and Fe-S cluster synthesis.

Table 2. Interaction between mitochondrial iron status and the Aft1/2p iron regulon

Action	Genes Targeted
Expression induced by Aft1p and/or Aft2p	MMT2, MRS4, ISU1
Deletion or repression induces Aft1p and/or Aft2p	ERV1, MRS3/4, YMH1/GGC1, NFS1, YFH1, ISU1/2, JAC1, GRX5
Overexpression induces Aft1p and/or Aft2p	MRS3/4

A recent report from Kaplan and his colleagues suggests that control of cellular iron status *via* Aft1/2p is signaled from the mitochondria, at least in part (Chen et al. 2004). These investigators monitored cytosolic iron by measuring the activity of Erg25-2p. As noted above, Erg25p is an ER diiron enzyme required for ergosterol biosynthesis. *ERG25-2* is a mutant allele that fails to activate under conditions of low cytosolic iron. In *yfh1*Δ mutants, the iron regulon is upregulated (by Aft1/2p) even though cytosolic iron is apparently unchanged as indicated by Erg25-2p activity. In addition, *nfs1* mutants that exhibit a strongly reduced Fe-S protein assembly, also exhibit an iron regulon hyper-activity. This work demonstrated also that in the face of this reduced Fe-S protein activity, loading the cytosol with iron by growth in high-iron media did not downregulate the iron regulon. This challenges the alternative model that Aft1/2p activity is dependent on cytosolic iron status *per se*. The model is important to this discussion because Fe-S protein activity, whether in the cytosol or the mitochondria, is dependent on mitochondrial iron. Therefore, the model proposed by Chen *et al.* holds that mitochondrial iron status is the key regulator of iron homeostasis at the plasma, vacuolar, and mitochondrial membranes.

Support for a model in which Fe-S cluster synthesis regulates cellular iron uptake can also be inferred from mutants in the putative mitochondrial iron transporters. *mrs3/4Δ* mutants have low mitochondrial iron and high plasma membrane iron uptake while cells overexpressing *MRS3/4* exhibit the opposite phenotypes. These data reinforce the idea that iron uptake at the plasma membrane responds to mitochondrial iron levels. Furthermore, deletion of the *ATM1* and *ERV1* genes, thought to mediate Fe-S export from mitochondria, results in high mitochondrial iron and upregulated plasma membrane iron uptake, reinforcing the inference that cytosolic Fe-S cluster level/activity is what determines uptake at the cellular level.

Recent results have highlighted also the interaction between mitochondrial and vacuolar iron status. As noted, deletion of the vacuolar iron importer *CCC1* results in accumulation of iron in the cytoplasm and growth sensitivity on high iron media (Li et al. 2001). Overexpression of *MRS3/4* in a *ccc1*Δ mutant rescues this growth defect and reduces cytosolic iron levels, presumably by pumping iron out of the cytosol and into mitochondria (Li and Kaplan 2004). Thus Mrs3/4p, the putative mitochondrial iron importers, operate on the same pool of cytosolic iron that is imported by Ccc1p. Another example of the interdependence of vacuolar and mitochondrial iron status is observed in *yfh1*Δ mutants. Over-production of Ccc1p can suppress the respiration defect of a *yfh1*Δ mutant, apparently by reducing the mitochondrial iron levels in the latter mutant (Chen and Kaplan 2000). Ccc1p, presumably by sequestering iron in the vacuole, appears to limit the amount of iron available for absorption by mitochondria so as to prophylactically deal with

iron-dependent mitochondrial damage. These results are consistent with a model of iron homeostasis in which mitochondria and the vacuole import iron from a common cytosolic pool and that altered activity in one compartment has a direct and compensatory effect on the other.

6 The nucleus

The expression of most iron metabolic functions in yeasts are regulated at the level of the transcription of genes encoding the proteins involved in yeast iron metabolism. Therefore, at least as the site of this regulation, the nucleus is an important organelle to consider in the delineation of cellular iron homeostasis. As much as is known about the transcription factors responsible for this regulation, as much is not known about the extent to which the nucleus is part of the overall iron trafficking pathway in the yeast cell. Specifically, do iron-responsive regulators sense iron in the nucleus or do they do so in some extra-nuclear compartment and then traffic the signal back to the promoter(s) they are responsible for modulating? As discussed more fully below, in the kingdom Fungi both general mechanisms of transcriptional regulation are employed, repression and activation. In *Ascomycota,* regulation of iron metabolism has been investigated in the orders *Saccharomycetales* (*S. cerevisiae*), *Schizosaccharomycetales* (*S. pombe*), *Sordariales* (*N. crassa*), and *Eurotiales* (*A. nidulans*); in *Basidiomycetes*, iron metabolism has been studied in *U. maydis* (order *Ustilaginales*). *Ascomycota* species exhibit both mechanisms that balance the expression of iron handling activities with environmental iron availability. The iron content of a fungal nucleus is not known, but, given the pro-oxidant potential of iron, particularly Fe^{2+}, teleologically this value should be very small and likely correspond only to protein-associated metal. In short, although the nucleus is at the center of the known regulatory mechanisms that control iron metabolism in Fungi, it is likely that nuclear-cytoplasmic communication in regards to this regulation is serviced by proteins and not by iron itself. This inference remains to be substantiated.

7 Regulation and integration

In as much as control of yeast iron metabolism results primarily from transcriptional regulation of genes encoding iron handling proteins, iron homeostasis ultimately depends on the activity of the transcriptional regulators involved (but not entirely, see below). As sound as this statement may be, it can be applied knowledgably to five yeasts and fungi (moulds) at the most as noted above. In fact, only in *S. cerevisiae* are the relationships between cell iron status and transcriptional controls on iron handling activities relatively well understood in terms of the cell conditions that lead to up or downregulation of the corresponding transcription factors. Certainly one of the reasons for this relative dearth of mechanistic insight

into these control processes is the fact that the homology among proteins linked to *iron-dependent* control of iron trafficking activities encoded in fungal genomes is not informative. For example, the two iron-responsive transcription factors found in *S. cerevisiae*, Aft1p and Aft2p, have little if any homology with fep1, a *S. pombe* protein that regulates expression of the Fet3p, Ftr1p orthologs, fio1 and fip1. Furthermore, BLAST searches using either of the Aft proteins as query sequence identified only two possible homologs: the protein encoded by the CAG59681 locus in *Candida glabrata* and the one encoded by the XP_453211 locus in *Kluveromyces lactis* both again of the order *Saccharomycetales*. The function of neither of the proteins encoded by these genes has been elucidated. Also, there are at least two totally different strategies of regulating iron uptake activities at the transcriptional level found in fungal species: activation *via* Aft1-like proteins, and repression *via* GATA-like factors, of which Fep1 is one example. While hindering understanding to some extent, this complexity does have the potential to yield exciting new insight into the integration of iron metabolism in fungi.

7.1 Iron regulation *via* GATA factors

Irrespective of mechanism, iron handling activities in *S. cerevisiae*, *S. pombe*, *C. albicans*, *A. nidulans*, and *N. crassa* appear to be or most clearly are regulated at the level of transcription by proteins whose activity as modulators of gene expression are responsive to the level of bioavailable iron. The principal transcription factor characterized in each one of the latter four organisms linked to regulation of iron-handling activities is a member of the GATA family of DNA-binding proteins (Lowry and Atchley 2000): Fep1 (*S. pombe*) (Pelletier et al. 2003, 2002), Sfu1p (*C. albicans*) (Lan et al. 2004); SREA (*A. nidulans*) (Haas et al. 1999; Oberegger et al. 2001, 2002b); and SRE (*N. crassa*) (Harrison and Marzluf 2002; Zhou et al. 1998; Zhou and Marzluf 1999). All four are homologs of the first of these regulators to be identified, the Urbs1 protein in the *Basidiomycetes*, *Ustilago maydis* (Voisard et al. 1993). These proteins all have a pair of Zn-finger motifs that are required for binding to their HGATAR *cis*-elements (where H stands for A, T or C and R stands for any purine). For example, in *S. pombe* Fep1 binds to 5'-(A/T)GATAA-3' sequences in the 5'-untranscribed regions of all of the known genes that encode iron uptake activities in this yeast: *frp1* (reductase), *fio1* (ferroxidase), *fip1* (permease), and the siderophore receptor genes, *str1-3* (Pelletier et al. 2003, 2002). A cartoon that illustrates a possible model for how Fep1 modulates the expression of these genes is given in Fig. 4.

How the DNA-binding (or protein binding) activity of these GATA factors is modulated by cell iron level is not known. Models are portrayed in the literature with Fe in some undefined association with the factor in the DNA-bound (repressing) form of the protein (*cf.* Pelletier et al. 2002). Electrophoretic mobility shift assays do indicate that recombinant Fep1 produced and purified in the presence of an iron chelator does not bind to its *cis*-element (Pelletier et al. 2002). This suggests that DNA-binding by Fep1, for example, is dependent on iron in some

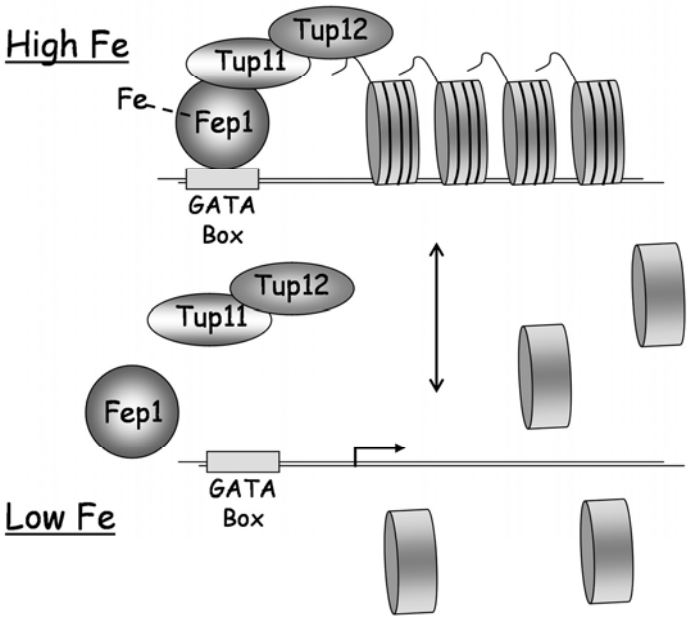


Fig. 4. Model for the mechanism of transcription regulation *via* the GATA protein, Fep1. Fep1 is a co-repressor of transcription of genes in *Schizosaccharomyces pombe* that encode Fe-handling activities such as the high affinity iron uptake complex of Fio1 and Fip1. Fep1 and its orthologs in other fungi bind to specific *cis* elements *via* a pair of Zn-finger motifs and recruit a repressor complex that in *S. pombe* includes Tup11/12. Tup11/12 in turn bind to the amino-terminii of histones H3 and H4 and thereby stabilize adjacent nucleosomes. In this model Fep1 binding (and transcriptional repression) occurs under a condition of iron hyperabundance, while debinding and transcription initiation occur in a condition of iron deficiency. The sensing mechanism that links cell iron status to GATA element binding by Fep1 and its orthologs has not been fully delineated.

fashion but there are no data that show unequivocally that any one of these factors actually binds iron, or that the chelator-treated form has stoichiometrically less iron than the DNA-binding species. On the other hand, there are some intriguing results that support the possibility that these specialized GATA factors bind some metal species, most likely iron or an iron-containing complex.

Marzluf and his co-workers have been investigating the function of the *Neurospora* GATA factor, SRE (Harrison and Marzluf 2002; Zhou et al. 1998; Zhou and Marzluf 1999). In their more recent work they targeted a Cys-containing motif for mutagenesis specifically to test whether it played a role in the iron-dependent transcriptional repression due to this protein. This motif (^{204}Cys-X$_5$-Cys$_{210}$) resides between the two Zn-finger elements that bind to the DNA; the C204S/C210S double mutant exhibited weak repressor activity *in vivo* and weaker DNA binding in the *in vitro* EMSA analysis. A compositional difference between the wild type and mutant recombinant protein was observed also, namely, the latter lacked the dis-

tinct absorbance envelope in the near UV (peak at 340 nm) and visible (peak at 440, shoulder at 540 nm) characteristic of the wild type SRE (Harrison and Marzluf 2002). Metal/prosthetic group analyses were not performed on these samples; the spectrum has features of both protein-heme and protein Fe-S complexes and so couldn't be assigned without some compositional data. Purification in the presence of an iron chelator was not performed in this case (as above) and so this correlation can't be used in determining what the WT protein has that the Cys mutant does not.

However, this Cys-motif most likely is critical to the iron regulation of this subfamily of GATA factors. The consensus amino acid sequence based on the five members discussed here (fep1, SRE, SREA, Urbs1, and Sfu1) is G(S/T)CPG(D/G)GXCNTGG. A BLAST search using the core element, GSCPGDGLCNGTGG, as query returned eleven subjects that included the five noted here; two orthologs (in *Candida* and *Ustilago*); and four additional candidates in four other fungi. Importantly, this query returned *no other* GATA factors; returned no subjects that weren't GATA factors; returned subject motifs that were complete in all cases; and returned sequences in which this motif fell between the two Zn-finger domains in the subject indicating that this is a conserved motif *specific* to those GATA proteins associated with fungal iron-dependent transcription repression.

At least in some cases, this protein functions along with a co-repressor or repressors, that is, these (and other) GATA factors function as part of a multi-protein complex supported by specific protein-protein interactions. In *S. pombe*, deletion of both *tup11* and *tup12* ablates iron-dependent, Fep1-mediated repression of *fio1* transcription (Pelletier et al. 2002). A similar result was reported for the *Candida TUP1* deletion mutant; transcript abundance for the ferric reductase (*CFL95*) and permease (*CaFTR1*) that in WT was strongly depressed at 3 μM culture iron, in the mutant remained unchanged at even 30 μM iron (Knight et al. 2002). Tup11 and Tup12, like the Tup1 protein in *S. cerevisiae*, together with Ssn6p bind to histones H3 and H4, recruit histone deacetylases and as a result of the resulting chromatin remodeling prevent the assembly of a transcription pre-initiation complex (Davie et al. 2003; Fagerstrom-Billai and Wright 2005; Smith and Johnson 2000). This multi-protein complex is itself recruited to the promoter region of the target gene by the *trans*-factor, in this case the GATA protein whose DNA binding is triggered by iron. These facts are represented in the model shown in Fig. 4.

The GATA protein, Cys-element Blast search noted above found no subjects in *S. cerevisiae*, nor in *C. glabrata* or *K. lactis*, the two fungi in which putative Aft1/2p homologs were identified. Consequently, fungi appear to use either the Aft1p or GATA protein mechanism of regulation of iron handling activities but not both. What is interesting about this choice is that Aft1/2p are transcriptional *activators*, not *trans*-factors that recruit the Tup1p/Ssn6p repressor complex, and so the choice of mechanism is quite fundamental. Silencing, which is what the GATA factor is doing under high iron, is strongly linked to differentiation, even in fungi, as in growth as a filamentous rather than a "budding" yeast; activation is more commonly geared towards response to an acute environmental and/or nutrient shift, although certainly the latter can induce a shift in fungal growth habit as

well. The Tup1p/Ssn6p complex is highly active in *S. cerevisiae*, targeting (repressing) >150 loci at last count (Gagiano et al. 2002). In other words, the repressive GATA mechanism serves fungi very well as a means of maintaining iron homeostasis. So an intriguing question is: Why Aft1p/Aft2p?

7.2 Iron regulation *via* Aft1 proteins

There are no data that demonstrate that Aft1p binding to DNA or its transcriptional activity is directly altered by iron as is the case for the copper-dependent transcription factor in *S. cerevisiae*, Mac1p (Yamaguchi-Iwai et al. 1997) and its homologs in *S. pombe*, Cuf1 (Labbe et al. 1999) and in *C. albicans*, CaMac1 (Marvin et al. 2004). Aft1p occupancy of its consensus *cis*-element, YRCACCCR, and, therefore, its transcriptional activity, appears to be controlled solely by its localization: in Fe-replete cells, Aft1p is cytoplasmic whereas in Fe-deficient ones it becomes localized in the nucleus (Yamaguchi-Iwai et al. 2002). How this trafficking is mediated by iron is not known, nor are there any data that indicate whether Aft1p binds iron or any iron species. Like the GATA factors, Aft1p contains a Cys-motif ($^{291}CDC_{293}$) that would be of little interest except that a point mutant that yields C291F renders Aft1p insensitive to cell iron giving the cell what is referred to as an $Aft1^{UP}$ phenotype. In such a strain, transcripts from the *FRE1*, *FET3*, and *FTR1* loci remain elevated at 100 μM Fe (Yamaguchi-Iwai et al. 1996) much as the homologous transcripts in the fep^- *S. pombe* strain do (Pelletier et al. 2002). This disregulation correlates with the iron-independent localization of $Aft1^{UP}$ to the nucleus (Yamaguchi-Iwai et al. 2002) and DNA *cis*-element occupancy (Yamaguchi-Iwai et al. 1996).

The Aft2p paralog, although significantly different in overall sequence, exhibits much the same features characteristic of Aft1p: CDC motif and $Aft2^{UP}$ allele phenotypes; recognition of the same or similar FeREs (for iron response element); and activation of numerous Aft1p-dependent loci (Rutherford et al. 2003, 2001). However, in addition to the latter gene profile are groups of loci activated solely or predominantly by Aft1p and those activated solely or predominantly by Aft2p (Rutherford et al. 2003). For example, Aft1p activates all seven of the *FRE* loci whereas Aft2p activates transcription only from *FRE1*. Aft1p appears to be the activator of choice for the siderophore receptors in *S. cerevisiae* (the *ARN* loci) whereas Aft2p is a more robust activator of the *FIT* loci that encode the cell wall proteins that bind siderophores thus increasing their effective concentration at the cell surface (above). How this transcriptional discretion is achieved is not altogether clear although it is likely due to a combination of slightly different affinities for variants of the canonical Aft *cis*-element and differential protein-protein interactions with as yet unidentified co-activators of the gene targets (Rutherford et al. 2003). One can appreciate that this discretion, whatever its molecular basis, is the answer to our Why Aft1p/Aft2p? question above: it appears to be a transcriptional activation mechanism that is both robust and selective, fitting the cell's response to a nutrient (iron) shift that allows for increase in one uptake system and not another; storage of iron in one compartment and not another; or the use of iron

for the assembly of one type of iron-containing prosthetic group and not another. Integrating this pattern of differential activation is what results in cellular iron homeostasis.

Indeed, *S. cerevisiae* regulates iron-handling and iron-dependent activities post-transcriptionally as well. Thiele and his co-workers have demonstrated that the Cth2 protein stimulates the turnover of as many as 84 transcripts under iron-limiting conditions, that is, under conditions of Aft1/2p activation (Puig et al. 2005). *CTH2* itself is a target of this activation indicating that the protein's function is to post-transcriptionally suppress the synthesis of proteins that utilize the iron that is limiting in the cell under these conditions. Consistent with this inference, of the 45 targeted transcripts encoding iron-related proteins, most encoded iron-utilizing enzymes or subunits of such enzymes, *e.g.*, many of the cytochrome *c* oxidase subunits, the diiron enzymes Erg25p and ribonucleotide reductase (Rnr2p and Rnr4p, above) and members of the Fe-S cluster biosynthetic pathway. Cth2p recognizes and binds to 5′-UUAUUUAU-3′ and/or 5′-UAUUUAUU-3′ sequences in the 3'-untranslated regions of these several transcripts typically doubling their turnover rate. Although this fold-change is far less robust than the increase in *FET3* or *FTR1* transcripts due to the transcriptional activation afforded by Aft1/2p, one can readily appreciate that a 50% reduction in iron-utilizing activities *globally* would significantly reduce the cell's overall iron requirement and thus make an important, albeit indirect, contribution to the maintenance of cellular iron homeostasis.

7.3 Sensing cellular iron status

Simply because what we know about iron uptake, intracellular compartmentalization, iron prosthetic group biosynthesis and regulation derives principally from studies in *S. cerevisiae*, here we assess how fungi sense their iron status by review of these data alone. The unique observations concerning mRNA turnover reviewed directly above are only one example of this fact. Iron homeostasis is achieved by balancing the uptake of the metal with the capacity of the intracellular milieu to store and or detoxify it while at the same time ensuring that there is sufficient metal in the right form in the right cell compartment for the assembly of iron-containing (enzyme) prosthetic groups. In this context, the cell's iron-handling or utilizing activities can be represented by five functional classes: 1) iron uptake; 2) iron storage and recycling; 3) iron import and trafficking; 4) iron prosthetic group assembly; and 5) iron end-users. Since the cell *needs* the latter activities irrespective of nutrient iron level, their expression (at the transcriptional level) is not likely to be regulated by iron; this does appear to be the case, *e.g.*, *RNR2* (ribonucleotide reductase) or *COX* loci (cytochrome oxidase) expression is not strongly iron-dependent (Shakoury-Elizeh et al. 2004; Foury and Talibi 2001). Therefore, the last, end-user group is what *depends* on iron homeostasis rather than playing a role in maintaining it. Similarly, one might expect heme synthesis or Fe-S cluster assembly to be relatively insensitive to iron status or to the activity of the iron-dependent regulators, Aft1/2p (in *S. cerevisiae*). This is true, but with

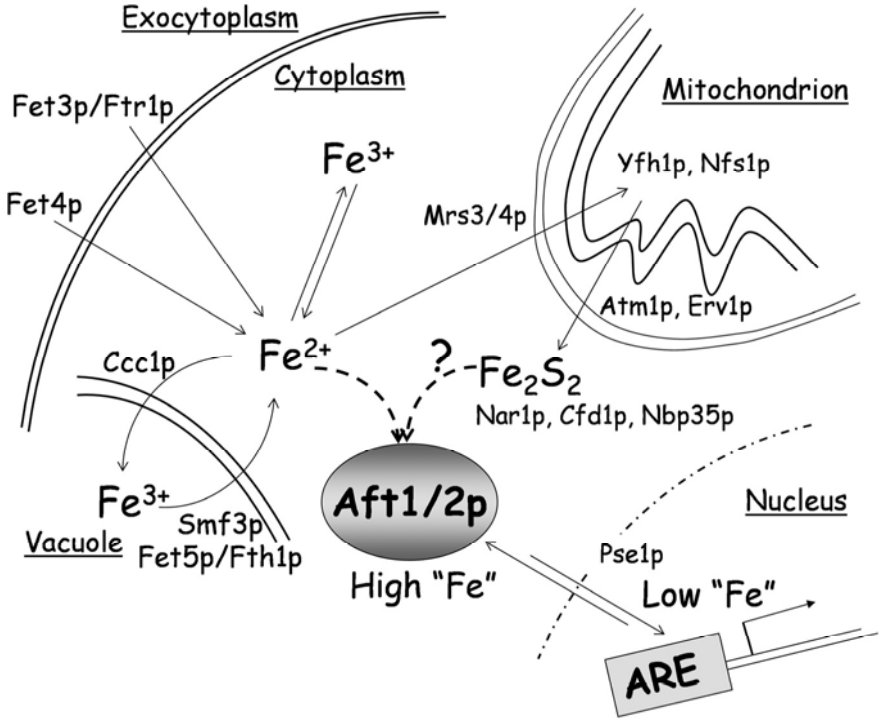


Fig. 5. Integrating iron metabolism in *Saccharomyces cerevisiae*. This figure assembles the specific Fe-handling activities associated with the various cell compartments found in this yeast and indicates their relationship to each other and to the iron-dependent regulator(s) of the expression of these activities, Aft1/2p. Newly-arrived iron likely enters a ferrous iron pool that is partitioned to either the mitochondrion for assembly of heme and Fe-S clusters, or to the vacuole for storage. Data with Fe^{2+} and Fe^{3+} chelators indicate that this cytoplasmic iron pool is redox labile as indicated. There is no evidence that Fe(II/III), per se, effluxes from the mitochondrion; rather, iron exits this organelle in either heme or Fe-S clusters. Iron uptake by the mitochondrion requires the activity of the Mrs3/4 protein pair while Fe-S cluster assembly (Yfh1p, Nfs1p) and export (Atm1p, Erv1p) depend on a host of gene products; mutation in any of these genes results in a deficit in cytoplasmic Fe-S cluster level. However, only mutations in genes encoding mitochondrial Fe-uptake (Mrs3/4p) or Fe-utilization (Yfh1p, Nfs1p) activities result in an apparent Fe-hyperabundance in the cell (cytoplasm). In parallel, loss of Ccc1p activity results in a deficit in Fe-accumulation in the vacuole and a corresponding apparent increase in cytoplasmic iron. In both of these cases, the Fe-hyperabundance in the cytoplasm is marked by a suppression of the transcriptional activation from the ARN loci, or Aft1p Regulon; this regulon is indicated generically in the figure by the ARE *cis*-element (Aft1p Response Element). Although the sensing mechanism that regulates the cytoplasmic-nuclear partitioning of Aft1p is not known (chaperoned by the Pse1p import receptor), data at this point indicate that iron in some form *other than* Fe-S is the likely signal.

exceptions: only *ISU1*/*2*, genes that encode the Fe-S cluster scaffolding proteins (above) are strongly induced under conditions that replicate cytoplasmic iron deficiency (Rutherford et al. 2003; Foury and Talibi 2001); this activation appears due to Aft2p alone (Rutherford et al. 2003).

On the other hand, genes encoding proteins involved in the transport of iron within the mitochondria (*MRS4*); or in the recycling of iron from the vacuole (*SMF3*) or from heme (*HMX1*); or in uptake of iron at the plasma membrane (above) are regulated by Aft1p, or Aft2p, or both (Rutherford et al. 2003, 2001; Shakoury-Elizeh et al. 2004; Foury and Talibi 2001). *HMX1* encodes a heme oxygenase homolog, an ER protein that facilitates the cell's use of heme as a source of iron (Protchenko and Philpott 2003). The Aft1/2p activation of genes encoding protein metabolism activities (ubiquitin conjugating enzymes, vacuolar proteases) is consistent with the notion that in conditions of low iron, the cell mobilizes to recycle (by degredation) what iron it can from endogenous iron-containing proteins and prosthetic groups (Foury and Talibi 2001; Shakoury-Elizeh et al. 2004).

This pattern of regulation does not explain the mechanism by which the activities of the *trans*-factors are modulated by iron status so as to maintain iron homeostasis, *i.e.* the mechanism by which the cytoplasmic-nuclear trafficking of Aft1p (and presumably, of Aft2p) is regulated. The context for this trafficking and its regulation is illustrated in Fig. 5. Obviously, understanding this mechanism is the key to an overall elucidation of iron homeostasis in *S. cerevisiae*. There are two general schemes: 1) a direct effect on the structure-function in Aft1/2p by association with iron or an iron-containing species; 2) a direct effect by such species on some accessory protein (an Aft1/2p chaperone or kinase, for example) whose activity modulates Aft protein localization. The latter mechanism would most reasonably involve a physical interaction of the modulator proteins with Aft1p; there are four reports that show or imply such a protein-protein interaction, two of which appear to bear on the question of Aft1p trafficking. Fragiadakis *et al.* have shown that an interaction between Aft1p and the structural protein, Nhp6p, recruits the co-repressor, Ssn6p, to the *FRE2* promoter. The assembly of this complex appears to lead to the full activation of this promoter under iron-limiting conditions (Fragiadakis et al. 2004). Expression from the *ARN2* locus also exhibits this Nhp6p, Ssn6p dependence. Rather than clarifying the mechanism of Aft1p trafficking, these observations only demonstrate another layer of complexity in regards to overall Aft1p-dependent gene regulation. Similarly, the report by Puig *et al.* that a Rpd3p-Sin3p histone deacetylase-associated protein, Cti6p, is required for growth of *S. cerevisiae* under iron-limited conditions only indicates the likely role for chromatin remodeling during the activation of Aft1/2p-dependent genes (Puig et al. 2004). This is not to discount these interesting results but rather to note that they do not address the specific question at hand.

Of more interest in this respect is the work of Casas *et al.* who observed that under some conditions a fraction of Aft1 protein in the cell was phosphorylated (Casas et al. 1997). Although this modification was not correlated with iron-dependent changes in the transcriptional activity, the data do indicate that Aft1p is substrate for some kinase(s) that itself is under metabolic control. This kinase has not been identified; however, data show that it is *not* Snf1p nor yeast PKA (Casas

et al. 1997). Yamaguchi-Iwai and her co-workers have presented data on the mechanisms that regulate the nucleo-cytoplasmic partitioning of Aft1p. Following up on their original report of this trafficking (Yamaguchi-Iwai et al. 2002), they demonstrated subsequently that with WT Aft1p, nuclear localization was associated with an interaction with the non-classical import receptor Pse1p (Kap121p), an association that was disrupted by Ran-GTP *in vitro* (Ueta et al. 2003). The trafficking into the nucleus was associated with a pair of nuclear localization sequences (NLS) in Aft1p each of which were recognized by the Pse1p receptor. This interaction was not directly iron-sensitive; however, and thus is not at the heart of the signal transduction mechanism that links iron status to Aft1p localization and gene activation.

There were two other facts in these papers that are significant. First, Casas *et al.* examined the transcriptional activity of *GAL1-HIS3* and *GAL1-lacZ* reporter loci in response to a fusion protein consisting of Aft1p and the DNA binding domain of Gal4p (Casas et al. 1997). They showed that this activity was iron-independent consistent with the inference that Aft1p-dependent transcription is regulated solely by the cytoplasmic-nuclear localization of this transcriptional activator. Second, Yamaguchi and co-workers also identified a nuclear export sequence (NES) in Aft1p; mutation of this sequence led to an $Aft1^{UP}$ phenotype like that observed with the CysXCys mutant reviewed above. This result suggests the possibility that Aft1p localization and hence its *trans*-activation is regulated at the level of nuclear export, but still doesn't address the question as to how this export activity might be modulated by iron status.

Put another way, what species/signal is this Aft1p system actually sensing (by "system," we emphasize not necessarily by Aft1p alone)? The possibilities are: 1) free, labile iron as described above; 2) a relatively stable iron chelate, *e.g.*, an Fe-S cluster or heme; or 3) cell redox status of which the Fe^{2+}/Fe^{3+} couple would be a part. These mechanisms, illustrated in Fig. 5, are not necessarily mutually exclusive signals; in any event, they would be found in the cytoplasm and/or nucleus which for small solutes like these are continuous compartments. The focus to date has been on the role of the CysXCys motif in a direct mechanism in which the signal is sensed by Aft1p (and a comparable role for the Cys-motif in the GATA factors discussed). This is a reasonable hypothesis in that it takes as paradigm the known transcription factors that respond directly to metal status by binding to the metal, *e.g.*, Mac1p and Ace1p, that bind and are regulated by Cu; Zap1p, a Zn sensor; and Hap1p that binds and is regulated by heme (Rutherford and Bird 2004). Addressing only the question of what species or cell state is sensed by Aft1p and not the question of how this signal alters the protein so as to cause its cellular redistribution, we have data that relate to all three of the possibilities listed above. First, the oxygen- and Fe^{2+}-chelator effects described by Hassett *et al*. (described above) suggested that Aft1p responded to Fe^{2+} and/or redox status (Hassett et al. 1998a). The key feature of these responses was that they were observed within 5 minutes, a time-frame consistent with a simple, direct binding/debinding mechanism and, given that a ferrous iron chelator, 2,2'-bipyridyl activated while oxygen inactivated transcription, the species involved certainly could be Fe^{2+}. This inference is consistent with the fact that the iron that recycles from the vacuole is

also likely to be Fe^{2+} since the Smf3 transporter and the Fet5p/Fth1p complex responsible for this efflux use Fe^{2+} as substrate (Portnoy et al. 2000) (Figs. 2 and 5).

On the other hand, Winge, Kaplan, and co-workers observed that in cells lacking Nfs1p (the mitochondrial cysteine desulfurase that supplies the sulfur used in Fe-S cluster biosynthesis, above) the Aft1p regulon was upregulated as in an $Aft1^{UP}$ strain (Chen et al. 2004). They inferred from this phenotype that Aft1p senses Fe-S clusters or a signal arising from an Fe-S cluster enzyme; given the cytoplasmic/nuclear locale, this would refer to non-mitochondrial species. This brings into play the Nar1 protein that Lill and his co-workers have shown is required for maturation of cytosolic/nuclear Fe-S proteins (Balk et al. 2004). An Fe-S protein itself, Nar1p is downstream from the *de novo* synthesis of Fe-S clusters that is reserved to the mitochondria; in other words, Nar1p functions as a cytosolic Fe-S cluster chaperone much as Ssq1p/Jac1p act as Fe-S chaperone in the maturation of mitochondrial Fe-S proteins (Muhlenhoff et al. 2003a; Voisine et al. 2001). As a result of this interdependence, depletion of Nar1p should result in an increase in un-chaperoned cytosolic Fe-S clusters; a decrease in cytosolic/nuclear Fe-S proteins; and a decrease in Nar1p (which is itself an Fe-S protein). One would expect that Nar1p would sample the same Fe-S cluster pool that Kaplan *et al.* propose is sensed by Aft1p, or be responsible for the maturation of the Fe-S proteins that regulate Aft1p if this is the mechanism. Thus, according to the model proposed (Chen et al. 2004) depletion of Nar1p should decrease Aft1p transcription if the *trans*-factor sensed accessible (un-chaperoned) Fe-S clusters, or increased its activity if it sensed (was downregulated) by either Fe-S proteins in general or in specific, *e.g.*, by Nar1p. In fact, depletion of Nar1p elicited no change in iron handling activities including iron uptake into the cell and mitochondrial iron homeostasis (Balk et al. 2004). Lastly, deletion of *CFD1* and *NBP35*, genes that encode proteins required for assembly of cytosolic Fe-S cluster-containing enzymes also, has no effect on expression from the Aft1/Aft2p regulon. Based on these finding, Lill and co-workers conclude that iron sensing by the Aft proteins is not linked to *assembly* of cytosolic Fe-S clusters; they do not and cannot exclude the possibility that the regulation of the iron regulon is linked to mitochondrially-generated clusters, however (Rutherford et al. 2005). In summary, the question mark on the role of Fe-S clusters in yeast iron homeostasis remains, as is indicated in Fig. 5.

Another observation of interest is that the $Aft1^{UP}$-dependent hyperactivation of the iron regulon is suppressed by deletion of *HEM1*, the gene encoding the first step in porphyrin (heme) biosynthesis (Crisp et al. 2003). In the *hem1*Δ strain, Aft1p nuclear localization and DNA binding are not effected, however, indicating that the defect in its transcriptional activity is due to the absence or inactivation of some other factor required for Aft1p function that itself is heme-dependent in some fashion. The work by the Alexandraki lab that demonstrated an essential interaction between Ssn6p and Aft1p while not obviously connected to this heme-dependence does lend some support to the idea that Aft1p relies on other co-activators (Fragiadakis et al. 2004). A complication in understanding the Aft1p phenotype in the *hem1*Δ background is the relatively well-characterized link between heme and Fe-S cluster biosynthesis (Lange et al. 2004). Again, rather than

illuminating the mechanism of Aft1p regulation, these results illustrate the proscription that a cell needs a relatively complex system in order to regulate a highly interdependent metabolic pathway – in this case, iron uptake, trafficking and utilization – and, at this point, we have yet to fully comprehend how this system works.

7.4 Achieving iron homeostasis

Since fungal cells don't efflux iron, cell iron homeostasis is achieved by matching the rate of cell growth and division with the rate of iron uptake. Calculations have indicated that the total iron in a yeast cell (*S. cerevisiae*) is ~60 μM assuming a cell volume of 60 μm^3 (Hassett et al. 1998a). This corresponds to ~3 pmol Fe/10^6 cells. Although the Fe uptake velocity depends on the growth conditions and exocytoplasmic [Fe], typical uptake of iron *via* the Fet3p/Ftr1p high-affinity transport system provides ~5 pmol Fe/10^6/h. Given that the doubling time of a wild type yeast strain in a complete medium is ~1 h, one can conclude that under these conditions the kinetic efficiency (K_M and k_{cat}) of this uptake system; its level of expression (its V_{max}); and the regulatory set-point in relationship to exocytoplasmic [Fe] and corresponding degree of transporter saturation are together matching Fe uptake with cell volume increase so as to maintain a constant total intracellular [Fe].

A similar balancing is on-going within the cell. Arguably, the most dramatic example of this is the subtle but in the aggregate quantitatively significant reduction in the cell's demand for Fe under conditions of iron limitation as a result of the twofold increase in the rate of degradation of nearly 100 transcripts recognized by Cth1p (Puig et al. 2005). The cross-talk between heme and Fe-S cluster biosynthesis is another example. A third example is the recycling of cell components including heme as a means of scavenging iron for maintenance of presumably the most essential iron-dependent functions. Lastly, the vacuole-cytoplasmic and mitochondrial-cytoplasmic Fe-cycling apparent in yeast represents what is likely to be the quantitatively most significant example of the intracellular mechanisms of iron homeostasis that supplement, if only on a short-term basis, the eventual increase in iron accumulation from the environment due to the *trans*-activation of iron uptake activities. An important aspect of this cellular reprogramming upon a shift from normo- to hyponutrient levels of iron is the transient cell cycle arrest in G_1 commonly seen as a first-line response to stressors in yeast. In terms of intracellular [Fe], slowing the rate of cell volume increase is equivalent to increasing the rate of iron uptake.

We are certainly far from knowing all of the details of this intricate but extraordinarily exact balancing act, but from what has been reviewed here the reader can appreciate what the significant unknowns are. Hopefully, this review of our current understanding of iron homeostasis in fungi will provide the spring-board for the future experiments that eventually will fully elucidate the mechanisms by which these and other eukaryotes match environmental iron availability to organ-

ismal iron requirement. As has been the case so far, what we learn from fungi will undoubtedly serve as a paradigm for what we ultimately know about ourselves.

Acknowledgement

The work on iron in yeast carried out in this laboratory, some of which has been described in this review, is supported by a grant from the National Institutes of Health, USA, DK53820.

References

Ali A, Zhang Q, Dai J, Huang X (2003) Calcein as a fluorescent iron chemosensor for the determination of low molecular weight iron in biological fluids. Biometals 16:285-293

Ardon O, Bussey H, Philpott C, Ward DM, Davis-Kaplan S, Verroneau S, Jiang B, Kaplan J (2001) Identification of a *Candida albicans* ferrichrome transporter and its characterization by expression in *Saccharomyces cerevisiae*. J Biol Chem 276:43049-43055

Ardon O, Nudelman R, Caris C, Libman J, Shanzer A, Chen Y, Hadar Y (1998) Iron uptake in *Ustilago maydis*: tracking the iron path. J Bacteriol 180:2021-2026

Askwith C, Eide D, Van Ho A, Bernard PS, Li L, Davis-Kaplan S, Sipe DM, Kaplan J (1994) The FET3 gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. Cell 76:403-410

Askwith C, Kaplan J (1997) An oxidase-permease-based iron transport system in *Schizosaccharomyces pombe* and its expression in *Saccharomyces cerevisiae*. J Biol Chem 272:401-405

Askwith C, Kaplan J (1998) Iron and copper transport in yeast and its relevance to human disease. Trends Biochem Sci 23:135-138

Balk J, Pierik AJ, Netz DJ, Muhlenhoff U, Lill R (2004) The hydrogenase-like Nar1p is essential for maturation of cytosolic and nuclear iron-sulphur proteins. EMBO J 23:2105-2115

Bard M, Bruner DA, Pierson CA, Lees ND, Biermann B, Frye L, Koegel C, Barbuch R (1996) Cloning and characterization of ERG25, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase. Proc Natl Acad Sci USA 93:186-190

Bode HP, Dumschat M, Garotti S, Fuhrmann GF (1995) Iron sequestration by the yeast vacuole. A study with vacuolar mutants of *Saccharomyces cerevisiae*. Eur J Biochem 228:337-342

Boukhalfa H, Crumbliss AL (2002) Chemical aspects of siderophore mediated iron transport. Biometals 15:325-339

Broadwater JA, Haas JA, Fox BG (1998) The fundamental, versatile role of diiron enzymes in lipid metabolism. Fett/Lipid 100:103-113

Buchanan SK, Smith BS, Venkatramani L, Xia D, Esser L, Palnitkar M, Chakraborty R, van der Helm D, Deisenhofer J (1999) Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. Nat Struct Biol 6:56-63

Bulteau AL, O'Neill HA, Kennedy MC, Ikeda-Saito M, Isaya G, Szweda LI (2004) Frataxin acts as an iron chaperone protein to modulate mitochondrial aconitase activity. Science 305:242-245

Casas C, Aldea M, Espinet C, Gallego C, Gil R, Herrero E (1997) The AFT1 transcriptional factor is differentially required for expression of high-affinity iron uptake genes in *Saccharomyces cerevisiae*. Yeast 13:621-637

Chen OS, Crisp RJ, Valachovic M, Bard M, Winge DR, Kaplan J (2004) Transcription of the yeast iron regulon does not respond directly to iron but rather to iron-sulfur cluster biosynthesis. J Biol Chem 279:29513-29518

Chen OS, Kaplan J (2000) CCC1 suppresses mitochondrial damage in the yeast model of Friedreich's ataxia by limiting mitochondrial iron accumulation. J Biol Chem 275:7626-7632

Clarke TE, Braun V, Winkelmann G, Tari LW, Vogel HJ (2002) X-ray crystallographic structures of the *Escherichia coli* periplasmic protein FhuD bound to hydroxamate-type siderophores and the antibiotic albomycin. J Biol Chem 277:13966-13972

Claros MG, Vincens P (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 241:779-786

Crisp RJ, Pollington A, Galea C, Jaron S, Yamaguchi-Iwai Y, Kaplan J (2003) Inhibition of heme biosynthesis prevents transcription of iron uptake genes in yeast. J Biol Chem 278:45499-45506

Dailey HA (2002) Terminal steps of haem biosynthesis. Biochem Soc Trans 30:590-595

Dailey HA, Dailey TA, Wu CK, Medlock AE, Wang KF, Rose JP, Wang BC (2000) Ferrochelatase at the millennium: structures, mechanisms and [2Fe-2S] clusters. Cell Mol Life Sci 57:1909-1926

Dailey HA, Finnegan MG, Johnson MK (1994) Human ferrochelatase is an iron-sulfur protein. Biochemistry 33:403-407

Dancis A, Roman DG, Anderson GJ, Hinnebusch AG, Klausner RD (1992) Ferric reductase of *Saccharomyces cerevisiae*: Molecular characterization, role in iron uptake and transcriptional control by iron. Proc Natl Acad Sci USA 89:3869-3873

Davie JK, Edmondson DG, Coco CB, Dent SY (2003) Tup1-Ssn6 interacts with multiple class I histone deacetylases *in vivo*. J Biol Chem 278:50158-50162

Davis-Kaplan SR, Askwith CC, Bengtzen AC, Radisky D, Kaplan J (1998) Chloride is an allosteric effector of copper assembly for the yeast multicopper oxidase Fet3p: an unexpected role for intracellular chloride channels. Proc Natl Acad Sci USA 95:13641-13645

De Luca NG, Wood PM (2000) Iron uptake by fungi: contrasted mechanisms with internal or external reduction. Adv Micro Physiol 43:39-74

de Silva DM, Askwith CC, Eide D, Kaplan J (1995) The FET3 gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. J Biol Chem 270:1098-1101

Diaz AR, Mansilla MC, Vila AJ, de Mendoza D (2002) Membrane topology of the acyl-lipid desaturase from *Bacillus subtilis*. J Biol Chem 277:48099-48106

Dix D, Bridgham J, Broderius M, Eide D (1997) Characterization of the FET4 protein of yeast. Evidence for a direct role in the transport of iron. J Biol Chem 272:11770-11777

Dix DR, Bridgham JT, Broderius MA, Byersdorfer CA, Eide DJ (1994) The FET4 gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae*. J Biol Chem 269:26092-26099

Dutkiewicz R, Schilke B, Cheng S, Knieszner H, Craig EA, Marszalek J (2004) Sequence-specific interaction between mitochondrial Fe-S scaffold protein Isu and Hsp70 Ssq1 is essential for their *in vivo* function. J Biol Chem 279:29167-29174

Eck R, Hundt S, Härtl A, Roemer E, Künkel W (1999) A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption. Microbiol 145:2415-2422

Eisendle M, Oberegger H, Buttinger R, Illmer P, Haas H (2004) Biosynthesis and uptake of siderophores is controlled by the PacC-mediated ambient-pH regulatory system in *Aspergillus nidulans*. Eukaryot Cell 3:561-563

Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik I (1997) Fluorescence analysis of the labile iron pool of mammalian cells. Anal Biochem 248:31-40

Fagerstrom-Billai F, Wright AP (2005) Functional comparison of the tup11 and tup12 transcriptional corepressors in fission yeast. Mol Cell Biol 25:716-727

Ferguson AD, Hofmann E, Coulton JW, Diederichs K, Welte W (1998) Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science 282:2215-2220

Foster LA (2002) Utilization and cell-surface binding of hemin by *Histoplasma capsulatum*. Can J Microbiol 48:437-442

Foury F, Roganti T (2002) Deletion of the mitochondrial carrier genes MRS3 and MRS4 suppresses mitochondrial iron accumulation in a yeast frataxin-deficient strain. J Biol Chem 277:24475-24483

Foury F, Talibi D (2001) Mitochondrial control of iron homeostasis. A genome wide analysis of gene expression in a yeast frataxin-deficient strain. J Biol Chem 276:7762-7768

Fragiadakis GS, Tzamarias D, Alexandraki D (2004) Nhp6 facilitates Aft1 binding and Ssn6 recruitment, both essential for FRE2 transcriptional activation. EMBO J 23:333-342

Frazzon J, Fick JR, Dean DR (2002) Biosynthesis of iron-sulphur clusters is a complex and highly conserved process. Biochem Soc Trans 30:680-685

Gagiano M, Bauer FF, Pretorius IS (2002) The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*. FEMS Yeast Res 2:433-470

Gaxiola RA, Yuan DS, Klausner RD, Fink GR (1998) The yeast CLC chloride channel functions in cation homeostasis. Proc Natl Acad Sci USA 95:4046-4050

Ge J, Perlstein DL, Nguyen HH, Bar G, Griffin RG, Stubbe J (2001) Why multiple small subunits (Y2 and Y4) for yeast ribonucleotide reductase? Toward understanding the role of Y4. Proc Natl Acad Sci USA 98:10067-10072

Georgatsou E, Alexandraki D (1994) Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Saccharomyces cerevisiae*. Mol Cell Biol 14:3065-3073

Gerber J, Muhlenhoff U, Lill R (2003) An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1. EMBO Rep 4:906-911

Gerber J, Neumann K, Prohl C, Muhlenhoff U, Lill R (2004) The yeast scaffold proteins Isu1p and Isu2p are required inside mitochondria for maturation of cytosolic Fe/S proteins. Mol Cell Biol 24:4848-4857

Greene JR, Brown NH, DiDomenico BJ, Kaplan J, Eide DJ (1993) The GEF1 gene of *Saccharomyces cerevisiae* encodes an integral membrane protein; mutations in which have effects on respiration and iron-limited growth. Mol Gen Genet 241:542-553

Haas H (2003) Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage. Appl Microbiol Biotechnol 62:316-330

Haas H, Schoeser M, Lesuisse E, Ernst JF, Parson W, Abt B, Winkelmann G, Oberegger H (2003) Characterization of the *Aspergillus nidulans* transporters for the siderophores enterobactin and triacetylfusarinine C. Biochem J 371:505-513

Haas H, Zadra I, Stoeffler G, Angermayr K (1999) The *Aspergillus nidulans* GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake. J Biol Chem 274:4613-4619

Halliwell B, Gutteridge JMC (1992) Biologically relevant metal ion-dependent hydroxyl radical generation - an update. Febs Letters 307:108-112

Harrison KA, Marzluf GA (2002) Characterization of DNA binding and the cysteine rich region of SRE, a GATA factor in *Neurospora crassa* involved in siderophore synthesis. Biochemistry 41:15288-15295

Hassett R, Kosman DJ (1995) Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. J Biol Chem 270:128-134

Hassett RF, Romeo AM, Kosman DK (1998a) Regulation of high affinity iron uptake in the yeast, *Saccharomyces cerevisiae*. Role of dioxygen and Fe(II). J Biol Chem 273:7628-7636

Hassett RF, Yuan DS, Kosman DJ (1998b) Spectral and kinetic properties of the Fet3 protein from *Saccharomyces cerevisiae*, a multinuclear copper ferroxidase enzyme. J Biol Chem 273:23274-23282

He Y, Alam SL, Proteasa SV, Zhang Y, Lesuisse E, Dancis A, Stemmler TL (2004) Yeast frataxin solution structure, iron binding, and ferrochelatase interaction. Biochemistry 43:16254-16262

Heymann P, Ernst JF, Winkelmann G (1999) Identification of a fungal triacetylfusarinine C siderophore transport gene (TAF1) in Saccharomyces cerevisiae as a member of the major facilitator superfamily. Biometals 12:301-306

Heymann P, Ernst JF, Winkelmann G (2000a) A gene of the major facilitator superfamily encodes a transporter for enterobactin (Enb1p) in *Saccharomyces cerevisiae*. Biometals 13:65-72

Heymann P, Ernst JF, Winkelmann G (2000b) Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 186:221-227

Heymann P, Gerads M, Schaller M, Dromer F, Winkelmann G, Ernst JF (2002) The siderophore iron transporter of *Candida albicans* (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. Infect Immun 70:5246-5255

Howard DH (2004) Iron gathering by zoopathogenic fungi. FEMS Immunol Med Microbiol 40:95-100

Hu CJ, Bai C, Zheng XD, Wang YM, Wang Y (2002) Characterization and functional analysis of the siderophore-iron transporter CaArn1p in *Candida albicans*. J Biol Chem 277:30598-30605

Huang M, Elledge SJ (1997) Identification of RNR4, encoding a second essential small subunit of ribonucleotide reductase in *Saccharomyces cerevisiae*. Mol Cell Biol 17:6105-6113

Huffman DL, O'Halloran TV (2001) Function, structure, and mechanism of intracellular copper trafficking proteins. Annu Rev Biochem 70:677-701

Huschka H, Naegeli HU, Leuenberger-Ryf H, Keller-Schierlein W, Winkelmann G (1985) Evidence for a common siderophore transport system but different siderophore receptors in *Neurospora crassa*. J Bacteriol 162:715-721

Kaplan J (2002) Mechanisms of cellular iron acquisition: another iron in the fire. Cell 111:603-606

Kim Y, Yun CW, Philpott CC (2002) Ferrichrome induces endosome to plasma membrane cycling of the ferrichrome transporter, Arn1p, in *Saccharomyces cerevisiae*. EMBO J 21:3632-3642

Kispal G, Csere P, Guiard B, Lill R (1997) The ABC transporter Atm1p is required for mitochondrial iron homeostasis. FEBS Lett 418:346-350

Kispal G, Csere P, Prohl C, Lill R (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. Embo J 18:3981-3989

Knight SA, Lesuisse E, Stearman R, Klausner RD, Dancis A (2002) Reductive iron uptake by *Candida albicans*: role of copper, iron and the TUP1 regulator. Microbiol 148:29-40

Knight SA, Sepuri NB, Pain D, Dancis A (1998) Mt-Hsp70 homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis. J Biol Chem 273:18389-18393

Kosman DJ (2003) Molecular mechanisms of iron uptake in fungi. Mol Microbiol 47:1185-1197

La Fontaine S, Quinn JM, Nakamoto SS, Page MD, Gohre V, Moseley JL, Kropat J, Merchant S (2002) Copper-dependent iron assimilation pathway in the model photosynthetic eukaryote *Chlamydomonas reinhardtii*. Eukaryot Cell 1:736-757

Labbe S, Pena MM, Fernandes AR, Thiele DJ (1999) A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. J Biol Chem 274:36252-36260

Labbe-Bois R (1990) The ferrochelatase from *Saccharomyces cerevisiae*. Sequence, disruption, and expression of its structural gene HEM15. J Biol Chem 265:7278-7283

Lan CY, Rodarte G, Murillo LA, Jones T, Davis RW, Dungan J, Newport G, Agabian N (2004) Regulatory networks affected by iron availability in *Candida albicans*. Mol Microbiol 53:1451-1469

Lange H, Kispal G, Lill R (1999) Mechanism of iron transport to the site of heme synthesis inside yeast mitochondria. J Biol Chem 274:18989-18996

Lange H, Lisowsky T, Gerber J, Muhlenhoff U, Kispal G, Lill R (2001) An essential function of the mitochondrial sulfhydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins. EMBO Rep 2:715-720

Lange H, Muhlenhoff U, Denzel M, Kispal G, Lill R (2004) The heme synthesis defect of mutants impaired in mitochondrial iron-sulfur protein biogenesis is caused by reversible inhibition of ferrochelatase. J Biol Chem 279:29101-29108

Lee J, Hofhaus G, Lisowsky T (2000) Erv1p from *Saccharomyces cerevisiae* is a FAD-linked sulfhydryl oxidase. FEBS Lett 477:62-66

Lesuisse E, Blaiseau P-L, Dancis A, Camadro J-M (2001) Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*. Microbiol 147:289-298

Lesuisse E, Knight SA, Camadro JM, Dancis A (2002) Siderophore uptake by *Candida albicans*: effect of serum treatment and comparison with *Saccharomyces cerevisiae*. Yeast 19:329-340

Lesuisse E, Lyver ER, Knight SA, Dancis A (2004) Role of YHM1, encoding a mitochondrial carrier protein, in iron distribution of yeast. Biochem J 378:599-607

Lesuisse E, Santos R, Matzanke BF, Knight SA, Camadro JM, Dancis A (2003) Iron use for haeme synthesis is under control of the yeast frataxin homologue (Yfh1). Hum Mol Genet 12:879-889

Lesuisse E, Simon-Casteras M, Labbe P (1998) Siderophore-mediated iron uptake in *Saccharomyces cerevisiae*: the SIT1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. Microbiol 144:3455-3462

Li L, Chen OS, McVey Ward D, Kaplan J (2001) CCC1 is a transporter that mediates vacuolar iron storage in yeast. J Biol Chem 276:29515-29519

Li L, Kaplan J (1997) Characterization of two homologous yeast genes that encode mitochondrial iron transporters. J Biol Chem 272:28485-28493

Li L, Kaplan J (2004) A mitochondrial-vacuolar signaling pathway in yeast that affects iron and copper metabolism. J Biol Chem 279:33653-33661

Lill R, Kispal G (2001) Mitochondrial ABC transporters. Res Microbiol 152:331-340

Lowry JA, Atchley WR (2000) Molecular evolution of the GATA family of transcription factors: conservation within the DNA-binding domain. J Mol Evol 50:103-115

Luk E, Jensen LT, Culotta VC (2003) The many highways for intracellular trafficking of metals. J Biol Inorg Chem 8:803-809

Martins LJ, Jensen LT, Simon JR, Keller GL, Winge DR (1998) Metalloregulation of FRE1 and FRE2 homologs in *Saccharomyces cerevisiae*. J Biol Chem 273:23716-23721

Marvin ME, Mason RP, Cashmore AM (2004) The CaCTR1 gene is required for high-affinity iron uptake and is transcriptionally controlled by a copper-sensing transactivator encoded by CaMAC1. Microbiol 150:2197-2208

Matzanke BF, Anemuller S, Schunemann V, Trautwein AX, Hantke K (2004) FhuF, part of a siderophore-reductase system. Biochemistry 43:1386-1392

Medlock AE, Dailey HA (2000) Examination of the activity of carboxyl-terminal chimeric constructs of human and yeast ferrochelatases. Biochemistry 39:7461-7467

Muhlenhoff U, Balk J, Richhardt N, Kaiser JT, Sipos K, Kispal G, Lill R (2004) Functional characterization of the eukaryotic cysteine desulfurase Nfs1p from *Saccharomyces cerevisiae*. J Biol Chem 279:36906-36915

Muhlenhoff U, Gerber J, Richhardt N, Lill R (2003a) Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. EMBO J 22:4815-4825

Muhlenhoff U, Richhardt N, Gerber J, Lill R (2002) Characterization of iron-sulfur protein assembly in isolated mitochondria. A requirement for ATP, NADH, and reduced iron. J Biol Chem 277:29810-29816

Muhlenhoff U, Stadler JA, Richhardt N, Seubert A, Eickhorst T, Schweyen RJ, Lill R, Wiesenberger G (2003b) A specific role of the yeast mitochondrial carriers MRS3/4p in mitochondrial iron acquisition under iron-limiting conditions. J Biol Chem 278:40612-40620

Napier I, Ponka P, Richardson DR (2004) Iron trafficking in the mitochondrion: novel pathways revealed by disease. Blood

Neilands JB (1995) Siderophores: structure and function of microbial iron transport compounds. J Biol Chem 270:26723-26726

Nelson N (1999) Metal ion transporters and homeostasis. EMBO J 18:4361-4371

Nguyen HH, Ge J, Perlstein DL, Stubbe J (1999) Purification of ribonucleotide reductase subunits Y1, Y2, Y3, and Y4 from yeast: Y4 plays a key role in diiron cluster assembly. Proc Natl Acad Sci USA 96:12339-12344

Oberegger H, Schoeser M, Zadra I, Abt B, Haas H (2001) SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*. Mol Microbiol 41:1077-1089

Oberegger H, Schoeser M, Zadra I, Schrettl M, Parson W, Haas H (2002a) Regulation of freA, acoA, lysF, and cycA expression by iron availability in *Aspergillus nidulans*. Appl Environ Microbiol 68:5769-5772

Oberegger H, Zadra I, Schoeser M, Abt B, Parson W, Haas H (2002b) Identification of members of the *Aspergillus nidulans* SREA regulon: genes involved in siderophore biosynthesis and utilization. Biochem Soc Trans 30:781-783

Pelletier B, Beaudoin J, Mukai Y, Labbe S (2002) Fep1, an iron sensor regulating iron transporter gene expression in *Schizosaccharomyces pombe*. J Biol Chem 277:22950-22958

Pelletier B, Beaudoin J, Philpott CC, Labbe S (2003) Fep1 represses expression of the fission yeast *Schizosaccharomyces pombe* siderophore-iron transport system. Nucleic Acids Res 31:4332-4344

Philpott CC, Protchenko O, Kim YW, Boretsky Y, Shakoury-Elizeh M (2002) The response to iron deprivation in *Saccharomyces cerevisiae:* expression of siderophore-based systems of iron uptake. Biochem Soc Trans 30:698-702

Portnoy ME, Liu XF, Culotta VC (2000) *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the nramp family of metal transporters. Mol Cell Biol 20:7893-7902

Protchenko O, Ferea T, Rashford J, Tiedeman J, Brown PO, Botstein D, Philpott CC (2001) Three cell wall mannoproteins facilitate the uptake of iron in *Saccharomyces cerevisiae*. J Biol Chem 276:49244-49250

Protchenko O, Philpott CC (2003) Regulation of intracellular heme levels by HMX1, a homologue of heme oxygenase, in *Saccharomyces cerevisiae*. J Biol Chem 278:36582-36587

Puig S, Askeland E, Thiele DJ (2005) Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. Cell 120:99-110

Puig S, Lau M, Thiele DJ (2004) Cti6 is an Rpd3-Sin3 histone deacetylase-associated protein required for growth under iron-limiting conditions in *Saccharomyces cerevisiae*. J Biol Chem 279:30298-30306

Radisky DC, B. SW, Emr SD, Kaplan J (1997) Characterization of VPS41, a gene required for vacuolar trafficking and high-affinity iron transport in yeast. Proc Natl Acad Sci USA 94:5662-5666

Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 284:805-808

Raguzzi F, Lesuisee E, Crichton RR (1988) Iron storage in *Saccharomyces cerevisiae*. FEBS Lett 231:253-258

Ramanan N, Wang Y (2000) A high-affinity iron permease essential for *Candida albicans* virulence. Science 288:1062-1064

Ramazzotti A, Vanmansart V, Foury F (2004) Mitochondrial functional interactions between frataxin and Isu1p, the iron-sulfur cluster scaffold protein, in *Saccharomyces cerevisiae*. FEBS Lett 557:215-220

Roman Dragos G, Dancis A, Anderson GJ, Klausner R, D. (1993) The fission yeast ferric reductase gene frp1 positive is required for ferric iron uptake and encodes a protein that is homologous to the gp91-phox subunit of the human NADPH phagocyte oxidoreductase. Mol Cell Biol 13:4342-4350

Rouault TA (2004) Microbiology. Pathogenic bacteria prefer heme. Science 305:1577-1578

Rutherford JC, Bird AJ (2004) Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. Eukaryot Cell 3:1-13

Rutherford JC, Jaron S, Ray E, Brown PO, Winge DR (2001) A second iron-regulatory system in yeast independent of Aft1p. Proc Natl Acad Sci USA 98:14322-14327

Rutherford JC, Jaron S, Winge DR (2003) Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. J Biol Chem 278:27636-27643

Rutherford JC, Ojeda L, Balk J, Muhlenhoff U, Lill R, Winge DR (2005) Activation of the iron regulon by the yeast Aft1/Aft2 transcription factors depends on mitochondrial, but not cytosolic iron-sulfur protein biogenesis. J Biol Chem published on-line

Santos R, Buisson N, Knight S, Dancis A, Camadro JM, Lesuisse E (2003) Haemin uptake and use as an iron source by *Candida albicans*: role of CaHMX1-encoded haem oxygenase. Microbiol 149:579-588

Sauer M, Hantke K, Braun V (1990) Sequence of the fhuE outer-membrane receptor gene of *Escherichia coli* K12 and properties of mutants. Mol Microbiol 4:427-437

Schroder I, Johnson E, de Vries S (2003) Microbial ferric iron reductases. FEMS Microbiol Rev 27:427-447

Schwappach B, Stobrawa S, Hechenberger M, Steinmeyer K, Jentsch TJ (1998) Golgi localization and functionally important domains in the NH2 and COOH terminus of the yeast CLC putative chloride channel Gef1p. J Biol Chem 273:15110-15118

Severance S, Chakraborty S, Kosman DJ (2004) The Ftr1p iron permease in the yeast plasma membrane: orientation, topology and structure-function relationships. Biochem J 380:487-496

Shakoury-Elizeh M, Tiedeman J, Rashford J, Ferea T, Demeter J, Garcia E, Rolfes R, Brown PO, Botstein D, Philpott CC (2004) Transcriptional remodeling in response to iron deprivation in *Saccharomyces cerevisiae*. Mol Biol Cell 15:1233-1243

Smith RL, Johnson AD (2000) Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. Trends Biochem Sci 25:325-330

Srinivasan C, Liba A, Imlay JA, Valentine JS, Gralla EB (2000) Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. J Biol Chem 275:29187-29192

Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A (1996) A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science 271:1552-1557

Stubbe J, Riggs-Gelasco P (1998) Harnessing free radicals: formation and function of the tyrosyl radical in ribonucleotide reductase. Trends Biochem Sci 23:438-443

Tan PK, Howard JP, Payne GS (1996) The sequence NPFXD defines a new class of endocytosis signal in *Saccharomyces cerevisiae*. J Cell Biol 135:1789-1800

Thomas F, Serratrice G, Beguin C, Aman ES, Pierre JL, Fontecave M, Laulhere JP (1999) Calcein as a fluorescent probe for ferric iron. Application to iron nutrition in plant cells. J Biol Chem 274:13375-13383

Timmerman MM, Woods JP (1999) Ferric reduction is a potential iron acquisition mechanism for *Histoplasma capsulatum*. Infect Immun 67:6403-6408

Timmerman MM, Woods JP (2001) Potential role for extracellular glutathione-dependent ferric reductase in utilization of environmental and host ferric compounds by Histoplasma capsulatum. Infect Immun 69:7671-7678

Ueta R, Fukunaka A, Yamaguchi-Iwai Y (2003) Pse1p mediates the nuclear import of the iron-responsive transcription factor Aft1p in *Saccharomyces cerevisiae*. J Biol Chem 278:50120-50127

Urbanowski JL, Piper RC (1999) The iron transporter Fth1p forms a complex with the Fet5 iron oxidase and resides on the vacuolar membrane. J Biol Chem 274:38061-38070

Van Ho A, Ward DM, Kaplan J (2002) Transition metal transport in yeast. Annu Rev Microbiol 56:237-261

Wang TP, Quintanar L, Severance S, Solomon EI, Kosman DJ (2003) Targeted suppression of the ferroxidase and iron trafficking activities of the multicopper oxidase Fet3p from *Saccharomyces cerevisiae*. J Biol Inorg Chem 8:611-620

Weissman Z, Kornitzer D (2004) A family of *Candida* cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. Mol Microbiol 53:1209-1220

Winkelmann G (2002) Microbial siderophore-mediated transport. Biochem Soc Trans 30:691-696

Voegtli WC, Ge J, Perlstein DL, Stubbe J, Rosenzweig AC (2001) Structure of the yeast ribonucleotide reductase Y2Y4 heterodimer. Proc Natl Acad Sci USA 98:10073-10078

Voisard C, Wang J, McEvoy JL, Xu P, Leong SA (1993) urbs1, a gene regulating siderophore biosynthesis in *Ustilago maydis*, encodes a protein similar to the erythroid transcription factor GATA-1. Mol Cell Biol 13:7091-7100

Voisine C, Cheng YC, Ohlson M, Schilke B, Hoff K, Beinert H, Marszalek J, Craig EA (2001) Jac1, a mitochondrial J-type chaperone, is involved in the biogenesis of Fe/S clusters in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 98:1483-1488

Vozza A, Blanco E, Palmieri L, Palmieri F (2004) Identification of the mitochondrial GTP/GDP transporter in *Saccharomyces cerevisiae*. J Biol Chem 279:20850-20857

Yamaguchi-Iwai Y, Dancis A, Klausner RD (1995) AFT1: A mediator of iron-regulated transcriptional control in *Saccharomyces cerevisiae*. EMBO J 14:1231-1239

Yamaguchi-Iwai Y, Serpe M, Haile D, Yang W, Kosman DJ, Klausner RD, Dancis A (1997) Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. J Biol Chem 272:17711-17718

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner RD (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. EMBO J 15:3377-3384

Yamaguchi-Iwai Y, Ueta R, Fukunaka A, Sasaki R (2002) Subcellular localization of Aft1 transcription factor responds to iron status in *Saccharomyces cerevisiae*. J Biol Chem 277:18914-18918

Yoon T, Cowan JA (2003) Iron-sulfur cluster biosynthesis. Characterization of frataxin as an iron donor for assembly of [2Fe-2S] clusters in ISU-type proteins. J Am Chem Soc 125:6078-6084

Yoon T, Cowan JA (2004) Frataxin-mediated iron delivery to ferrochelatase in the final step of heme biosynthesis. J Biol Chem 279:25943-25946

Yun C-W, Bauler M, Moore RE, Klebba PE, Philpott CC (2001) The role of the FRE family of plasma membrane reductases in the uptake of siderophore-iron in *Saccharomyces cerevisiae*. J Biol Chem 276:10218-10223

Yun C-W, Tiedeman J, Moore RE, Philpott CC (2000) Siderophore-iron uptake in *Saccharomyces cerevisiae*. Identification of ferrichrome and fusarinine transporters. J Biol Chem 275:16354-16359

Zhou L, Marzluf GA (1999) Functional analysis of the two zinc fingers of SRE, a GATA-type factor that negatively regulates siderophore synthesis in *Neurospora crassa*. Biochemistry 38:4335-4341

Zhou L-W, Haas H, Marzluf GA (1998) Isolation and characterization of a new gene, sre, which encodes a GATA-type regulatory protein that controls iron transport in *Neurospora crassa*. Mol Gen Gen 259:532-540

Abbreviations

PM: plasma membrane
FOB: ferrioxamine B
FC: ferrichrome
TAF: triacetylfusainine C
GFP: green fluorescent protein
SGD: *Saccharomyces* Genome Database (http://www.yeastgenome.org/)
ER: endoplasmic reticulum
BIP: 2,2'-bipyridyl
DFO: desferrioxamine
BPS: bathophenanthroline disulfonic acid
WT: wild type.

Kosman, Daniel
Department of Biochemistry, The University at Buffalo, Department of Biochemistry, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214, USA
camkos@buffalo.edu
Kwok, Ernest
Department of Biochemistry, The University at Buffalo, Department of Biochemistry, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214, USA

Copper in mammals: mechanisms of homeostasis and pathophysiology

Michael A. Cater and Julian F.B. Mercer

Abstract

The ability of mammals to tightly regulate systemic copper levels is vital for health as demonstrated by the severity of the genetic copper deficiency and copper toxicity disorders, Menkes disease and Wilson disease, respectively. Analysis of these genetic disorders has led to a substantial increase in the understanding of the role of copper in health and disease. The isolation of the genes involved in these diseases and use of yeast mutants with altered copper and iron homeostasis has revealed a range of molecular mechanisms governing copper homeostasis. These mechanisms include regulation of cellular copper uptake and efflux and involve the use of chaperones for safe intracellular copper distribution. Here we provide an overview of the physiological role of copper and the molecular mechanisms regulating systemic and cellular copper levels in mammals. Furthermore, we discuss the pathophysiological mechanisms and consequences of copper deficiency/overload in relation to disease.

1 The biochemical properties of copper

Copper exists physiologically in two redox states, as cuprous Cu^{1+} (reduced) or cupric Cu^{2+} (oxidized) and can interchange between these forms by accepting or donating an electron. This allows the cation to participate in biochemical reactions as a reducing or oxidizing agent (Alberts et al. 1995). In mammals there are over 30 known proteins that bind copper (Solioz 1998), and many of these proteins are enzymes that utilize copper as a cofactor in single-electron-reactions. Several of the important copper dependent enzymes are shown in Table 1. Despite the oxidative capacity of copper being essential for various enzymatic reactions, this property also makes the cation potentially toxic. Ionic copper can catalyse the production of free radicals, in particular, the highly reactive hydroxyl radical through Fenton chemistry, which subsequently can damage lipids, proteins, DNA and other biomolecules (Yoshida et al. 1993). Therefore, it is imperative that the level of copper in the body is strictly regulated and that the delivery of copper to enzymes that require the cation occurs in a manner that avoids oxidative damage.

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_101 / Published online: 11 August 2005

Table 1. Mammalian copper-dependent proteins

Common name	Major localization	Enzymatic function	Consequence of deficiency/defect
Ceruloplasmin	Plasma	Converts ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) through ferroxidase activity	Defective iron transport and metabolism, anemia, haemosiderosis
Lysyl Oxidase	Extracellular fluid, cartilage, bone and blood	Connective tissue synthesis (cross-linking of collagen and elastin)	Connective and skeletal tissue defects resulting in arterial weakness, bladder diverticulae, loose skin and joints, osteoporosis, emphysema
Tyrosinase	Melanocytes of eye and skin	Pigment (melanin) synthesis	Depigmentation
Dopamine-β-hydroxylase	Catecholamine storage vesicles in neuron synapses	Neurotransmitter synthesis, conversion of dopamine to acetylcholine (noradrenaline)	Hypothalamic imbalance resulting in hypothermia, anorexia, respiratory failure, somnolence, dehydration, ataxia
Cu/Zn superoxide dismutase (SOD)	The cytoplasm and mitochondria	Free radical detoxification, dismutation of superoxide radicals	Oxidative stress to cellular component resulting in central nervous system degeneration and mitochondrial defects
Cytochrome c oxidase	Inner mitochondrial membrane	Electron-transport enzyme	Deficient energy (ATP) production, altered nerve conduction, myopathy, ataxia, seizures

Adapted from (Pena *et al.*, 1999)

2 Physiological copper homeostasis

The extensive early literature on the physiology of copper can now begin to be interpreted given the recent discoveries of the molecular components of the copper homeostatic mechanisms. Here we present only a summary of the overall process of copper transport, for more detailed information, the early work is well summarized in a number of reviews (Evans 1973; Linder 1991; Danks 1995). More recently, reviews incorporating the molecular advances have appeared (Vulpe and Packman 1995; Pena et al. 1999; Harris 2000).

2.1 Copper absorption from the diet

The copper content of the human body ranges from 50-120mg (Sass-Kortsak 1965) with the average adult containing 75mg (Tipton and Cook 1963). Copper balance studies in volunteer human subjects have indicated a minimum requirement of 1.5-2mg per day (Lonnerdal 1996). The absorption of dietary copper occurs predominantly in the duodenum where the cation is transferred across the brush border into the cells of the intestinal mucosa (enterocytes) (Van Campen 1971). The bioavailability of copper from the diet depends on a variety of factors including the presence of other metals and dietary components (Linder et al. 1999). The amount of copper absorbed from the diet is regulated to a limited extent by absorption increasing when copper ingestion is low and decreasing with high dietary intakes (Turnlund et al. 1998). The mechanism of uptake across the apical surface of the enterocyte (from intestinal lumen) has not been established. One candidate molecule in mammals is Ctr1, a ubiquitously expressed transmembrane protein, which is known to be involved in copper uptake in a range of cells (Moller et al. 2000; Kuo et al. 2001). Another candidate transporter in the brush border is DMT1 (Nramp2), a divalent metal transporter that has been shown to mediate the cellular uptake of Cu^{2+}, Fe^{2+}, Zn^{2+}, and Mn^{2+} (Gunshin et al. 1997). However, DMT1 has been reported to mediate copper uptake only when sufficient amounts of the cation are present and/or when other metal cations are absent. Recent data from Knopfel and colleagues however, provide evidence that DMT1 is an ATP-dependent high affinity copper transporter in rat intestines and these authors suggest this may be the primary uptake mechanism (Knopfel et al. 2005). Copper that enters the intestinal mucosal cells is transferred across the basolateral membrane into the hepatic-portal circulation for systemic uptake. This step requires energy and is mediated by ATP7A, the protein affected in the X-linked copper deficiency disorder, Menkes disease (see Section 4.1).

2.2 Copper in the general circulation

The level of copper in the serum of adults is maintained at approximately 17-27μM (Versieck and Cornelis 1980). Plasma copper is bound to albumin, a macroglobulin termed transcuprein, and ceruloplasmin (Owen 1965; Harris and Sass-Kortsak 1967; Goode et al. 1989; Linder et al. 1998). Although albumin is by far the most abundant protein, the majority of serum copper is coordinated with ceruloplasmin (~70%) (Wirth and Linder 1985). Only 12% of serum copper is bound to albumin and transcuprein and traces are associated with a variety of enzymes, such as clotting factors, and low-molecular-weight proteins. The liver is the initial repository for newly absorbed copper and most of this copper is either secreted into plasma bound to ceruloplasmin or excreted in bile (Section 2.3). The role of ceruloplasmin in copper homeostasis is controversial. Given that the majority of plasma copper is bound to ceruloplasmin and that specific receptors for ceruloplasmin have been identified on the plasma membranes of various non-hepatic cell types, suggests that ceruloplasmin may play a role in the distribution of cop-

per to peripheral tissue (Barnes and Frieden 1984; Kataoka and Tavassoli 1985; Orena et al. 1986). There is also evidence that ceruloplasmin plays a role in copper delivery to the rat fetus (Lee et al. 1993). However, aceruloplasminemia patients display an iron accumulation phenotype and do not show any symptoms of disturbed copper homeostasis, which is strong evidence that ceruloplasmin is not an essential copper transport protein (Harris et al. 1995; Yoshida et al. 1995; Meyer et al. 2001). Furthermore, a ceruloplasmin knockout mouse also manifest defects in iron and not copper homeostasis (Harris et al. 1999). Therefore, the role of ceruloplasmin as a copper transporter is unclear and if indeed involved then its function seems redundant. Similarly, systemic copper distribution in patients with analbuminemia and in Nagase rats lacking albumin is not compromised (Vargas et al. 1994; Watkins et al. 1994), indicating that albumin is also not an essential component for copper transportation in the blood. Thus, it appears that the distribution of copper to extrahepatic tissues involves a number of molecules, which may play complementary roles to provide effective copper distribution.

2.3 Copper excretion

The liver maintains copper balance in the body by regulating the amount of copper released in the bile. About 50% of newly absorbed dietary copper is taken up by the liver within 10 minutes of entry into the hepatic-portal circulation (Sass-Kortsak 1965). Copper uptake by hepatocytes is a carrier-mediated process not dependent on metabolic energy (Schmitt et al. 1983). If the uptake of copper is excessive, the surplus is secreted in the bile. Of all the body fluids, the bile has the highest copper concentration and it has been estimated that between 0.5mg to 1.5mg of copper is eliminated daily into the gastrointestinal tract (Winge and Mehra 1990). Copper excreted into bile is in a non-absorbable form, preventing reuptake by intestinal enterocytes (Gross et al. 1989; Danks 1995). The key molecule that regulates copper excretion into bile is ATP7B, the protein affected in the copper toxicosis disorder, Wilson disease (see Section 4.3). The importance of the biliary excretion pathway for copper elimination is demonstrated by the massive accumulation of copper in the liver of Wilson disease patients. This excess copper is bound principally to metallothioneins and also accumulates in lysosomes (Cox 1995). Copper is also eliminated in sweat and urine, but under normal circumstances the amounts are too small to contribute significantly to copper homeostasis. Therefore, the body is reliant on the processes of dietary uptake and biliary excretion to maintain homeostatic copper levels (Turnlund et al. 1998).

3 Cellular copper homeostasis

All organisms have developed mechanisms to supply copper to dependent enzymes without damaging cellular constituents. An elegant and intricate network of proteins involved in moving copper into and out of cells and between subcellular

compartments has been identified. Yeast, in particular *Saccharomyces cerevisiae*, has been used extensively to study and identify key proteins involved in cellular copper transport and because copper transport mechanisms have been conserved throughout evolution, the identification of proteins important in yeast copper homeostasis has been followed rapidly by the isolation of the mammalian orthologues. In most cases the mammalian orthologue when expressed in yeast can functionally replace the yeast protein, providing a useful system for functional studies on human proteins involved in copper metabolism. Details of the yeast intracellular copper transport system are provided in Chapter 2.

The current knowledge of intracellular copper transport in mammalian cells is summarized in Figure 1. Copper entry into the cell across the plasma membrane is mediated by the Ctr1 protein (Zhou and Gitschier 1997). Cytoplasmic copper is thought to be in the Cu^{1+} state and a reductase (copper reductases have been identified in rat hepatocytes and yeast) may be required with Ctr1 for cellular copper uptake (Dancis et al. 1994; Knight et al. 1996). Topological studies have revealed that human Ctr1 (hCtr1) has three transmembrane regions with a hydrophilic, histidine and methionine-rich N-terminal domain exofacial to the cell (Klomp et al. 2003). Histidines and methionines can readily bind copper and there is evidence that hCtr1 forms a channel by existing in a trimeric state, through which copper is thought to traverse the membrane (Eisses and Kaplan 2002; Klomp et al. 2002; Lee et al. 2002). When cells are exposed to excessive amounts of copper, hCtr1 internalises from the plasma membrane and is degraded in endosomal compartments, consistent with a mechanism that regulates copper intake (Petris et al 2003). The hCtr1 gene is expressed in all tissues so far examined with the highest level found in the liver where it may be responsible for the rapid uptake of dietary copper (Zhou and Gitschier 1997). The importance of Ctr1 in mammalian development is illustrated by the embryonic death of mice homozygous for a *Ctr1* knockout (Lee et al. 2001). A second potential copper-importer, hCtr2 was identified through sequence homology with hCtr1. The sequence of hCtr2 is more similar to yeast Ctr2p, suggesting that it may function as a low-affinity copper importer like the yeast counterpart. In support of this hypothesis, hCtr2 was found insufficient to complement the functional defects in a yeast c*tr1 ctr3* double knockout mutant strain, whereas hCtr1 being a high-affinity copper importer can rescue copper uptake when expressed in the same strain (Zhou and Gitschier 1997).

Copper imported by Ctr1 is delivered to specific destinations within the cell by small chaperone proteins. The literature on chaperones has been thoroughly reviewed by Field and colleagues (Field et al. 2002). Three such chaperones have so far been identified and are orthologues of those previously discovered in yeast (see Chapter 2). Nothing is known about how copper is transferred from Ctr1 to the chaperones, but this step is a potential regulation point, i.e. one that determines the pattern of distribution of copper within the cell. The COX17 chaperone (Cox17p in yeast) transports copper to the mitochondria for incorporation into cytochrome c oxidase (Srinivasan et al. 1998). The mammalian COX17 has been shown to dock in the mitochondrial intermembrane space, suggesting the direct copper transfer between chaperone and cytochrome c oxidase (Maxfield et al. 2004). The

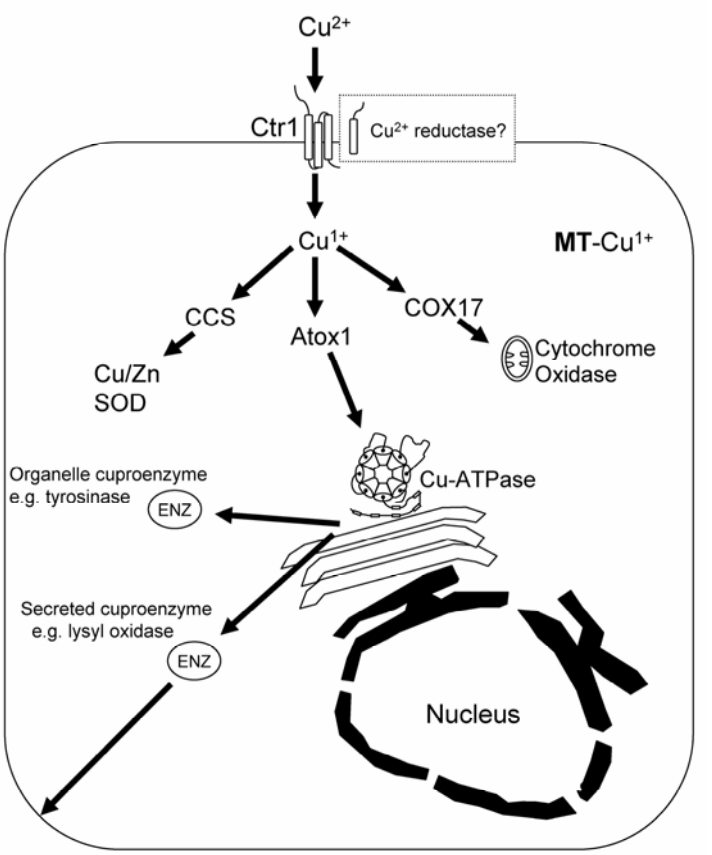


Fig. 1. The copper transport pathway in mammalian cells. Copper enters the cell via the plasma membrane copper importer Ctr1; Cu^{2+} is thought to be reduced to Cu^{1+} by membrane reductases. Upon entry into the cell, copper is distributed to three copper chaperones, CCS, COX17, and ATOX, that deliver the cation to Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase at the mitochondria or to the copper-ATPases (ATP7A or ATP7B depending on cell type) at the *trans*-Golgi network (TGN). The copper-ATPases pump copper into the TGN, where the cation is incorporated into secreted cuproenzymes, such as lysyl oxidase. ATP7A traffics between the TGN and the plasma membrane, and ATP7B traffics between the TGN and post-Golgi vesicular compartments (not shown). If intracellular copper levels increase, the copper-ATPases are predominantly found at their post-Golgi localization, allowing cellular efflux or vesicular sequestration of the excess copper cations. If copper-ATPase mediated efflux is insufficient to maintain low cytoplasmic copper levels, metallothioneins (MT) are induced and bind the excess copper.

second cytosolic copper chaperone CCS (known as either CCS or LYS7 in yeast), transports copper to copper/zinc superoxide dismutase (SOD). A direct interaction between CCS and SOD has been demonstrated and their intracellular localization is identical, with a nuclear and cytoplasmic distribution (Casareno et al. 1998).

Mutation of the copper binding site in SOD (H48F) prevents copper incorporation and abrogates the interaction with CCS (Torres et al. 2001). CCS knockout mice have a marked reduction in SOD activity and their phenotype is similar to the *sod1* knockout animals in that they display increased sensitivity to oxidant challenge (Wong et al. 2000). The third copper chaperone, Atox1 (also called HAH1) delivers copper to the Menkes (ATP7A) and Wilson (ATP7B) proteins through direct interaction. These interactions occur through homologous copper binding sites on the donor (Atox1) and recipient (ATP7A or ATP7B) proteins and are dependent on copper (Larin et al. 1999; Walker et al. 2002). The phenotype of the *Atox1* null mice is consistent with its role in delivery of copper to the copper-ATPases. The mutant mice display about a 50% reduction in liver and brain copper levels and die soon after birth (Hamza et al. 2001). They are also hypopigmented, suggesting that Atox1 is required for delivery of copper to tyrosinase, a step that also requires Atp7A (Petris et al. 2000).

The ATP7A and ATP7B proteins are closely related copper-transporting P-type ATPases that have both biosynthetic and protective roles in cellular copper homeostasis (see Section 5.2). Under normal copper conditions, both copper-ATPases reside at the *trans*-Golgi network (TGN) of the cell, where the cation is incorporated into various copper-dependent enzymes such as lysyl oxidase in fibroblasts (mediated by ATP7A) or ceruloplasmin in hepatocytes (mediated by ATP7B). When copper levels in the cell start to rise, a protective mechanism is activated that enables the cell to reduce and maintain safe intracellular copper levels. This mechanism involves the copper-induced redistribution of the copper-ATPases to the plasma membrane (as is the case for ATP7A) or to vesicular compartments (as is the case for ATP7B) of the cell. In these locations, the excess copper can be expelled directly across the membrane or sequestered into vesicles for detoxification. If the intracellular copper levels exceed the efflux capacity of the cell, expression of metallothioneins is induced and these small cysteine-rich proteins sequester the excess copper (Mercer et al. 2003). The effects of copper imbalance are dramatically illustrated in two human genetic disorders, Menkes and Wilson disease, in which ATP7A and ATP7B respectively are affected (see Section 4.1 and 4.3).

An enigmatic, yet apparently important, intracellular participant in copper homeostasis was revealed by analysis of the Bedlington terrier, a dog breed in which an autosomal recessive copper toxicosis is common. Affected dogs accumulate massive amounts of copper in their livers, in a manner reminiscent of Wilson disease. The affected gene (*MURR1, or COMMD1*) was isolated by positional cloning (Klomp et al. 2003) and has been shown to interact with ATP7B (Tao et al. 2003), but its function in copper homeostasis remains elusive as it has no obvious copper binding sites. MURR1 is in fact a multifunctional protein and it has been shown to inhibit nuclear factor κB, a transcription factor involved in immunity, apoptosis, cell cycle regulation and oncogenesis (Burstein et al. 2005). Why mutation of this molecule produces a copper-specific disease in dogs remains a mystery.

4 Genetic diseases of copper homeostasis

As with genetic studies in yeast, the analysis of the human genetic disorders of copper homeostasis allowed the identification of the copper ATPases, ATP7A, and ATP7B that are pivotal players in the maintenance of systemic copper balance. Establishing that Menkes and Wilson disease were disorders of copper homeostasis revealed for the first time the importance of copper in human health and the necessity for tight regulation of copper status.

4.1 Menkes disease

Menkes disease (MD) is an X-linked recessive disorder of copper metabolism first described as a degenerative condition of the central nervous system by John Menkes in 1962 (Menkes et al. 1962), but the link with copper was not discovered until 1972 (Danks et al. 1972a; Danks et al. 1972b). The incidence of MD is estimated at being between 1/100,000 and 1/298,000 in the total population (Kaler 1994). Classical MD symptoms include severe neurological degeneration (mental retardation), skeletal and connective tissue defects, pili torti (twisting of hair shafts) and hypopigmentation (Menkes et al. 1962; Kaler 1994). Severe neurological abnormalities cause affected individuals to suffer seizures, the frequency of which increase until death occurs usually between three and four years of age (Danks 1995).

The recognition that MD was a copper deficiency condition eventuated by the realization that patients abnormal hair (pili torti) was similar to that seen in copper-deficient sheep ('Steely-wool') and arterial abnormalities resembled that seen in copper-deficient pigs (Danks et al. 1972a). MD was first referred to in the literature as 'Kinky hair syndrome' because of the unusual hair (Menkes et al. 1962). The analysis of the copper level in the liver and serum of seven affected individuals confirmed copper deficiency and the cause was recognized as being an impairment of intestinal copper absorption and subsequent transportation around the body (Danks et al. 1972a). Despite the overall copper deficiency, copper accumulates in intestinal and kidney cells, suggesting that the defective product in MD patients would normally facilitate the transport of this trapped copper to other tissues.

The affected gene in MD patients was isolated by positional cloning in 1993 by three independent groups; all used an X:2 translocation found in a rare female to track the gene. The translocation actually disrupted the Menkes gene. The gene is referred to as *ATP7A* or *MNK* (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993). The protein encoded by *ATP7A* shows significant homology with a family of transmembrane cation pumps, known as P-type ATPases (see Section 5.1). Given that P-type ATPases are involved in the transport of cations across membranes, the ATP7B protein was proposed to encode a copper transporter required for the efflux of copper from cells (Vulpe et al. 1993). This copper efflux role is consistent with the abnormally low copper efflux capacity found in Menkes patient fibroblasts (Camakaris et al. 1980) and the enhanced copper efflux capacity

due to amplification of the Menkes gene found in copper-resistant Chinese hamster ovary cells (Camakaris et al. 1995).

The clinical features of MD can be explained by the reduced activity of copper-dependent enzymes as described in Table 1. However, the extent of copper deprivation on the brain is most devastating. The brain is severely copper deficient in Menkes patients due to the requirement for ATP7A for passage of copper across the blood-brain barrier, which compounds the overall deficiency due to reduced intestinal absorption of copper (Kodama 1993). Abnormalities in arterial development, including elongation and irregularities in wall thickness, due to lysyl oxidase deficiency sometimes lead to fatal arterial aneurisms (Danks 1995). Milder variants of the MD have been reported, including occipital horn syndrome (OHS) and mild-Menkes. OHS patients are not significantly mentally retarded and have pronounced connective and skeletal tissue defects; fatal aneurisms are common in this group. The skeletal defects produce occipital exostoses which give rise to the syndrome's name and are due to lysyl oxidase deficiency. Mild-Menkes patients have less severe symptoms than those with the classical form, with cerebellar ataxia and moderate developmental delay being the predominant features (Procopis et al. 1981). Both disease variants are caused by mutations in *ATP7A*. In the case of OHS, splice site mutations which allow production of a small amount of normal protein appear to be the consistent mutation type (Das et al. 1994). Mild- Menkes disease in one case has been shown to be due to a missense mutation that is predicted to reduce but not abolish the activity of ATP7A (Ambrosini and Mercer 1999). The distinct clinical presentations between classical MD, OHS, mild-Menkes can been explained by a combination between the effect of the *ATP7A* mutation on the copper transport activity, the amount of residual protein and the intracellular location of ATP7A (Mercer 2001).

Treatment of Menkes disease by administration of copper salts or complexes (copper-histidine) has not been very successful. Although four patients have survived longer following treatment, none have their symptoms completely corrected, and in most cases the connective tissue defects become predominant (Christodoulou et al. 1998). Many patients succumb despite early treatment, and it has been proposed by Kaler that the responding patients must have some residual ATP7A activity to allow copper delivery to the brain when the small intestine is bypassed (Kaler 1996).

4.2 Mouse models of Menkes disease

The mottled mouse mutants were first identified by the mottled coat of the female heterozygotes, and were used by Mary Lyon in formulating her model of X-inactivation in females (Lyon 1962). These mice were proposed to be models for MD, when they were shown to have a similar defect in copper distribution to human patient babies and this resulted in severe neurological abnormalities (Hunt 1974). The validity of the mottled mice as Menkes models was confirmed when abnormalities in murine *Atp7a* gene were described in several different mottled mutants (Levinson et al. 1994; Mercer et al. 1994). The range of phenotypes dis-

played by males carrying various mutant alleles is even more diverse than the allelic variation in human patients. Most notably there is a class of *Atp7a* mutants in mice that die in utero, which is not the case in humans; these are all predicted to be due to null mutations in *Atp7a*. The same type of mutation does not cause prenatal death in humans; instead classical Menkes disease is the result. This difference suggests that mice have a more critical need for copper during development than humans, or that humans have alternative sources of transporting copper across the placenta (Mercer et al. 1999). The latter hypothesis appears unlikely as the placenta from Menkes babies show a similar copper accumulation phenotype to that of the brindled mouse placenta (Horn et al. 1978; Mann et al. 1980).

The brindled mouse is the closest model to Menkes disease, affected males are hypopigmented and die around fifteen days postnatal. A six base pair deletion was found in *Atp7a* in this mouse, but as there is no frame shift normal amounts of Atp7a are produced, but the loss of two amino acids has a severe effect on the function of the protein (Grimes et al. 1997). Interestingly the brindled mouse responds to copper therapy provided that copper is administered prior to 10 days of life (Mann et al. 1979). This observation may support Kaler's contention that some residual ATP7A activity is needed for a Menkes patient to respond to copper therapy (Kaler 1996).

The blotchy mouse is a good model for occipital horn syndrome and like the human disease the mutation is a mild splice site variant (Das et al. 1995) that allows some normal splicing and presumably some normal protein is produced (La Fontaine et al. 1999). The viable brindled mouse has very similar features to mild-Menkes patients and a missense mutation that is predicted to reduce the rate of protein catalytic activation (copper translocation) (see Section 5) has been described, which is the type of mutation that might be expected to produce a mild phenotype (Cecchi et al. 1997). We have previously proposed a model that relates the phenotype of the mottled mutants to the residual activity of Atp7a and proposed threshold requirements for copper at different stages of mouse development (Mercer 1998).

4.3 Wilson disease

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism that is characterized primarily by copper toxicosis of the liver and/or neurological damage. The disease was first recognized in 1912 by Samuel Alexander Kinnear Wilson, who reported several cases of a new familial disorder displaying degradation of the lenticular nuclei (brain division; includes basal ganglia) associated with hepatic cirrhosis at autopsy (Wilson 1912). The worldwide frequency of WD is 1/30,000 with a carrier (heterozygote) frequency of 1/90 (Scheinberg and Sternlieb 1984). In 1993 the gene defective in WD, *ATP7B*, was cloned and shown to encode a novel member of the family of cation-transporting P-type ATPase (Bull et al. 1993; Tanzi et al. 1993; Yamaguchi et al. 1993). The main functions of the ATP7B protein are to deliver copper to ceruloplasmin (Terada et al. 1998) and to mediate the excretion of excess copper into bile (Hung et al. 1997). WD pa-

tients display normal intestinal absorption of copper (which is ATP7A-mediated) coupled with reduced biliary excretion. This leads to copper constantly accumulating in the body, with subsequent liver overload. For instance, the average adult hepatic-copper levels generally do not exceed 50μg/g dry weight, whereas patients with WD accumulate greater than 250μg/g and can reach up to 3000μg/g (Roberts and Cox 1998).

WD patients can present hepatic disease anywhere between their second and sixth decade of life. It appears that more severe mutations may predispose to an earlier onset of hepatic disease (Wilson et al. 2000). Hepatitis, hepatic failure and cirrhosis are sequential, and extrahepatic conditions can also manifest. These include primarily neurological degenerative symptoms such as behavioural disturbances (depression and schizophrenia), dysarthria (speech impediment), and Parkinsonism (motor disorders and tremors) and are possibly caused from the accumulation of copper in the brain subsequent to the cation being released from the damaged liver (Danks 1995). These neurological symptoms reflect the changes in the basal ganglia observed at autopsy, which include cavitary degradation, gliosis, and neuronal loss (Culotta and Gitlin 2000). Neurological symptoms occur in about 60% of patients with affected individuals generally older than those presenting with only liver disease and are most often diagnosed in their third of fourth decade of life. The incorporation of copper into ceruloplasmin is also reduced in most (~85%) WD patients with low circulating levels of holo-ceruloplasmin used as a biochemical marker in diagnosis (Culotta and Gitlin 2000). Copper may also deposit in the descemet's membrane of the cornea and can be visualized as Kayser-Fleischer rings (Ghosh et al. 2004).

Analysis of patient mutations has revealed an enormous heterogeneity, with a small number of frequent mutations that are population specific and a much greater number of rare individual alleles. Over 100 different mutations have been characterized in patients from varying ethnic origins. Of these, more than half are missense mutations, while the remainder involve small deletions or insertions (25%), splice site abnormalities (10%) and nonsense mutations (10%) (Cox 1997). Of the common mutations, H1069Q accounts for about 40% of the alleles found in populations of Northern European origin (Cox 1997), while A778L has been identified in about 30% of the alleles in Asian populations (Kim et al. 1998). The degree of allelic heterogeneity accounts, at least in part, for the enormous clinical variation observed in patients. As expected with the large number of different mutations, the majority of affected individuals are compound heterozygotes, making correlation between genotype and phenotype very difficult to study (Thomas et al. 1995). The marked difference in clinical variation among affected sibs and identical twins indicates that additional genetic and environmental factors significantly influence the overall clinical phenotype (Thomas et al. 1995).

The goal of treatment in WD is to restore normal systemic copper levels through chelation therapy, which is directed at either removing or detoxifying accumulated copper. D-penicillamine is possibly the most effective copper-chelator currently used, although the exact mechanism of how D-penicillamine results in detoxification and elimination of copper remains unclear. Long term treatment using D-penicillamine has been shown to promote urinary copper excretion and

neuroimaging in such patients reveals a decrease in copper related abnormalities; which correlates with clinical improvement (Schlaug et al. 1996). If diagnosed early, patients will be asymptomatic within 4 months after starting D-penicillamine treatment and subsequently will be placed on maintenance therapy at half the initial dose (Schilisky 1996). WD patients must also restrict their copper intake and definitely avoid foods rich in copper. Dietary supplementation with Zn can be used to blocks intestinal absorption of copper as an adjunct therapy (Brewer et al. 1990; Brewer 2000).

4.4 Rodent models for Wilson disease

The Long-Evans Cinnamon (LEC) rat is an inbred mutant strain that has been proposed to be the closest animal model of WD. The LEC rat orthologue of *ATP7B* (A*tp7b*) contains a partial (900bp) deletion at the 3' end rendering the protein non-functional (Wu et al. 1994). Furthermore, the *Atp7b* transcript in the LEC rat is undetectable by Northern blot, suggesting that the deletion causes instability in the mRNA transcribed (Yamaguchi et al. 1994). The LEC rats display hepatic copper accumulation, reduced biliary copper excretion, reduced levels of plasma copper, and a remarkable decrease in serum ceruloplasmin activity with these symptoms analogous to those found in patients with WD (Li et al. 1991; Sugawara et al. 1991, 1993; Yamada et al. 1993). Hepatocyte injury due to copper toxicity is also the major manifestation in both. However, there are some major differences in the clinical manifestations between the LEC rat and WD patients. While about 60% of WD patients display neurological abnormalities, these are rarely observed in the LEC rat and although very few patients with WD are known to develop liver cancer, more than 90% of LEC rats develop liver cancer after their first year of life. The increased susceptibility to hepatic carcinoma has been shown to be independent to copper accumulation and is thought to be an autosomal dominant trait of the LEC rats (Hattori et al. 1995).

The Toxic Milk (tx) mouse is an unusual animal model for WD. A point mutation (causes M1356V) in the *ATP7B* orthologue (*atp7b*) in this mouse line causes classical WD symptoms, including hepatic copper accumulation and reduced levels of holoceruloplasmin, but also seemingly makes dam's milk toxic (Rauch 1983; Biempica et al. 1988; Theophilos et al. 1996). Pups suckling on mutant dams die from copper deficiency due to inadequate copper levels in milk (Rauch 1983). The tx mouse has been demonstrated to be a true model of WD when a mutation was found that resulted in a missense mutation in a highly conserved region of the eighth transmembrane domain of Atp7b (Theophilos et al. 1996). The specific affect of the mutation on milk copper is seemingly because Atp7b is required for copper delivery to milk in the mammary gland (Ackland et al. 1999).

4.5 Possible genetic copper toxicity conditions

Copper toxicity in humans is rare because dietary intake is usually low and removal of excess copper from the body by biliary excretion is very efficient. A group of childhood copper toxicity conditions are known and appear to be a result of an autosomal recessive mutation coupled with high intake of dietary copper (for example in milk or water contaminated with copper). The first such condition recognized is known as Indian Childhood Chirrosis (ICC) and was linked to boiling milk in brass vessels (O'Neill and Tanner 1989). Such children accumulate large amounts of copper in the liver, develop cirrhosis, liver failure, and unless treated with D-penicillamine, die during infancy (Bhusnurmath et al. 1991). Analysis of families with ICC indicated an autosomal recessive mode of inheritance and it is now thought that this disorder arises from a combination of environmental (copper exposure) and genetic factors (Pandit and Bhave 1996). Further support for the genetic basis of these copper-associated childhood cirrhoses comes from the analysis of a very similar (possibly identical) condition to ICC reported from Austria. In this case, the children died because of consumption of milk that had been boiled in copper pots. A detailed analysis of the pedigrees involved showed a clear pattern of autosomal recessive inheritance (Muller et al. 1996). The gene affected in this disorder has not been identified, however, the disease has been shown not to be a variant of WD (Wijmenga et al. 1998).

5 Mechanistic and cellular aspects of the copper-ATPases (ATP7A and ATP7B)

5.1 The P-type ATPase family of proteins and cation translocation

The Menkes protein, ATP7A was the first heavy metal P-type ATPase described in mammals (Vulpe et al. 1993). Many other members of this family of enzymes had been previously well studied and this has allowed conclusions to be drawn regarding the mechanism of action of the mammalian copper-ATPases. In general, P-type ATPases are multipass transmembrane proteins that function to translocate cations across membranes using the energy derived from ATP hydrolysis. More than 100 P-type ATPases have been identified and occur in a wide range of organisms including bacteria, yeast, plants, and mammals. Prominent eukaryotic members of the family include the Ca^{2+}-ATPase of the plasma membrane and the sarcoplasmic reticulum (SR), which regulates cellular levels of Ca^{2+} and mediates muscle contractions; and the Na^{+}/K^{+}-ATPases that are involved in cellular volume regulation, membrane potentiality and Na^{+}-dependent nutrient uptake (reviewed in Moller et al. 1996).

Although the similarity in the overall sequence between P-type ATPases is often low, each member has several conserved regions which permits the homologous alignment between members (Palmgren and Axelsen 1998). Both ATP7A and ATP7B contain these invariant regions, which include the GDGIND motif

(ATP binding site), the TGEA motif (phosphatase domain) and a phosphorylation domain (DKTGT(I,L)T) (Scarborough 1999). A common feature of all P-type ATPases is a 'core' comprising a hydrophilic head that protrudes into the cytosol, which contains the phosphorylation and ATP-binding site, and a smaller cytosolic region (exposing the phosphatase domain) located in the N-terminal part of the protein. These cytosolic (hydrophilic) regions are linked to the membrane by a number of membrane traverses (usually 8) that are assumed to be involved in the formation of an intramembranous channel, but with differences in organization between members. A suggested linear and three-dimensional representation of the structure of the copper-ATPases is shown in Figure 2, which was based on the proposed model for ATP7A (Mercer and Camakaris 1997).

Copper transporting P-type ATPase form a subfamily termed CPX type ATPases. Members of the subfamily are characterized by containing in addition to the common features of P-type ATPases, a variable number of putative heavy metal binding sites within the N-terminus and a conserved Cys-Pro-X motif (X=Cys, His or Ser) in the proposed cation transduction channel (refer to Fig. 2) (Solioz and Vulpe 1996). The prominent feature of the CPX type ATPases is the presence of a CPX motif within a predicted transmembrane region. This motif is situated precisely 43 amino acids upstream of the phosphorylation domain and in most family members the X represents a cysteine. However, several bacterial heavy metal transporters have a CPS or CPH in place of the CPC (Solioz and Vulpe 1996). This motif is critical for the function of both copper-ATPases, with mutation of the first cysteine in ATP7A (C1000R) known to cause Menkes disease (Tumer et al. 1999) and mutation of the second cysteine in ATP7B (C985Y) known to cause Wilson disease (Hass et al. 1999). In ATP7B, mutation of both cysteines to serines (CPC-SPS) has been shown to inhibit copper-translocation (Forbes and Cox 1998) and intracellular trafficking (Forbes and Cox 2000). Given that cysteines have been demonstrated to coordinate copper, it has been proposed that the CPC motif facilitates the transduction of copper through the lipid membrane.

Following the discoveries of the Na^+/K^+-ATPase (Skou 1957) and the SR Ca^{2+}-ATPase (Hasselbach and Mankinose 1961) numerous studies have been employed especially on these two enzymes to characterize the intermediate steps and mechanism of cation translocation by P-type ATPases (reviewed in Moller et al. 1996 and Scarborough 1999). The reaction mechanism can be explained in terms of a four step process: E_1 ,E_1P ,E_2P ,E_2, E_1. (reviewed for the Cu-ATPases by Voskoboinik et al. (2002). Initially the cytosolic hydrophilic head containing the GDGIND motif (ATP-binding site) becomes active in response to the binding of the cation(s). For both ATP7A and ATP7B, copper is delivered to highly conserved N-terminal motifs by Atox1, which is described in Section 5.3. Upon activation, ATP hydrolysis occurs resulting in the phosphorylation of an invariant aspartic acid residue (γ-phosphate transfer) in the phosphorylation domain (DKTGT(I,L)T). The aspartate phosphorylation is of the 'high energy type' and can be dephosphorylated by ADP with reformation of ATP. In step (2) the phosphorylation drives conformational changes in the protein leading to the translocation of the bound cations across the membrane. At this stage, the covalently bound

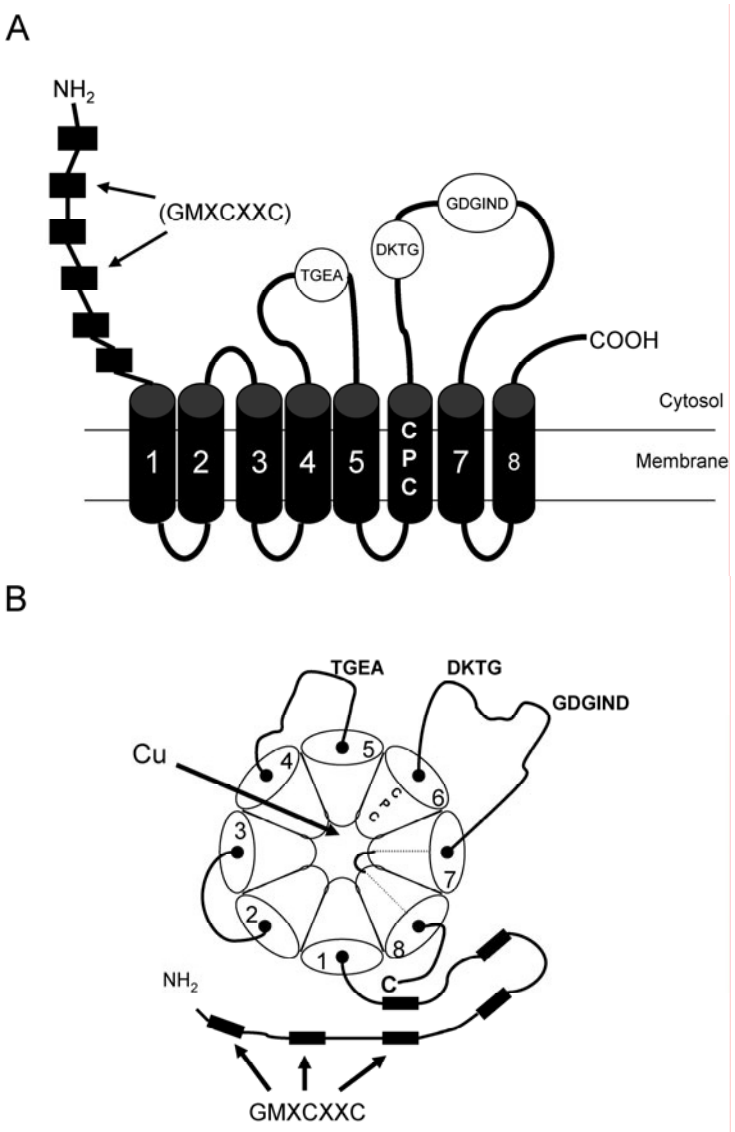


Fig. 2. Schematic representations of ATP7A and ATP7B. ATP7A and ATP7B contain all the conserved domains typical of P-type ATPases, including eight putative transmembrane domains, an ATP binding domain (GDGIND), a phosphorylation domain (DKTG) and a phosphatase domain (TGEA). In addition to these features, the Cu-ATPases also contain six copper binding sites (GMXCXXC) within the N-terminus and an intramembranous transduction motif (CPC) believed essential for the copper translocation process. (A) Linearized schematic showing the eight transmembrane domains thought to form an cation transduction channel through which copper crosses the membrane as shown in (B).

phosphate converts into a 'low-energy type' (E_2P), non-reactive with ADP. The release of the cation possibly takes place concurrently, or immediately following the E_1P to E_2P transition. In the third step the acyl-phosphate is hydrolysed by the phosphatase domain before the pump is returned back to its original conformation to allow further cation binding and translocation. For several P-type ATPases the last step often involves the transfer of other cation in the opposite direction such as $2K^+$ for Na^+/K^+-ATPase.

The current knowledge on the mechanism for ATP7A-mediated copper translocation conforms to the 'P-type ATPase translocation model' explained above. The transient phosphorylation of the invariant aspartate in the DKTGT(I,L)T domain has been shown to be essential for the copper translocation activity of ATP7A (Petris et al. 2002), with phosphorylation dependent on the presence of copper, ATP and being ADP sensitive (Voskoboinik et al. 1998, 2001). Mutation of the phosphatase domain (TGEA) causes ATP7A to be fixed in a hyperphosphorylated intermediate, consistent with the requirement of autohydrolysis of the acyl-phosphate for reaction turnover (Petris et al. 2002). Furthermore, a structural homologue of P_i, orthovanadate, has a strong inhibitory effect on the acyl-phosphorylation of ATP7A, with this agent known to perturb acyl-phosphate formation of P-type ATPases (Voskoboinik et al. 2001). Recently X-ray crystallography has provided an elegant picture of the Ca ATPases at various stages of its reaction cycle (Toyoshima et al. 2004).

5.2 Cell biology of ATP7A and ATP7B

The two mammalian copper-ATPases are very similar in amino acid composition and are likely to have evolved from a single ancestral copper-ATPase to perform specific functions in different cell types. Immunocytochemical analysis of mammalian cell lines expressing either ATP7A or ATP7B has revealed that both proteins reside at the final compartment of the Golgi apparatus, the *trans*-Golgi network (TGN) (Petris et al. 1996; Dierick et al. 1997; Hung et al. 1997; Schaefer et al. 1999a; Forbes and Cox 2000; Roelofsen et al. 2000). At this location, both proteins translocate copper into the TGN lumen for incorporation into copper-dependent enzymes as they migrate through the secretory pathway (Terada et al. 1998; Petris et al. 2000). However, as noted in Section 3, when mammalian cell lines are exposed to elevated levels of copper both ATP7A and ATP7B undergo intracellular redistribute and traffic from the TGN to either the plasma membrane (ATP7A) or to cytosolic vesicular compartments (ATP7B) (Petris et al. 1996; Hung et al. 1997).

A fundamental difference between ATP7A and ATP7B, which most likely accounts for their need in different cell types, is only recognized when polarized cells are exposed to elevated levels of copper. In epithelial cells, such as Madin-Darby canine kidney (MDCK) cells, ATP7A relocalizes from the TGN to the basolateral membrane (Greenough et al. 2004). Copper-induced trafficking of ATP7A to the basolateral surface has also been shown *in vivo*, in intestinal mucosal cells (enterocytes) of a transgenic mouse expressing human ATP7A (François

Monty, unpublished data). Basolateral localization is consistent with the role of ATP7A in translocating copper across the enterocyte membrane into the hepatic-portal circulation. However, when polarized hepatocytes are exposed to elevated levels of copper, ATP7B traffics to vesicles that are situated in close proximity to the apical membrane both *in vivo* (Schaefer et al. 1999a; Schaefer et al. 1999b) and *in vitro* (Schaefer et al. 1999a; Roelofsen et al. 2000). ATP7B is presumably required to traffic toward the apical (canalicular) membrane of hepatocytes to mediate the excretion of excess copper into bile. Therefore, the type of copper-ATPase expressed in a given polarized cell type may depend on which cell surface copper is required to traverse; basolateral translocation requires ATP7A and apical translocation ATP7B. Consistent with this idea, both copper-ATPases are expressed in cell types where copper is required to be actively-translocated across both the apical and basolateral membranes. For instance, both proteins are expressed in breast alveoli cells where basolateral efflux is presumably required to reduce excess intracellular copper and apical efflux is required for copper delivery to milk (Ackland et al. 1999). Similarly, in the syncytiotrophoblasts of the placenta, bi-directional copper efflux is required to maintain sufficient and safe levels of copper in the developing fetus, and both ATP7A and ATP7B are expressed (Hardman et al. 2004). It has been postulated that ATP7A is required for copper delivery into the fetal circulation and ATP7B returns excess copper across the apical surface to the maternal circulation (Hardman et al. 2004). In non-polarized cell types ATP7A traffics to the entire cellular circumference (Petris et al. 1996), while ATP7B to a dispersed population of cytosolic vesicles (Hung et al. 1997).

5.3 Role of the N-terminal metal binding domains of the copper-ATPases

Within the N-termini of ATP7A and ATP7B there are six well conserved sequences each of approximately 70 residues, of which the GMXCXXC sequence is the most prominent feature (X represents any amino acid). These regions fold to form metal binding domains with the GMXCXXC sequence exposed at the top. The three dimensional structure of the 4th metal binding domain of ATP7A shows a well ordered structure with the cation bound to the two cysteines at one end of the domain (Gitschier et al. 1998). The N-terminal domains of human ATP7B and ATP7A have the capacity to bind six atoms of copper (Lutsenko et al. 1997). Interestingly, not all orthologues of ATP7A and ATP7B contain six metal binding sites. Bacterial copper transporters possess a single metal binding site (CopA) (Odermatt et al. 1993), the yeast protein contains two metal binding sites (*Ccc2*p), and the orthologue in the nematode (*C. elegans*) has three (Sambongi et al. 1997). Considering that these proteins are still capable of transporting copper, it is not understood why there are six metal binding sites in the mammalian orthologues when one seems to be sufficient (Strausak et al. 1999; Forbes and Cox 2000). One possibility is that multiple metal binding sites have a function in addition to that of direct copper transport, perhaps increasing in number to take on further functions,

such a sequestration of copper, as an adaptation for multi-cellular organisms (Strausak et al. 1999).

The function of the N-terminal copper-binding domain of the copper-ATPases has been the subject of several studies. The N-terminal metal binding domain has been shown to receive copper from the cytosolic chaperone Atox1 and cooperatively bind copper prior to transport (Hung et al. 1998; Larin et al. 1999; Walker et al. 2002). The addition of copper-loaded Atox1 to membrane preparations corresponded with the acyl-phosphorylation (catalytic activation) of ATP7B, with the number of molecules phosphorylated proportionate to the concentration of copper-Atox1 added.

Yeast devoid of *Ccc2*p expression are defective in high-affinity iron uptake because copper is not incorporated into the ferroxidase Fet3 (see Chapter 2). *Ccc2*p is the yeast orthologue of the mammalian copper-ATPases (ATP7A and ATP7B) and expression of either ATP7A or ATP7B in Δ*ccc2 S. cerevisiae* can complement the iron-deficiency phenotype of these cells (Hung et al. 1997; Forbes and Cox 1998; Payne and Gitlin 1998). Complementation of Δ*ccc2* with ATP7A and ATP7B N-terminal mutants has provided a useful system for determining the importance of individual metal binding sites in the copper-translocation activity of these proteins. Several reports have indicated that the role of the six N-terminal metal binding sites in copper-translocation has been highly conserved between the copper-ATPases, with metal binding sites five or six (those closest to transmembrane domain 1) shown essential to support the translocation activity of ATP7A (Mercer et al. 2003) and ATP7B (Forbes et al. 1999; Cater et al. 2004).

Another proposed function for the N-terminal copper-binding domain is that the metal binding motifs individually or collectively act as a copper sensor, regulating the subcellular localization of the copper-ATPase in accordance with the copper status of the cell (Strausak et al. 1999; Cater et al. 2004). Recently, the copper-induced trafficking of both copper-ATPases has been associated with their catalytic cycle (Petris et al. 2002). Formation of the acyl-phosphorylated intermediate (catalytic activation) is required for trafficking of ATP7A to the plasma membrane (Petris et al. 2002), while mutation of the phosphatase domain (TGEAAA) in ATP7B, which is required for hydrolysis of the acyl-phosphate, caused ATP7B molecules to localize constitutively in vesicles (Petris et al. 2002). Consistent with this idea, the same N-terminal metal binding sites required for the copper translocation activity of both copper-ATPases (sites five or six), are required for both proteins to undergo intracellular trafficking (Strausak et al. 1999; Cater et al. 2004). However, how acyl-phosphorylation (catalytic activation) translates into movement of the protein in the cell is not known.

6 Brain copper and neurodegenerative diseases

While the importance of regulating copper levels in the brain is dramatically illustrated by the severe symptoms associated with Menkes and Wilson disease, little is known about copper homeostatic mechanisms in the brain in comparison to pe-

ripheral tissues. Copper must be actively transported across the blood brain barrier and this task is accomplished by the Menkes protein (ATP7A), thus explaining the severe copper deficiency in the brain of MD patients. In the brindled mouse mutant (a model of MD, see Section 4.2) copper was found to accumulate in the cells of the blood brain barrier, consistent with a block in transport due to ATP7A being inactive (Kodama 1993). The severe copper deficiency and subsequent neurological problems in the brains of MD patients is thought to be primarily caused by a reduction of cytochrome c oxidase activity and therefore generation of ATP (Kaler 1994).

Copper has recently been implicated in the pathogenesis of several other neurological disorders including, Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jacob disease, and amyotrophic lateral sclerosis (Bush 2000), illustrating the importance of understanding copper homeostasis in the brain. In Alzheimer's disease (AD) the pathological hallmarks involve a marked accumulation of the extracellular amyloid-β (Aβ) protein (plaques) and intraneuronal tangles in the neocortex. Aβ is produced by the cleavage of the amyloid precursor protein (APP) and it has been established that copper and zinc play important roles in promoting the aggregation and toxicity of Aβ *in vitro* (Jobling et al. 2001; White et al. 2001). In the cerebral amyloid plaques of patients with AD, copper and zinc concentrations are enriched (~300%) and both metals (but no other metals) directly coordinate to Aβ subunits (via histidine side chains) and co-purify with Aβ from post-mortem brains (Rogers et al. 2002). In addition, copper and zinc chelators have been shown to dissolve Aβ in post-mortem brains of patients (Barnham et al. 2003). Recent studies have also indicated that potentially there is a physiological role for Aβ or APP in exporting copper from neurons. APP knockout mice have elevated levels of copper in the brain (White et al. 1999), whereas, APP transgenic mice (overexpressing APP) have decreased brain copper levels (Phinney et al. 2003). It is has been reported that in AD patient brains there is decreased copper and copper-dependent enzymatic activities and that elevated copper concentrations inhibit amyloid accumulation in APP transgenic mice (Bush 2003). These symptoms could relate to the overproduction of APP in the diseased state and subsequent increased copper efflux from neurons. However, there is currently no clear mechanistic explanation for the involvement of copper (and zinc) in APP/Aβ pathology.

Transmissible spongiform encephalopathies, also referred to as Prion diseases, are characterized by the deposition of an abnormal isoform of the prion protein (PrPC) in the brain (Brown 2004). The prion protein has the capacity to bind copper and possibly plays a role in copper transport in the brain (Brown et al. 1997). The copper-binding activity of the normal prion protein is lost during is conversion to the aggregated abnormal amyloid form (PrPSc). Copper had also been shown to modulate expression levels and increase the release of the prion protein in cell culture neuronal models (Massimino et al. 2005).

7 Conclusions

In recent years, the analysis of the genetic disorders that affect copper in man and model organisms such as yeast has provided a dramatic increase in the understanding of the role of copper in health and disease. These advances have taken the field of copper homeostasis into a new era of molecular understanding of how cells and organisms obtain this potentially dangerous essential element without damage. The more recent advances in the neurobiology of copper are showing that, far from being a minor study affecting only rare diseases, the field of copper biology is of great importance in disorders that are major health challenges, such as Alzheimer's disease. Hopefully all the outstanding molecular analysis in a range of organisms will produce innovative solutions to these health problems, as well as solving the mysteries of copper homeostasis.

References

Ackland ML, Anikijenko P, Michalczyk A, Mercer JFB (1999) Expression of Menkes copper-transporting ATPase, MNK, in the lactating human breast: possible role in copper transport into milk. J Histochem Cytochem 47:1553-1561

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1995) Molecular Biology of the Cell, 3rd edn. Garland Publishing Inc., New York & London

Ambrosini L, Mercer JFB (1999) Defective copper-induced trafficking and localization of the Menkes protein in patients with mild and copper-treated classical Menkes disease. Hum Mol Genet 8:1547-1555

Barnes G, Frieden E (1984) Ceruloplasmin receptors of erythrocytes. Biochem Biophys Res Commun 125:157-162

Barnham K, Ciccotosto G, Tickler A, Ali F, Smith D, Williamson N, Lam Y-H, Carrington D, Tew D, Kocak G, Volitakis I, Separovic F, Barrow C, Wade J, Masters C, Cherny R, Curtain C, Bush A, Cappai R (2003) Neurotoxic, redox-competent Alzheimer's ß-amyloid is released from lipid membrane by methionine oxidation. J Biol Chem 278:42959-52965

Biempica L, Rauch H, Quintana N, Sternlieb I (1988) Morphological and chemical studies on a murine mutation (toxic milk mice) resulting in hepatic copper toxicosis. Lab Invest 59:500-508

Brewer GJ (2000) Recognition, diagnosis and management of Wilson's disease. Proc Soc Exp Biol Med 223:39-46

Brewer GJ, Yuzbasiyan-Gurkan V, Dick R (1990) Zinc therapy of Wilson's disease VIII: dose response studies. J Trace Elem Exp Med 3:227-234

Brown D (2004) Metallic prions. Biochem Soc Symp 71:193-202

Brown D, Qin K, Herms J, Madlung A, Manson J, Strome R, Fraser P, Kruck T, von Bohlen A, Schulz-Schaeffer W, Giese A, Westaway D, Kretzschmar H (1997) The cellular prion protein binds copper in vivo. Nature 390:684-687

Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DC (1993) The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nat Genet 5:327-337

Burstein E, Hoberg J, Wilkinson A, Rumble J, Csomos R, Komarck C, Maine G, Wilkinson J, Mayo M, Duckett C (2005) COMMD proteins: A novel family of structural and functional homologs of MURR1. J Biol Chem 280:22222-22232

Bush A (2003) The metallobiology of Alzheimer's disease. Trends Neurosci 26:207-214

Bush AI (2000) Metals and neuroscience. Curr Opin Cell Biol 4:184-191

Camakaris J, Ackland L, Danks D (1980) Abnormal copper metabolism in cultured fibroblasts from patients with Wilson's disease. J Inherit Metab Dis 3:155-157

Camakaris J, Petris MJ, Bailey L, Shen P, Lockhart P, Glover TW, Barcroft CL, Patton J, Mercer JFB (1995) Gene amplification of the Menkes (MNK; ATP7A) P-type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. Hum Mol Genet 4:2117-2123

Casareno RLB, Waggoner D, Gitlin JD (1998) The copper chaperone CCS directly intereacts with copper/zinc superoxide dismutase. J Biol Chem 273:23625-23628

Cater M, Forbes JR, La Fontaine S, Cox DC, Mercer JFB (2004) Intracellular trafficking of the human Wilson protein: the role of the six N-terminal metal binding sites. Biochem J 380:805-813

Cecchi C, Biasotto M, Tosi M, Avner P (1997) The *mottled* mouse as a model for human Menkes disease: identification of mutations in the *Atp7a* gene. Hum Mol Genet 6:425-433

Chelly J, Tumer Z, Tonnesen T, Petterson A, Ishikawa-Brush Y, Tommerup N, Horn N, Monaco AP (1993) Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. Nat Genet 3:14-19

Christodoulou J, Danks DM, Sarkar B, Baerlocher KE, Casey R, Horn N, Tumer Z, Clarke JT (1998) Early treatment of Menkes disease with parenteral copper-histidine: long-term follow-up of four treated patients. Am J Med Genet 76:154-164

Cox DW (1995) Genes of the copper pathway. Am J Hum Genet 56:828-834

Cox DW (1997) Molecular approaches to inherited liver disease. Focus on Wilson disease. J Gastroenterol Hepatol:S251-S255

Culotta VC, Gitlin JD (2000) Disorders of copper transport. In: Scriver CR, Beaudet AL, Sly WM, Valle D (eds) The metabolic and molecular basis of inherited disease, 8th edition edn. McGraw-Hill, New York, pp 3105-3126

Dancis A, Yuan DS, Haile D, Askwith C, Eide D, Moehle C, Kaplan J, Klausner RD (1994) Molecular characterisation of a copper transport protein in *S. cerevisiae*: An unexpected role for copper in iron transport. Cell 76:393-402

Danks DM (1995) Disorders of copper transport. In: Scriver CR, Beaudet AL, Sly WM, Valle D (eds) The metabolic and molecular basis of inherited disease, 7th edn. McGraw-Hill, New York, pp 2211-2235

Danks DM, Cambell PE, Walker J, Smith J, Stevens BJ, Gillespie JM, Blomfield J, Turner B (1972a) Menkes' kinky-hair syndrome. Lancet 1:1100-1102

Danks DM, Campbell PE, Stevens BJ, Mayne V, Cartwright E (1972b) Menkes' kinky hair syndrome. Pediatrics 50:188-201

Das S, Levinson B, Vulpe C, Whitney S, Gitschier J, Packman S (1995) Similar splicing mutations of the Menkes/mottled copper-tranporting ATPase gene in occipital horn syndrome and the blotchy mouse. Am J Hum Genet 56:570-579

Das S, Levinson B, Whitney S, Vulpe C, Packman S, Gitschier J (1994) Diverse mutations in patients with Menkes disease often lead to exon skipping. Am J Hum Genet 55:883-889

Dierick HA, Adam AN, Escara-Wilke JF, Glover TW (1997) Immunocytochemical localization of the Menkes copper transporter (ATP7 A) to the trans compartment of the Golgi complex. Hum Mol Genet 6:409-416

Eisses JF, Kaplan JH (2002) Molecular characerization of hCTR1, the human copper uptake protein. J Biol Chem 277:29162-29171

Evans GW (1973) Copper homeostasis in the mammalian system. Physiol Rev 53:535-569

Field L, Luk E, Culotta V (2002) Copper chaperones: personal escorts for metal ions. J Bioenerg Biomembr 34:373-379

Forbes JR, Cox DW (1998) Functional characterization of missense mutations in ATP7B: Wilson disease mutation or normal variant? Am J Hum Genet 63:1663-1674

Forbes JR, Cox DW (2000) Copper-dependent trafficking of Wilson disease mutant ATP7B proteins. Hum Mol Genet 9:1927-1935

Forbes JR, Hsi G, Cox DW (1999) Role of the copper-binding domain in the copper transport function of ATP7B, the P-type ATPase defective in Wilson disease. J Biol Chem 274:12408-12413

Ghosh JB, Chakrabarty S, Singh AK, Gupta D (2004) Wilson's disease-Unusual features. Indian. J Pediatr 71:937-938

Gitschier J, Moffat B, Reilly D, Wood WI, Fairbrother WJ (1998) Solution structure of the fourth metal-binding domain from the Menkes copper-transporting ATPase. Nat Struct Biol 5:47-53

Goode CA, Dinh CT, Linder MC (1989) Mechanism of copper transport and delivery in mammals: review and recent findings. Adv Exp Med Biol 258:131-144

Greenough M, Pase L, Voskobionik I, Petris M, Wilson Obrien A, Camakaris J (2004) Signals regulating trafficking of the Menkes (MNK; ATP7A) copper translocating P-type ATPase in polarized MDCK cells. Am J Physiol Cell Physiol 287:1463-1471

Grimes A, Hearn C, Lockhart P, Newgreen D, Mercer FBJ (1997) Molecular basis of the brindled mouse mutant (Mo(br)): a murine model of Menkes disease. Hum Mol Genet 6:1037-1042

Gross JBJ, Myers BM, Kost LJ, Kuntz SM, LaRusso NF (1989) Biliary copper excretion by hepatocyte lysosomes in the rat. J Clin Invest 83:30-39

Gunshin H, Mackenzie B, Erger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388:482-488

Hamza I, Faisst A, Prohaska J, Chen J, Gruss P, Gitlin JD (2001) The metallochaperone Atox1 plays a critical role in perinatal copper homeostasis. Proc Natl Aacd Sci USA 98:6848-6852

Hardman B, Manuelpillai U, Wallace ME, Vaan de Waasenberg S, Cater M, Mercer FBJ, Ackland ML (2004) Expression and localization of the Menkes and Wilson copper transporting ATPases in human placenta. Placenta 24:512-517

Harris DIM, Sass-Kortsak A (1967) The influence of amino acids on copper uptake by rat liver slices. J Clin Invest 46:659-667

Harris ED (2000) Cellular copper transport and metabolism. Annu Rev Nutr 20:291-310

Harris ZL, Durley AP, Man TK, Gitlin JD (1999) Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci USA 96:10812-10817

Harris ZL, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RT, Giltin JD (1995) Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. Proc Natl Acad Sci USA 92:2539-2543

Hass R, Gutierrez-Rivero B, Knoche J, Boker K, Manns MP, Schmidt HH (1999) Mutation analysis in patients with Wilson diease: identification of 4 novel mutations. Hum Mutat 14:88

Hasselbach W, Mankinose M (1961) [The calcium pump of the "relaxing granules" of muscle and its dependence of ATP-splitting]. Biochem Z 333:518-528

Hattori A, Sawaki M, Enomoto K, Tsuzuki N, Isomura H, Kojima T, Kamibayashi Y, Sugawara N, Sugiyama T, Mori M (1995) The high hepatocarcinogen susceptibility of LEC rats is genetically independent of abnormal copper accumulation in the liver. Carcinogenesis 16:491-494

Horn N, Heydorn K, Damsgaard E, Tygstrup I, Vestermark S (1978) Is Menkes syndrome a copper storage disorder? Clin Genet 14:186-187

Hung IH, Casareno RLB, Labesse G, Mathews FS, Gitlin JD (1998) HAH1 is a copper-binding protein with distinct amino acid residues mediating copper homeostasis and antioxidant defense. J Biol Chem 272:1749-1754

Hung IH, Suzuki M, Yamaguchi Y, Yuan DS, Klausner RD, Gitlin JD (1997) Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. J Biol Chem 272:21461-21466

Hunt DM (1974) Primary defect in copper transport underlies mottled mutants in the mouse. Nature (Lond) 249:852-854

Jobling M, Huang X, Stewart L, Barnham K, Curtain C, Volitakis I, Perugini M, White A, Cherny R, Masters C, Barrow C, Collins S, Bush A, Cappai R (2001) Copper and zinc binding modulates the aggregation and neurotoxic properties of the prion peptide PrP106-126. Biochemistry 40:80738084

Kaler SG (1994) Menkes disease. Adv Pediatr 41:262-303

Kaler SG (1996) Menkes disease mutations and response to early copper histidine treatment. Nat Genet 13:21-22

Kataoka M, Tavassoli M (1985) Identification of ceruloplasmin receptors on the surface of human blood monocytes, granulocytes, and lymphocytes. Exp Hematol 13:806-810

Kim EK, Yoo OJ, Song KY, Yoo HW, Choi SY, Cho SW, Hahn SH (1998) Identification of three novel mutations and a high frequency of the Arg778Leu mutation in Korean patients with Wilson disease. Hum Mutat 11:275-278

Klomp A, Van de Sluis B, Klomp L, Wijmenga C (2003) The ubiquitously expressed MURR1 protein is absent in canine copper toxicosis. J Hepatol 39:703-709

Klomp AEM, Juijn JA, Van Der Gun LTM, Van Den Berg IET, Berger R, Klomp LW (2003) The N-terminus of the human copper transporter (hCTR!) is located extracellularly, and interacts with itself. Biochem J 370:881-889

Klomp AEM, Tops BBJ, Van Den Berg IET, Berger R, Klomp LWJ (2002) Biochemical characterization and subcellular localization of human copper transporter 1 (hCTR1). Biochem J 364:497-505

Knight S, Labbe S, Kwon LF, Kosman DJ, Theile DJ (1996) A widespread transposable element masks expression of a yeast copper transport gene. Genes Dev 10:1917-1929

Knopfel M, Smith C, Solioz M (2005) ATP-driven copper transport across the intestinal brush border membrane. Biochem Biophys Res Commun 330:645-652

Kodama H (1993) Recent developments in Menkes disease. J Inherit Metab Dis 16:791-799

Kuo Y-M, Zhou B, Cosco D, Gitschier J (2001) The copper transporter CTR1 provides as essential function in mammalian embryonic development. Proc Natl Aacd Sci USA 98:6836-6841

La Fontaine S, Firth SD, Lockhart PJ, Brooks H, Camakaris J, Mercer JFB (1999) Intracellular localization and loss of copper responsiveness of Mnk, the murine homologue of the Menkes protein, in cells from blotchy (*Mo^{blo}*) and brindled (*Mo^{br}*) mouse mutants. Hum Mol Genet 8:1069-1075

Larin D, Mekios C, Das K, Ross B, An-Suei Y, Gilliam TC (1999) Characterization of the interaction between the Wilson and Menkes disease proteins and the cytoplasmic copper chaperone, HAH1P*. J Biol Chem 274:28497-28504

Lee J, Pena MMO, Nose Y, Thiele DJ (2002) Biochemical characterisation of the human copper transporter Ctr1. J Biol Chem 277:4380-4387

Lee J, Prohaska J, Thiele D (2001) Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc Natl Acad Sci USA 98:6842-6847

Lee S, Lancey R, Montaser A, Madani N, Linder M (1993) Ceruloplasmin and copper transport during the latter part of gestation in the rat. Proc Soc Exp Biol Med 203:428-439

Levinson B, Vulpe C, Elder B, Martin C, Verley F, Packman S, Gitschier J (1994) The mottled gene is the mouse homologue of the Menkes disease gene. Nat Genet 6:369-373

Li Y, Togashi Y, Sato S, Emoto T, Kang J-H, Takeichi N, Kobayashi H, Kojima Y, Une Y, Uchino J (1991) Spontaneous hepatic copper accumulation in long-evans cinnamon rats with hereditary hepatitis: a model of Wilson's disease. J Clin Invest 87:1858-1861

Linder M, Wooten L, Cerveza P, Cotton S, Shulze R, Lomeli N (1998) Copper transport. Am J Clin Nutr 67 (5 suppl):965S-971S

Linder MC, Lomeli NA, Donley S, Mehrbod F, Cerveza P, Cotton S, Wooten L (1999) Copper transport in mammals. Kluwer Acedemic/Plenum Publishers, New York

Lonnerdal B (1996) Bioavailability of copper. Am J Clin Nutr 63:821S-828S

Lyon MF (1962) Sex chromatin and gene action in the mammalian X-Chromosome. Am J Hum Genet 14:135-148

Mann J, Camakaris J, Danks D (1980) Copper metabolism in mottled mouse mutants. Defective placental transfer of 64Cu to foetal brindled (Mobr) mice. Biochem J 186:629-631

Mann JR, Camakaris J, Danks DM, Walliczek EG (1979) Copper metabolism in mottled mouse mutants: copper therapy of brindled (Mo^{br}) mice. Biochem J 180:605-612

Massimino T, Griffoni M, Salvato C, Tomasi V, Spisni E (2005) Extracellular copper ions regulate prion protein (PrPC) expression and metabolism in neuronal cells. FEBS Lett 579:741-744

Maxfield AB, Heaton DN, Winge DR (2004) Cox17 is functional when tethered to the mitochondrial inner membrane. J Biol Chem 279:5072-5080

Menkes JH, Alter M, Steigleder GK, Weakley DR, Sung JH (1962) A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. Pediatrics:764-779

Mercer FBJ (2001) The molecular basis of copper-transport diseases. Trends Mol Med 7:64-69

Mercer JFB (1998) Menkes syndrome and animal models. Am J Clin Nutr 5:1027S-1028S

Mercer JFB, Ambrosini L, Horton S, Gazeas S, Grimes A (1999) Animal models of Menkes disease. Adv Exp Biol Med 448:97-108

Mercer JFB, Barnes N, Steverson J, Strausak D, Llanos R (2003) Copper-induced trafficking of the Cu-ATPases: A key mechanism for copper homeostasis. Biometals 16:175-184

Mercer JFB, Camakaris J (1997) Menkes and Wilson's diseases: Genetic disorders of copper transport. Metal Ions Gen Reg:250-276

Mercer JFB, Grimes A, Ambrosini L, Lockhart P, Paynter JA, Dierick H, Glover TW (1994) Mutations in the murine homologue of the Menkes gene in dappled and blotchy mice. Nat Genet 6:374-378

Mercer JFB, Livingston J, Hall BK, Paynter JA, Begy C, Chandrasekharappa S, Lockhart P, Grimes A, Bhave M, Siemenack D, Glover TW (1993) Isolation of a partial candidate gene for Menkes disease by postional cloning. Nat Genet 3:20-25

Meyer LA, Durley AP, Prohaska JR, Harris ZL (2001) Copper transport and metabolism are normal in aceruloplasminemic mice. J Biol Chem 276:36857-36861

Moller LB, Petersen C, Lund C, Horn N (2000) Characterization of the hCTR1 gene: genomic organization, functional expression, and identification of a highly homologous processed gene. Gene 257:13-22

Muller T, Feichtinger H, Berger H, Muller W (1996) Endemic Tyrolean infantile cirrhosis: an ecogenetic disorder. Lancet 347:877-880

Odermatt A, Suter H, Krapf R, Solioz M (1993) Primary structure of two P-type ATPases involved in copper homeostasis in *E. hirae*. J Biol Chem 268:12775-12779

Orena SJ, Goode CA, Linder MC (1986) Binding and uptake of copper from ceruloplasmin. Biochem Biophys Res Commun 139:822-829

Owen CAJ (1965) Metabolism of radiocopper (Cu64) in the rat. Am J Physiol 209:900-904

Palmgren MG, Axelsen KB (1998) Evolution of P-type ATPases. Biochim Biophys Acta 1365:37-45

Payne AS, Gitlin JD (1998) Functional expression of the Menkes disease protein reveals common biochemical mechanisms among the copper-transporting P-type ATPases. J Biol Chem 273:3765-3770

Pena MMO, Lee J, Thiele DJ (1999) A delicate balance: Homeostatic control of copper uptake and distribution. J Nutr 129:1251-1260

Petris MJ, Mercer JFB, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J (1996) Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. EMBO J 15:6084-6095

Petris MJ, Strausak D, Mercer JFB (2000) The Menkes copper transporter is required for the activation of tyrosinase. Hum Mol Genet 9:2845-2851

Petris MJ, Voskoboinik I, Cater M, Smith K, Kim B-E, Llanos RM, Strausak D, Camakaris J, Mercer JFB (2002) Copper-regulated trafficking of the Menkes disease Copper ATPase is associated with formation of a phosphorylated catalytic intermediate. J Biol Chem 277:46736-46742

Phinney A, Drisaldi B, Schmidt S, Lugowski S, Coronado V, Liang Y, Horne P, Yang J, Sekoulidis J, Coomaraswamy J, Chishti M, Cox D, Mathews P, Nixon R, Carlson G, George-Hyslop P, Westaway D (2003) In vivo reduction of amyloid-{beta} by a mutant copper transporter. Proc Natl Acad Sci USA 100:14193-14198

Procopis P, Camakaris J, Danks DM (1981) A mild form of Menkes steely hair syndrome. J Pediatr 98:97-99

Rauch H (1983) Toxic milk, a new mutation affecting copper metabolism in the mouse. J Hered 74:141-144

Roberts EA, Cox DW (1998) Wilson disease. Baillieres Clin Gastroenterol 12

Roelofsen H, Wolters H, Luyn MJAV, Miura N, Kuipers F, Vonk RJ (2000) Copper-induced apical trafficking of ATP7B in polarized hepatoma cells provides a mechanism for biliary copper excretion. Gastroenterology 119:782-793

Rogers J, Randall J, Eder P, Huang X, Bush A, Tanzi R, Venti A, Peyton S, Giordano A, Greig N, Sarang S, Gullans S (2002) Alzheimer's disease drug discovery targeted to the APP mRNA 5' untranslated region. J Mol Neurosci 19:77-82

Sambongi Y, Wakabayashi T, Yoshimizu T, Omote H, Oka T, Futai M (1997) *Caenorhabditis elegans* cDNA for a Menkes/Wilson disease gene homologue and its function in a yeast CCC2 gene deletion mutant. J Biochem 121:1169-1175

Sass-Kortsak A (1965) Copper metabolism. Adv Clin Chem 8:1-67

Scarborough GA (1999) Structure and function of the P-type ATPases. Curr Opin Cell Biol 11:517-522

Schaefer M, Hopkins RG, Failla MA, Gitlin JD (1999a) Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. Am J Physiol 276:G639-G646

Schaefer M, Roelofsen H, Wolters H, Hofmann WJ, Müller M, Kuipers F, Stremmel W, Vonk RJ (1999b) Localization of the Wilson's disease protein in human liver. Gastroenterology 117:1380-1385

Scheinberg IH, Sternlieb I (1984) Wilson's disease. Saunders, Philadelphia

Schilisky ML (1996) Wilson disease: genetic basis of copper toxicity and natural history. Semin Liver Dis 16:83-95

Schlaug G, Hefter H, Engelbrecht V, Kuwert T, Arnold S, Stockin G, Seitz RJ (1996) Neurological impairment and recovery in Wilson's disease: evidence by PET and MRI. J Neurol Sci 136:129-139

Schmitt R, Darwish H, Cheney J, Ettinger M (1983) Copper transport kinetics by isolated rat hepatocytes. Am J Physiol 224:183-192

Skou JC (1957) The influence of some cations on an adenosin triphosphatase from peripheral nerves. Biochim Biophys Acta 23:394-401

Solioz M (1998) Copper homeostasis by CPX-type ATPases. The new subclass of heavy metal P-type ATPase. Adv Mol Cell Biol 23A:167-203

Solioz M, Vulpe C (1996) CPX-type ATPases: a class of P-type ATPases that pump heavy metals. Trends Biochem Sci 21:237-241

Srinivasan C, Posewitz MC, George GN, Winge DR (1998) Characterization of the Copper Chaperone Cox17 of *Saccharomyces cerevisiae*. Biochemistry 37:7572-7577

Strausak D, Fontaine SL, Hill J, Firth SD, Lockhart PJ, Mercer JFB (1999) The role of GMXCXXC metal binding sites in the copper-induced redistribution of the Menkes protein. J Biol Chem 274:11170-11177

Sugawara N, Li D, Sugawara C, Miyake H (1993) Decrease in biliary excretion of copper in Long-Evans Cinnamon (LEC) rats causing spontaneous hepatitis due to a gross accumulation of hepatic copper. Res Comm Chem Path Pharmacol 81:45-52

Sugawara N, Sugawara C, Sato M, Katakura M, Takahashi H, Mori M (1991) Copper metabolism in LEC rats aged 30 and 80 days old: induction of Cu-metallothionein and status of zinc and iron. Res Comm Chem Path Pharmacol 72:353-362

Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B, Romano DM, Parano E, Pavone L, Brzustowicz LM, Devoto M, Peppercorn J, Bush AI, Sternlieb I, Pirastu M, Gusella JF, Evgrafov O, Penchaszadeh GK, Honig B, Edelman IS, Soares MB,

Scheinberg IH, Gilliam TC (1993) The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat Genet 5:344-350

Tao T, Liu F, Klomp L, Wijmenga C, Gitlin J (2003) The copper toxicosis gene product Murr1 directly interacts with the Wilson disease protein. J Biol Chem 278:41593-41596

Terada K, Nakako T, Yang X-L, Iida M, Aiba N, Minamiya Y, Nakai M, Sakaki T, Miura N, Sugiyama T (1998) Restoration of holoceruloplasmin synthesis in LEC rat after infusion of recombinant adenovirus bearing WND cDNA. J Biol Chem 273:1815-1820

Theophilos MB, Cox DW, Mercer JFB (1996) The toxic milk mouse is a murine model of Wilson disease. Hum Mol Genet 5:1619-1624

Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW (1995) The Wilson disease gene: spectrum of mutations and their consequences. Nat Genet 9:451

Tipton IH, Cook MJ (1963) Trace elements in human tissue. Past II. Adult subjects from the United States. Health Phys 9:103-145

Torres AS, Petri V, Rae TD, O'Halloran TV (2001) Copper stabilizes a hetrodimer of the yCCS metallochaperone and interacts with superoxide dismutase. J Biol Chem 276:38410-38416

Toyoshima C, Nomura H, Tsuda T (2004) Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. Nature 432:361-368

Tumer Z, Moller LB, Horn N (1999) Mutation spectrum of ATP7A, the gene defective in Menkes disease. Adv Exp Med Biol 448:83-95

Turnlund JR, Keyes WR, Peiffer GL, Scott KS (1998) Copper absorption, excretion and retention by young men consuming low dietary copper determined by using the stable isotope 65Cu. Am J Clin Nutr 67:1219-1225

Van Campen DR (1971) Absorption of copper from the gastrointestinal tract. In: Skoryna SC, Waldron-Edwards D (eds) Intestinal absorption of metal ions, trace elements and radionuclides. Pergamon, Oxford, pp 211-227

Vargas E, Shoho AR, Linder MC (1994) Copper transport in the Nagase analbuminemic rat. Am J Physiol 267:G259-G269

Versieck J, Cornelis R (1980) Normal levels of trace elements in human blood plasma or serum. Anal Chim Acta 116:217-254

Voskoboinik I, Brooks H, Smith S, Shen P, Camakaris J (1998) ATP-dependent copper transport by the Menkes protein in membrane vesicles isolated from cultured Chinese hamster ovary cells. FEBS Letters 438:178-182

Voskoboinik I, Camakaris J, Mercer JF (2002) Understanding the mechanism and function of copper P-type ATPases. In: Valentine J, Gralla E (eds) Adv. Protein Chemistry. Elsevier Science, pp 123-150

Voskoboinik I, Mar J, Strausak D, Camakaris J (2001) The regulation of catalytic activity of the Menkes copper-translocating P-type ATPase. J Biol Chem 276:28620-28627

Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat Genet 3:7-13

Vulpe CD, Packman S (1995) Cellular copper transport. Annu Rev Nutr 15:293-322

Walker JM, Tsivkovskii R, Lutsenko S (2002) Metallochaperone Atox1 transfers copper to the NH_2-terminal domain of the Wilson's disease protein and regulates its catalytic activity. J Biol Chem 277:27953-27959

Watkins S, Madison J, Galliano M, Minchiotti L, Putnam FW (1994) A nucleotide insertion and frameshift cause analbuminemia in an Italian family. Proc Natl Acad Sci USA 91:2275-2279

White A, Huang X, Jobling M, Barrow C, Beyreuther K, Masters C, Bush A, Cappai R (2001) Homocysteine potentiates copper and amyloid beta peptide-mediated toxicity in primary neuronal cultures: possible risk factors in the Alzheimer's-type neurodegenerative pathways. J Neurochem 76:1509-1520

White AR, Reyes R, Mercer JFB, Camakaris J, Zheng H, Bush AI, Multhaup G, Beyreuther K, Masters CL, Cappai R (1999) Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice. Brain Res 842:439-444

Wijmenga C, Muller T, Murli IS, Brunt T, Feichtinger H, Schonitzer D, Houwen RH, Muller W, Sandkuijl LA, Pearson PL (1998) Endemic Tyrolean infantile cirrhosis is not an allelic variant of Wilson's disease. Eur J Hum Genet 6:624-628

Wilson DC, Phillips MJ, Cox DW, Roberts EA (2000) Severe hepatic Wilson's disease in preschool-aged children. J Pediatr 137:719-722

Wilson KSA (1912) Progressive lenticular degeneration. A familial nervous disease associated with cirrhosis of the liver. Brain 34:295-506

Winge DR, Mehra RK (1990) Host defences against copper toxicity. In: Richter GW, Solez K, Aisen P, Cohen G (eds) Transition Metal Toxicity. Academic Press, San Diego, pp 47-83

Wirth PL, Linder MC (1985) Distribution of copper among components of human serum. JNCL 75:277-284

Wong PC, Waggoner D, Subramaniam JR, Tessarollo L, Bartinakas TB, Culotta VC, Price DL, Rothstein J, Gitlin JD (2000) Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. Proc Natl Aacd Sci USA 97:2886-2891

Wu J, Forbes JR, Chen HS, Cox DW (1994) The LEC rat has a deletion in the copper transporting ATPase homologous to the Wilson disease gene. Nat Genet 7:541-545

Yamada T, Agui T, Suzuki Y, Sato M, Matsumoto K (1993) Inhibition of the copper incorporation into ceruloplasmin leads to the deficiency in serum ceruloplasmin activity in Long-Evans Cinnamon mutant rat. J Biol Chem 268:8965-8971

Yamaguchi Y, Heiny ME, Gitlin JD (1993) Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease. Biochem Biophys Res Com 197:271-277

Yamaguchi Y, Heiny ME, Shimizu N, Aoki T, Gitlin JD (1994) Expression of the Wilson disease gene is deficient in the Long-Evans Cinnamon rat. Biochem J 301:1-4

Yoshida K, Furihata K, Takeda S, Nakamura A, Yamamoto K, Morita H, Hiyamuta S, Ikeda S, Norikazu S, Yanagisawa N (1995) A mutation in the ceruloplasmin gene is associated with systemic hemosiderosis in humans. Nat Genet 9:267-272

Yoshida Y, Furuta S, Niki E (1993) Effects of metal chelating agents on the oxidation of lipids induced by copper and iron. Biochim Biophys Acta 1210:81-88

Zhou B, Gitschier J (1997) h*CTR1*: A human gene for copper uptake identified by complementation in yeast. Proc Natl Aacd Sci USA 94:7481-7486

Cater, Michael A.
Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, Burwood, Victoria Australia

Mercer, Julian F.B.
Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, Burwood, Victoria Australia
jmercer@deakin.edu.au

Zn^{2+}, a dynamic signaling molecule

Michal Hershfinkel

Abstract

Zinc is essential for cell proliferation thereby promoting growth and development, yet a rise of intracellular zinc is a leading cause of neuronal cell death in excitotoxic syndromes. While pervious studies have addressed mostly the structural role of zinc as a cofactor of numerous enzymes and zinc finger proteins, recent data suggest that zinc is acting as a signaling molecule. Despite the accumulating knowledge on the transporters, which are shown to maintain cellular and subcellular zinc homeostasis, the mechanisms by which they function are much less understood. Changes in extracellular or intracellular zinc trigger the activation of major signaling pathways, partially mediated by a specific zinc sensing receptor, which are linked to either cell growth or cell death. These proteins, which are regulated by zinc, will be the subject of this review. The major challenges in future studies will be to reveal the cellular network of zinc signaling and their links to cellular zinc homeostasis.

1 Zinc in health and disease

Zinc, is an essential trace element for cell growth and development and its deficiency leads to abnormal growth (Evans 1986; Vallee and Falchuk 1993; Sandstead et al. 1998; MacDonald 2000). Among the most well known syndromes associated with zinc deficiency is growth arrest, improper development of the brain, loss of taste and smell, attenuated wound healing, and impaired immune response (Prasad 1998; Hambidge 2000; Komai et al. 2000; MacDonald 2000; Sandstead 2000; Scott and Koski 2000; Wapnir 2000; Bhatnagar and Taneja 2001). Zinc deficiency has been also linked to retarded development of the male reproductive system (El-Tawil 2003). The most severe manifestation of zinc deficiency is Acrodermatitis enteropathica that is a genetic disorder linked to improper zinc uptake leading to severe skin lesions, diarrhea, and subsequently brain damage (Perafan-Riveros et al. 2002). While this disorder was described many years ago, it has been only recently that the genes and cellular mechanisms underlying this disorder were identified (Wang et al. 2002, 2004b).

While earlier works focused on pathophysiological aspects of zinc deficiency, during the past three decades it has been repeatedly demonstrated that excessive rise in cellular zinc, particularly in brain cells, may also be harmful (Assaf and Chung 1984; Sloviter 1985; Danscher et al. 1997; Suh et al. 2000; Weiss et al.

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_95 / Published online: 2 August 2005

2000; Takeda 2001; Frederickson 2003; Sensi and Jeng 2004). Zinc, endogenously released, has been shown to induce neuronal cell death following its permeation into neurons. Such neuronal cell death is associated with ischemia, where certain brain regions such as the CA1 and CA3 regions of the hippocampus and neocortical layers 3, 5, and 6 are considered particularly vulnerable (Choi 1996; Choi and Koh 1998; Suh et al. 1999; Wei et al. 2004). The role of zinc in cytotoxicity has been first implied by the depletion of presynaptic zinc from the mossy fibers of the hippocampus followed by its appearance in post synaptic neurons destined to death (Frederickson et al. 1988, 1989; Lee et al. 2002a). Neurons could be rescued by the application of extracellular zinc chelators such as Ca-EDTA prior to the insult or by blocking the Ca/kainite AMPA channels, a major zinc permeation pathway to neurons (Koh et al. 1996; Sensi et al. 1999; Yin et al. 2002; Wei et al. 2004). Recent data suggest that not only the "free" synaptic zinc is linked to neuronal cell death but suggest a role for zinc that is released from intracellular pools (Lee et al. 2003; Sensi and Jeng 2004). Studies employing a zinc transporter knockout model in which synaptic zinc is depleted have also shown zinc-dependent neuronal death, highlighting the role of intracellular zinc pools in zinc dependent cell death (Cole et al. 2000; Lee et al. 2000). Zinc linked neuronal cell death is also occurring in epilepsy and traumatic brain injury (Buhl et al. 1996; Nagatomo et al. 1998; Suh et al. 2000). Finally, zinc has been recently linked to the formation of β-amyloid senile plaques and its chelation using clioquinol resulted in reduction of the number and size of the senile plaques (Cuajungco et al. 2000; Cherny et al. 2001). Indeed, using the same chelator decreased the synaptic and vesicular zinc pools in the brain and pancreas respectively (Nitzan et al. 2003).

In pancreatic islets of Langerhans zinc is co-released with insulin in concentrations similar to the synaptic zinc in the brain (Gee et al. 2002). Although zinc deficiency may increase β-cells apoptosis and its presence enhances proliferation (Kato et al. 1997; Schott-Ohly et al. 2004) intracellular accumulation of zinc has been suggested to affect β-cell death, in models of diabetes type 1, and contribute to the destruction of the islet (Apostolova et al. 1997; Kim et al. 2000b).

The importance of zinc in development and the pathophysiologies linked to its excess are well documented (Table 1), however, the cellular mechanisms linking the changes in zinc to cell fate are much less understood. In recent years, genes and proteins linked to zinc homeostasis were identified. Lethal milk syndrome in mice, for example, has been associated with a mutation of the zinc transporter, ZnT-4. In this syndrome, maternal milk does not contain adequate zinc levels and the pups die of zinc deficiency unless supplemented with zinc (Huang and Gitschier 1997). In humans, the ZIP4 gene, a zinc transporter involved in epithelial zinc absorption, has been linked to the human acrodermatitis enteropathica (Kury et al. 2002; Wang et al. 2002). This review will, therefore, focus on advances of our understanding of zinc transport and signaling mechanisms, which are linked to the physiology and pathophysiology of zinc homeostasis.

Table 1. Common syndromes linked to zinc excess or deficiency.

Excess of zinc		
Neuronal cell death	Associated with ischemia, epilepsy and traumatic brain injury.	(Choi 1996; Choi and Koh 1998; Suh et al. 1999; Wei et al. 2004)
Formation of β-amyloid senile plaques (synaptic zinc)	Treatment with a chelator reduces the formation of plaques.	(Cuajungco et al. 2000; Cherny et al. 2001; Maynard et al. 2005)
Pancreatic β-cell death	Zinc has been implicated in induction of cell proliferation, yet it is also linked to β-cell death in diabetes type-1 models.	(Apostolova et al. 1997; Kim et al. 2000b)
Prostate cancer	Zinc levels in the seminal fluid are among the highest in the body. Supplementary intake of zinc is associated with prostate carcinogenesis.	(Liang et al. 1999; Feng et al. 2002; Leitzmann et al. 2003; Costello et al. 2004; Franklin et al. 2005)
Zinc deficiency		
Growth arrest	Zinc deficiency leads to general growth arrest.	(Evans 1986; Vallee and Falchuk 1993; Sandstead et al. 1998; MacDonald 2000)
Brain development	Zinc deficiency during embryogenesis is linked to malformations. During adulthood zinc deficiency is linked to low cognitive performance.	(Krebs 2000; Sandstead 2000; Sandstead et al. 2000; Bhatnagar and Taneja 2001)
Loss of taste and smell	Zinc deficiency is linked to taste disorders, and xerostomia.	(Frederickson et al. 1987; Olmez et al. 1988; Ohara et al. 1995; Komai et al. 2000; Tanaka 2002)
Attenuated wound healing	Zinc deficiency is linked to skin lesions and zinc is added to enhance wound healing.	(Andrews and Gallagher-Allred 1999; Barceloux 1999; Kudravi and Reed 2000; Tenaud et al. 2000; Perafan-Riveros et al. 2002)
Impaired immune response	Zinc deficiency is linked to impaired immune response probably linked to attenuated proliferation and function of immune cells.	(Prasad 1998; Budinger and Hertl 2000; Mocchegiani and Muzzioli 2000; Scott and Koski 2000)
Retarded development and dysfunction of male reproductive system	Zinc deficiency is linked to underdeveloped testis, and interference in testosterone production. Zinc protects against lead and cadmium toxicity of the testis.	(Favier 1992; Vallee and Falchuk 1993; Om and Chung 1996; Batra et al. 1998)
Diarrhea	Zinc treatment reduces the duration and severity of diarrhea in children	(Hambidge 1992; Sazawal et al. 1995; Hambidge and Krebs 1999; Bhatnagar et al. 2004)

2 Mechanisms of cellular zinc homeostasis

Zinc ions lack direct redox activity and are thus much less damaging than other micronutrients such as iron and copper. It is, therefore, not surprising that zinc has evolutionary evolved as a cofactor for numerous enzymes, playing a catalytic or structural role (Vallee and Falchuk 1993). Zinc finger motifs are indeed the most abundant motifs in transcription factors. Because of the specific and strong binding of zinc to these domains, it was considered a rather inert intracellular ion, which mainly carries structural roles. In recent years, zinc pools are emerging as highly dynamic and are shown to be regulated by various signaling pathways involved in both physiological and pathophysiological roles.

2.1 Cellular zinc pools

Proteins such as zinc fingers are considered to bind a very significant amount of cellular zinc, and this is considered a tightly bound pool, which is least available for cellular signaling (Vallee and Falchuk 1993). Another group of zinc binding proteins are the metallothioniens (MT), this pool has been shown to release zinc upon various stimuli, and most prominent is the activation of the NO pathway (Maret 1995, 2003; Pearce et al. 2000; St Croix et al. 2002; Lee et al. 2003). Recent studies suggest that not only that the cytosolic MTs release zinc but interestingly MTs that are binding zinc translocate, upon cellular signaling, into the nucleus and provide a targeted zinc pool to specific organelles (Spahl et al. 2003). While zinc released from MTs have been mostly implicated in cell death, other studies have suggested that physiological signaling, not followed by cell damage, also leads to release of zinc from this pool. For example, it has been demonstrated that intracellular calcium rise, mediated by metabotropic receptors or calcium influx, followed by the generation of NO, resulted in the release of zinc from MTs (Pearce et al. 2000). Yet, calcium rise in cells is a general signaling pathway that, via generation of NO, may mediate intracellular zinc release following metabotropic stimuli.

The dynamic nature of the interaction of zinc with MTs raises the intriguing possibility that the zinc finger proteins, which are still considered a rigid pool of non-releasable zinc, may also be dynamically regulated (Berg and Shi 1996; Kroncke 2001). Considering the fundamental and diverse roles of the zinc fingers in gene expression such regulation, if indeed demonstrated, may have novel physiological implication.

The pool of zinc with the highest turnover is what was called "the chelatable zinc pool", which is composed of zinc ions that are packed into vesicles, and released during neuronal activity or secretion in the pancreas and salivary gland (Assaf and Chung 1984; Frederickson et al. 1987, 2003; Weiss et al. 2000; Kristiansen et al. 2001; Sensi and Jeng 2004). While the role of the released zinc during excitotoxic syndromes has been thoroughly studied, the role of secreted zinc during non-pathophysiological ("normal") activity is less understood. In the brain, the released zinc is interacting with receptors such as the NMDA, GABA, and

glycine thereby regulating the neuronal activity (Buhl et al. 1996; Paoletti et al. 1997; Han and Wu 1999; Choi et al. 2001; Hosie et al. 2003). Zinc inhibits both the GABA and NMDA pathways and therefore may tilt the balance between inhibitory and excitatory neuronal transmission. Indeed, on one hand, zinc released from aberrantly sprouting of mossy fibers has been shown to induce epileptic seizures by GABA inhibition (Cole et al. 2000), on the other hand, zinc chelation or deficiency leads to an increased incidence of seizures (Dominguez et al. 2003; Blasco-Ibanez et al. 2004). This is one example for the signaling role of zinc, which is more than a structural element in the packaging of hormones and neurotransmitters.

2.2 Mammalian zinc transport

The dynamic and intricate nature of cellular zinc homeostasis described above must be controlled by zinc transporters that are involved in transport of zinc across the cell membrane and into organelles. Indeed the large number of genes that have been implicated in cellular zinc transport underscore the importance of cellular zinc homeostasis. This is mediated by several families of zinc transporters, the most prominent are ZIP (SLC39), which is largely involved in accumulation of zinc in the cytosol and ZnT (SLC30), which is largely involved in lowering cytosolic zinc levels (Gaither and Eide 2001a; Eide 2004; Palmiter and Huang 2004). Other ion permeation pathways, which are considered non-specific for zinc are also playing a role in zinc transport, most notable are the DMTs (divalent-metal transporters) and L-type calcium channels (Atar et al. 1995; Canzoniero et al. 1997; Sensi et al. 1997; Kim et al. 2000a; Rolfs and Hediger 2001). In this review, I will mainly focus on the two major families of zinc transporters the ZIP and the ZnT transporter families (Fig. 1).

2.2.1 ZnT family

The ZnT family is a rapidly growing family of zinc transporter proteins, which consists up to date of ten members, the ZnT1-10. The expression of many of them is regulated by dietary zinc. The proteins of this family share a putative 6-transmembrane domain and a histidine rich region, located usually between the IV and V transmembrane domains, which is suggested to be involved in zinc binding and transport (Palmiter and Huang 2004). The ZnT5 c-terminal domain resembles other members of the family, yet, it has a much larger n-terminus and a putative larger number of transmembrane domains (Kambe et al. 2002). The ZnT proteins have been shown to lower intracellular zinc either by sequestration into intracellular organelles or via plasma membrane. The mechanisms by which these proteins are catalyzing zinc transport are not yet fully elucidated, although the phenotypes for their knockouts are clearly linked to cellular zinc homeostasis.

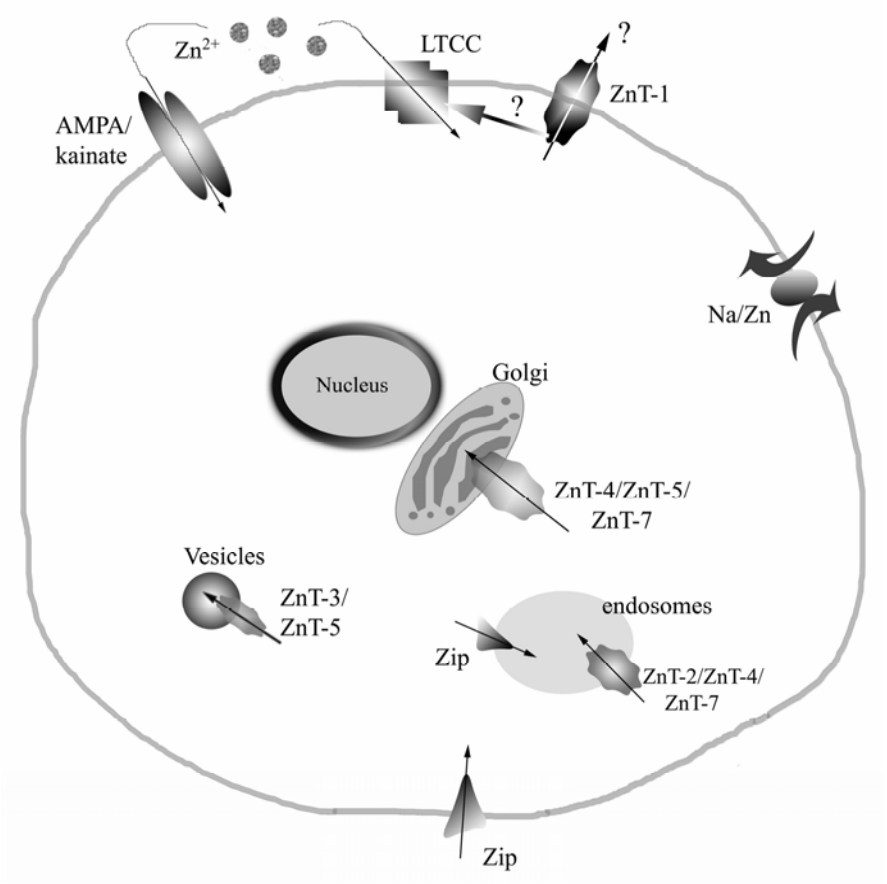


Fig. 1. A schematic representation of the major cellular zinc transporters described in Section 2.2. Note that the Zip transporters may translocate upon changes in zinc levels.

ZnT-1, the ubiquitously expressed protein of this family, is the only one found on the plasma membrane (Palmiter and Findley 1995). ZnT-1 was the first member of the ZnT family that was discovered by complementation studies in baby hamster kidney cell line (BHK) that is hypersensitive to zinc toxicity. This protein was later shown to confer resistance against zinc toxicity also in neurons and glial cells (Palmiter and Findley 1995; Nolte et al. 2004; Palmiter 2004). In the mouse brain, ZnT-1 is localized in regions where intense zinc homeostasis is occurring mostly in regions rich in synaptic zinc (Sekler et al. 2002). Interestingly, the expression of the ZnT-1 protein is correlated with the appearance of the synaptic zinc (Nitzan et al. 2002). Other organs in which the ZnT-1 is highly expressed are also linked to zinc homeostasis such as the villi of the small intestine, kidney, and placenta (McMahon and Cousins 1998). Knockout of ZnT-1 is lethal leading to early embryonic death at day 9 of the pregnancy (Andrews et al. 2004). The lethality has been linked to impairment of zinc transport since ZnT-1 mRNA expression

in WT embryos is particularly high in the visceral yolk sac and in regions, which form the placenta (Andrews et al. 2004). The ZnT-1 was first suggested to act as a zinc extruder due to its ability to lower zinc toxicity, yet a counter ion or ATP dependence has not been demonstrated (Palmiter and Findley 1995; Palmiter 2004). Other studies have indicated, however, that the ZnT-1 downregulates intracellular zinc accumulation via regulation of the voltage gated calcium channels (Nolte et al. 2004; Segal et al. 2004). The L-type subtype of these channels are a major route of zinc entry into cells; by downregulation of zinc influx through the L-type calcium channel, ZnT-1 may confer resistance against massive zinc permeation. Interestingly, recent results show that zinc-dependent interaction of ZnT-1, and its *C. elegans* homologue CDF-1, with Raf-1 leads to the regulation of ERK pathway (Jirakulaporn and Muslin 2004). Hence the role of ZnT-1 as a regulator of cellular signaling or a transporter *per se* remains an open and intriguing question.

The ZnT-3 has been extensively described although its mechanism of ion transport has not been elucidated (Palmiter et al. 1996; Lee et al. 2000). This protein has been suggested to transport zinc into synaptic vesicles of glutamatergic neurons, indeed ZnT-3 knockout mice are deficient of the synaptic zinc (Cole et al. 1999). Yet, the phenotype of these mice was apparently normal with no significant abnormalities (Cole et al. 1999). Furthermore, electrophysiological recordings from hippocampal slices did not monitor any functional impairment (Cole et al. 2001). Even more surprising is the fact that the ZnT-3 knockout mice suffer from neuronal zinc toxicity following ischemia (Lee et al. 2000). More recent studies, focusing on epilepsy models have found that the ZnT-3 knockout mice are more vulnerable to seizures (Cole et al. 2000). The synaptically released zinc has been linked to the pathogenesis of Alzheimer's disease, as the ZnT-3 knockout mice were less vulnerable to the accumulation of amyloid senile plaques (Lee et al. 2002b). Interestingly in the ZnT-3 knockouts, the gender difference between male and female in the susceptibility to plaque formation has disappeared.

The ZnT-5 protein is abundantly expressed in the pancreatic β-cells and is located on the insulin secretory vesicles and the Golgi apparatus (Kambe et al. 2002; Devergnas et al. 2004). The ZnT-5 was first suggested to play a role, similar to that of the ZnT-3, in the loading of zinc into the insulin secretory granules in these cells. ZnT-7, which is found on the same secretory vesicles, is presumably playing a similar role (Kirschke and Huang 2003). The localization of these proteins on the Golgi apparatus may further suggest a general role for these transporters in transferring zinc into zinc-dependent enzymes such as has been shown for the alkaline-phosphatases (ALPs) (Suzuki et al. 2005). A knockout model of ZnT-5 showed a phenotype of osteopenia and more than 60% of the male mice died of bradyarrhythmias (Inoue et al. 2002). These phenotypes were suggested to be related to the regulation of the expression of genes encoding for response to stress such as immediate-early response factors and heat shock proteins. Again, the mechanism by which the ZnT-5 affects these proteins remains to be elucidated.

The ZnT-2, ZnT-4 and ZnT-7 proteins are localized on intracellular organelles, late endosomes for the ZnT-2 and Golgi apparatus for ZnT-4 and ZnT-7. The ZnT-4 protein is localized mainly in vesicle of kidney and mammary gland cells although it is also found in intestinal cells and the brain. Mutation of the mouse

ZnT-4 protein was linked to the lethal milk syndrome and thus was suggested to regulate zinc sequestration into endosomes in mammary gland epithelia (Huang and Gitschier 1997). This protein is probably not involved in the human form of milk zinc deficiency (Michalczyk et al. 2003).

2.2.2 Zip family

The Zip family of proteins, which includes 14 known members, which are encoded by the human genome, is also involved in transport of zinc across cellular membranes (Eide 2004). Partial dependence on HCO_3^- for zinc transport mediated by Zip2 has been shown, suggesting it is a symporter (Gaither and Eide 2000). It is not clear, however, if this mode of transport is shared by other members of the Zip family. It has been demonstrated that the Zip proteins, in contrast to the ZnTs, mostly increase cytoplasmic zinc along the gradient of zinc concentrations.

The Zip proteins are predicted to have 8 transmembrane domains. Most members of the family have a loop region between transmembrane domains III and IV, which contains a histidine rich domain that varies in its length and sequence (Dufner-Beattie et al. 2003).

Zip1 is ubiquitously expressed in many tissues, and its function is linked to influx of zinc into the cytoplasm as monitored using ^{65}Zn and antisense (Gaither and Eide 2001b). Zip1 has been shown to be involved in epithelial vectorial zinc transport in the intestine and prostate (Franklin et al. 2003). Zip1 was localized to the plasma membrane and to intracellular membrane compartments (Wang et al. 2004a). This may be explained by the recent results indicating that the cellular distribution of the Zip1 is highly dynamic and the proteins translocate rapidly upon changes in extracellular zinc concentration. The translocation of the members of the Zip family seems a general phenomenon that may regulate the zinc transport by these proteins (Dufner-Beattie et al. 2004).

Zip2 is probably the most sensitive gene to zinc depletion, and its expression is downregulated by zinc supplementation (Cao et al. 2001). Zip2 was studied in mononuclear cells of peripheral blood in which the depletion of intracellular zinc using TPEN reduced cell viability.

The only member of this family, which has been directly linked to zinc deficiency syndromes, is the Zip4. Several mutations have been mapped in Acrodermatitis enteropathica patients who suffer from zinc malabsorption (see Section 1). Supplementation of zinc, however, overcomes the symptoms of zinc deficiency suggesting that other proteins are able to compensate and provide the necessary zinc uptake. Zip4 is expressed in small intestine, colon, and kidney together with the Zip5 protein (Dufner-Beattie et al. 2004). While in the small intestine, zinc is absorbed; most of the zinc loss is via urine in which zinc levels are also affected by dietary zinc. Both Zip4 and Zip5 proteins have been shown to translocate from apical to basolateral membranes of intestinal cells upon exposure to different dietary zinc conditions (Dufner-Beattie et al. 2004; Kim et al. 2004). Yet, only Zip4 expression level is affected by extracellular zinc concentrations.

Finally, a subfamily within the Zip transporters is the LZT (LIV-1 zinc transporters) family. Unlike other members of the Zip family the LIV-1 contains a met-

alloprotease motif, which is a hallmark of the LZT family (Taylor and Nicholson 2003). Several Zip genes have been associated with this family, the first was Zip6 (LIV-1) (Taylor et al. 2003). LIV-1 expression is stimulated by estrogen in breast cancer cells, but it is also expressed in normal mammary gland, prostate, placental cells, and the brain. This protein is located at the plasma membrane and has been shown to act as a zinc-influx transporter, yet its localization in lamellopodia taken together with the unique metalloprotease motif suggests its has also metalloprotease role (Taylor et al. 2003). The LIV-1 has been identified in breast cancer cells and its intriguing involvement in both zinc homeostasis linked to cell growth and metalloprotease activity linked to metastasis formation may point to the important role, which the LZT family members may play in cancer.

The Zip8 protein, interestingly, is involved in both zinc and cadmium transport (Begum et al. 2002; Dalton et al. 2005). The cadmium transport mediated by this protein links the Zip8 to cadmium toxicity in mice.

2.2.3 Zinc transport by other pathways

Zinc transport across plasma membranes is also mediated by non-specific zinc transporters. A mechanism for lowering zinc in neurons was suggested to be mediated by the Na^+/Ca^{2+} exchangers (Sensi et al. 1997). Other studies, however, have suggested that this zinc efflux is mediated in a Na^+-dependent manner by a highly specific Na^+/Zn^{2+} exchanger in neurons, yet, its gene has not been identified (Ohana et al. 2004). Other zinc transport mechanisms responsible for lowering intracellular zinc and maintaining the steep zinc gradient across the membrane, not yet identified, may have intriguing physiological and pathophysiological implications.

2.3 Zinc signaling

The steep gradient of zinc concentration across cellular plasma membrane (6 orders of magnitude) in some tissues may exceed that of calcium (Outten and O'Halloran 2001). The dynamic changes in extracellular zinc concentration upon its release in organs such as the brain, pancreas, and salivary gland suggest that this ion may also have a signaling role. Indeed in recent years, it has been established that zinc also acts as a second messenger and a signaling ion (Fig. 2).

2.3.1 Intracellular zinc

Although intracellular zinc is essential for the activity of numerous proteins, its role in induction of apoptosis by generation of ROS and mitochondrial membrane depolarization has been shown repetitively. This dual role of zinc in enhancing of cell growth on one hand and inducing cell death on the other seems to be dependent on a fine balance of its cellular level (Sakabe et al. 1998; Takeda et al. 2004).

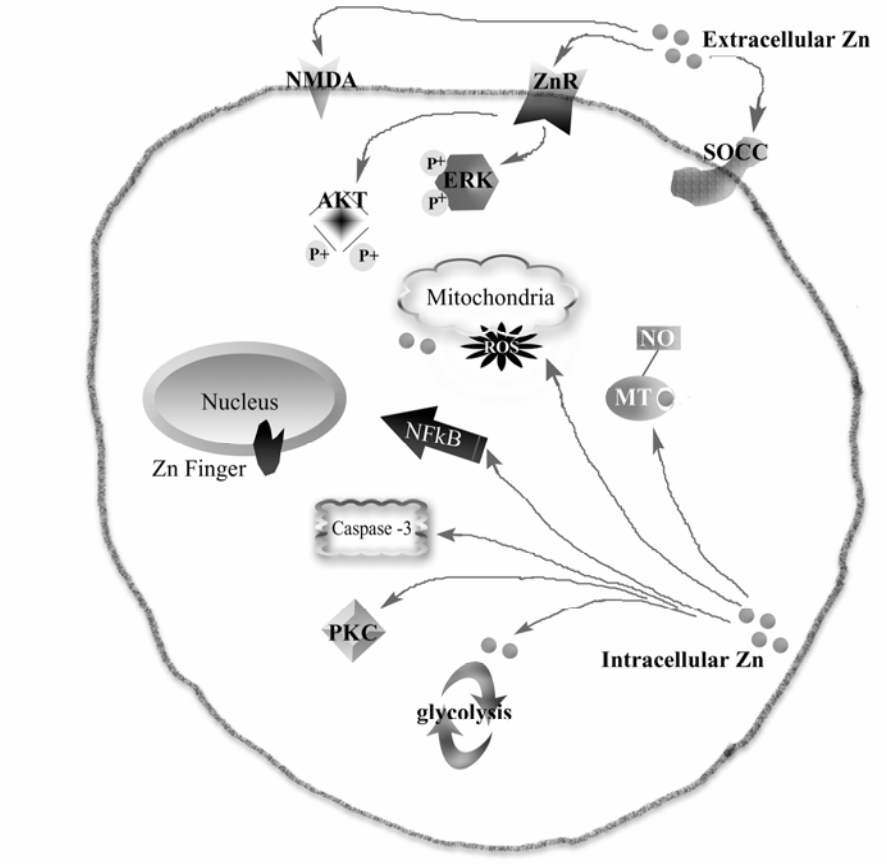


Fig. 2. A schematic representation of signaling pathways regulated by changes in extracellular or intracellular zinc.

Zinc is well known for its role in inducing proliferation and its role in enhancing wound healing has been documented for many years while zinc deficiency is linked to apoptosis (MacDonald et al. 1998, 2000; Thornton et al. 1998; Verstraeten et al. 2004). The cellular signaling such as ERK have been suggested to be activated by zinc and link changes in zinc to cell proliferation (Park et al. 2002). Further, cellular zinc deficiency is correlated with reduction of cell number, as a consequence of apoptosis mediated by activation of cell death mediators such as caspase-3 (Kolenko et al. 2001; Truong-Tran et al. 2002). Apoptosis and cellular zinc deficiency have been also associated with activation of various members of the PKC family, PKCα downregulation and PKCδ activation, both which subsequently trigger cell death (Noh et al. 1999; Chou et al. 2004). Activation of PKCδ alters the mitochondrial membrane potential and thus activates cytochrome c and AIF (Apoptosis induced factor). The immune system is particularly vulner-

able to zinc deficiency, and its activity during prolonged zinc deficiency is decreased due to lower lymphopoesis and B and T cell death (Fraker et al. 2000). Interestingly, in mast cells, where zinc is accumulated in granules, it has been shown that the different pools of zinc act separately. Depletion of the intracellular using a strong zinc chelator (TPEN) renders cells more susceptible to caspase activation by toxins while depletion of only the granular pools renders them resistant to toxins (Ho et al. 2004). The protective effects of zinc have also been shown in renal tubular cells, in which zinc inhibited apoptosis by attenuating cytochrome-c induced caspase activation triggered by ATP-depletion (Wei and Dong 2004). Another important pathway, which is triggered by zinc to inhibit apoptosis, is the NFkB. The translocation of NFkB to the nucleus is an antiapoptotic signal, which has been shown to be zinc dependent (Ho and Ames 2002; Ho et al. 2004). It has been further shown that the MTs regulate the NFkB translocation (Koropatnick 2004). It was also suggested that zinc deficiency followed by impairment of the cytoskeletal structure leads to the attenuation of NFkB translocation (Mackenzie et al. 2002).

While reduction of intracellular zinc renders the cells susceptible to cell death, excessive rise of cellular zinc has been repeatedly shown to be toxic. This is most notable in neurons where extracellular zinc levels are very high during synaptic release and the cells are highly permeable to zinc via the L-type voltage channels and Ca-AMPA/kainate receptors (Sensi et al. 1999; Kim et al. 2000a; Snider et al. 2000; Weiss and Sensi 2000; Jia et al. 2002). The zinc, which permeates the cells, interferes with the gylcolytic pathways in neurons by directly inhibiting the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) a key enzyme in the glycolysis pathway (Sheline et al. 2000). Such inhibition is particularly crucial during ischemia when the neurons are already depleted of ATP and the activity of the oxidative phosphorylation is impaired. This pathway was recently also demonstrated to play a role in β-cell death, which are similarly exposed to toxic zinc (Kim et al. 2000b). The zinc in these cells is co-released with insulin. In models of type-I diabetes, further work has indeed demonstrated that the intracellular effect of zinc was mediated by its effect on glycolysis, as pyruvate could rescue the zinc-containing cells (Chang et al. 2003). Zinc also affects the mitochondria, elegant studies by Sensi et al. have shown that permeation of zinc into neurons interferes with mitochondrial membrane potential and leads to enhanced generation of ROS, followed by cell death (Jiang et al. 2001). Further studies have shown that not only zinc permeation leads to the mitochondrial damage but also release of zinc from metallothioniens is involved in the impairment of mitochondrial function and cell death (Sensi et al. 2003). A pathway, which involves the production of ROS in breast cancer cells induced by zinc, has been demonstrated to be mediated by the p53, zinc finger protein, and cells that were deficient of this protein were less susceptible to zinc toxicity (Ostrakhovitch and Cherian 2005). The mechanism linking zinc to p53 activity has not been elucidated. Biochemical analysis of the signaling linking the rise of intracellular zinc to cell death revealed that also the NO pathway, by generation of peroxynitrite, leads to release of intracellular zinc (Zhang et al. 2004). The intracellular zinc then triggers mitochondrial damage,

which is followed by activation of P38 MAP kinase, and the activation of the K^+ channel culminating in neuronal death (Aizenman et al. 2004).

Although many of the signaling molecules regulated by zinc among them P53 and PKC express zinc finger domains, it is not clear what is the physiological significance of these domains, and most importantly if changes in intracellular zinc are sensed by these domains, thereby, regulating the protein activity.

The emerging picture regarding the role of intracellular zinc affecting cell fate indicates that many cell types are sensitive to changes in the levels of intracellular zinc. On one hand, zinc deficiency leads to apoptosis mediated by the PKC and NFkB pathways, on the other hand, zinc permeation or release from intracellular pools leads to cell death by interfering with glycolysis and mitochondrial functions leading to the activation of stress signaling such as P38 MAP kinase.

2.3.2 Extracellular zinc

Zinc gradients across cellular membranes and changes in its intracellular levels, as described above, may potentially lead to cell death. Therefore, it is not surprising if much of the zinc signaling is mediated by extracellular sites. Specific zinc binding sites are present on major membrane transporters and receptors most notable the dopamine transporter, NMDA, glycine, GABA and the purinergic receptors. High affinity binding site has also been recently identified on the store-operated channel (SOC), and like the effects on the NMDA receptor it is shown to be regulated by changes in redox potential (Gore et al. 2004). Such regulation of the SOC or NMDA channels may play a role in reshaping intracellular calcium signals and thereby regulation of numerous cellular processes including synaptic transmission, secretion and proliferation.

While many of the observations on the effect of zinc have been attributed to direct interaction of zinc with the signaling molecules more recent studies have suggested that the effect may be related to extracellular zinc. Such effect of zinc has been demonstrated for the regulation of the MAP kinase pathway and the PI3 kinase pathway, both linking zinc to its well known role of enhancing cellular proliferation (Park et al. 2003). Another important target for zinc is the EGF receptor, which has been shown to be transactivated by the zinc-dependent activation of Src-kinase (Wu et al. 1999, 2002; Samet et al. 2003). Interestingly, zinc has been recently shown to activate the EGF receptor by a pathway involving the activation of metalloproteinases, which leads to release of heparin-binding EGF (Wu et al. 2004). Activation of metalloproteinases by extracellular zinc has also been shown to activate the tropomyosin-related kinase, Trk, in neurons by releasing pro-BDNF (Hwang et al. 2005).

Altogether, the activity of extracellular zinc has been related to the regulation of major signaling pathways, yet, what links extracellular zinc to these pathways is poorly understood.

Extracellular zinc has a well documented role of enhancing epithelial cells growth (MacDonald 2000). Indeed, zinc deficiency has been linked to diarrhea and failure of wound healing, which are linked to epithelial cell loss. Extracellular zinc in colonocytes, keratinocytes, and salivary gland cells has been shown to

trigger a rise in intracellular calcium, which is mediated by activation of a Gαq-coupled receptor (Hershfinkel et al. 2001; Maret 2001). This activity is highly specific to zinc and although a receptor has no been cloned yet, it is likely a member of the cation sensing, G-protein coupled receptors, family although it is distinct from the Ca sensing receptor. Thus, intracellular calcium rise has been suggested to be triggered by a zinc sensing receptor (ZnR). Recently, it has been further demonstrated that in colonocytes the ZnR triggers the activation of the MAP kinase and the PI3 kinase pathways and the subsequent upregulation of the Na^+/H^+ exchange mediated by NHE1 (Azriel-Tamir et al. 2004). Thus, the ZnR is one candidate for linking the changes in extracellular zinc to the known regulation of signaling pathways involved in cell proliferation.

3 Future directions

It is becoming apparent that zinc is a signaling molecule acting through diverse signaling pathways. The dynamics of both intracellular and extracellular zinc pools may result in cell proliferation but on the other hand may trigger cell death and apoptosis. While in recent years a considerable body of information has been gained on the signaling molecules affected by zinc, a detailed "macro map" integrating the effects on the signaling pathways is far from being complete. Such knowledge will require the use of genomic and proteomic approaches, which will monitor the effects of zinc on patterns of gene expression and kinase regulation. Initial work toward this direction using a genomic screening approach demonstrated that zinc activates multiple genes in yeast and the silencing of a specific zinc-sensitive protein allowed identification of its targets (Lyons et al. 2000). A similar approach has been used in mammalian cells and demonstrated the change of multiple genes in response to dietary zinc (Cousins et al. 2003). Yet, the important role of the translocation of zinc-dependent proteins will have to be addressed. In addition, conventional biochemical experiments will be required to elucidate the mechanisms by which zinc is affecting signaling pathways. Although numerous proteins, which are involved in zinc homeostasis have been cloned, or functionally identified, the knowledge on the mechanism by which they regulate zinc homeostasis is lacking. Finally, the link between the combined activity of these proteins and the pathophysiologies, which are associated to zinc homeostasis are only starting to come into sight. The emerging highly specific and sensitive zinc monitoring tools and the silencing RNA approach that can be specifically used to target genes in cell cultures and *in vivo* will enable direct assessment of the function and the physiological implication of the zinc homeostatic system.

References

Pal S, He K, Aizenman E (2004) Nitrosative stress and potassium channel-mediated neuronal apoptosis: is zinc the link? Pflugers Arch 448:296-303

Andrews GK, Wang H, Dey SK, Palmiter RD (2004) Mouse zinc transporter 1 gene provides an essential function during early embryonic development. Genesis 40:74-81

Andrews M, Gallagher-Allred C (1999) The role of zinc in wound healing. Adv Wound Care 12:137138-

Apostolova MD, Choo KH, Michalska AE, Tohyama C (1997) Analysis of the possible protective role of metallothionein in streptozotocin-induced diabetes using metallothionein-null mice. J Trace Elem Med Biol 11:1-7

Assaf SY, Chung SH (1984) Release of endogenous Zn2+ from brain tissue during activity. Nature 308:734-736

Atar D, Backx PH, Appel MM, Gao WD, Marban E (1995) Excitation-transcription coupling mediated by zinc influx through voltage-dependent calcium channels. J Biol-Chem 270:2473-2477

Azriel-Tamir H, Sharir H, Schwartz B, Hershfinkel M (2004) Extracellular zinc triggers ERK-dependent activation of Na+/H+ exchange in colonocytes mediated by the zinc-sensing receptor. J Biol Chem 279:51804-51816

Barceloux DG (1999) Zinc. J Toxicol Clin Toxicol 37:279-292

Batra N, Nehru B, Bansal MP (1998) The effect of zinc supplementation on the effects of lead on the rat testis. Reprod Toxicol 12:535-540

Begum NA, Kobayashi M, Moriwaki Y, Matsumoto M, Toyoshima K, Seya T (2002) Mycobacterium bovis BCG cell wall and lipopolysaccharide induce a novel gene, BIGM103, encoding a 7-TM protein: identification of a new protein family having Zn-transporter and Zn-metalloprotease signatures. Genomics 80:630-645

Berg JM, Shi Y (1996) The galvanization of biology: a growing appreciation for the roles of zinc. Science 271:1081-1085

Bhatnagar S, Bahl R, Sharma PK, Kumar GT, Saxena SK, Bhan MK (2004) Zinc with oral rehydration therapy reduces stool output and duration of diarrhea in hospitalized children: a randomized controlled trial. J Pediatr Gastroenterol Nutr 38:34-40

Bhatnagar S, Taneja S (2001) Zinc and cognitive development. Br J Nutr 85:S139-145

Blasco-Ibanez JM, Poza-Aznar J, Crespo C, Marques-Mari AI, Gracia-Llanes FJ, Martinez-Guijarro FJ (2004) Chelation of synaptic zinc induces overexcitation in the hilar mossy cells of the rat hippocampus. Neurosci Lett 355:101-104

Budinger L, Hertl M (2000) Immunologic mechanisms in hypersensitivity reactions to metal ions: an overview. Allergy 55:108-115

Buhl EH, Otis TS, Mody I (1996) Zinc-induced collapse of augmented inhibition by GABA in a temporal lobe epilepsy model. Science 271:369-373

Canzoniero LM, Sensi SL, Choi DW (1997) Measurement of intracellular free zinc in living neurons. Neurobiol Dis 4:27279-5

Cao J, Bobo JA, Liuzzi JP, Cousins RJ (2001) Effects of intracellular zinc depletion on metallothionein and ZIP2 transporter expression and apoptosis. J Leukoc Biol 70:559-566

Chang I, Cho N, Koh JY, Lee MS (2003) Pyruvate inhibits zinc-mediated pancreatic islet cell death and diabetes. Diabetologia 46:1220-1227. Epub 2003 Jul 1224

Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, Barnham KJ, Volitakis I, Fraser FW, Kim Y, Huang X, Goldstein LE, Moir RD, Lim JT, Beyreuther K, Zheng H ,Tanzi RE, Masters CL, Bush AI (2001) Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. Neuron 30:665-676

Choi DW (1996) Zinc neurotoxicity may contribute to selective neuronal death following transient global cerebral ischemia. Cold Spring Harb Symp Quant Biol 61:385-387

Choi DW, Koh JY (1998) Zinc and brain injury. Annu Rev Neurosci 21:347-375

Choi Y, Chen HV, Lipton SA (2001) Three pairs of cysteine residues mediate both redox and zn2+ modulation of the nmda receptor. J Neurosci 21:392-400

Chou SS, Clegg MS, Momma TY, Niles BJ, Duffy JY, Daston GP, Keen CL (2004) Alterations in protein kinase C activity and processing during zinc-deficiency-induced cell death. Biochem J 383:63-71

Cole TB, Martyanova A, Palmiter RD (2001) Removing zinc from synaptic vesicles does not impair spatial learning, memory, or sensorimotor functions in the mouse. Brain Res 891:253-265

Cole TB, Robbins CA, Wenzel HJ, Schwartzkroin PA, Palmiter RD (2000) Seizures and neuronal damage in mice lacking vesicular zinc. Epilepsy Res 39:153-169

Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD (1999) Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proc Natl Acad Sci USA 96:1716-1721

Costello LC, Feng P, Milon B, Tan M, Franklin RB (2004) Role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve. Prostate Cancer Prostatic Dis 7:111-117

Cousins RJ, Blanchard RK, Moore JB, Cui L, Green CL, Liuzzi JP, Cao J, Bobo JA (2003) Regulation of zinc metabolism and genomic outcomes. J Nutr 133:1521S-1526S

Cuajungco MP, Faget KY, Huang X, Tanzi RE, Bush AI (2000) Metal chelation as a potential therapy for Alzheimer's disease. Ann NY Acad Sci 920:292-304

Dalton TP, He L, Wang B, Miller ML, Jin L, Stringer KF, Chang X, Baxter CS, Nebert DW (2005) Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. Proc Natl Acad Sci USA 102:3401-3406. Epub 2005 Feb 3418

Danscher G, Jensen KB, Frederickson CJ, Kemp K, Andreasen A, Juhl S, Stoltenberg M, Ravid R (1997) Increased amount of zinc in the hippocampus and amygdala of Alzheimer's diseased brains: a proton-induced X-ray emission spectroscopic analysis of cryostat sections from autopsy material. J Neurosci Methods 76:53-59 issn: 0165-0270

Devergnas S, Chimienti F, Naud N, Pennequin A, Coquerel Y, Chantegrel J, Favier A, Seve M (2004) Differential regulation of zinc efflux transporters ZnT-1, ZnT-5 and ZnT-7 gene expression by zinc levels: a real-time RT-PCR study. Biochem Pharmacol 68:699-709

Dominguez MI, Blasco-Ibanez JM, Crespo C, Marques-Mari AI, Martinez-Guijarro FJ (2003) Zinc chelation during non-lesioning overexcitation results in neuronal death in the mouse hippocampus. Neuroscience 116:791-806

Dufner-Beattie J, Kuo YM, Gitschier J, Andrews GK (2004) The adaptive response to dietary zinc in mice involves the differential cellular localization and zinc regulation of the zinc transporters ZIP4 and ZIP5. J Biol Chem 279:49082-49090. Epub 42004 Sept 49089

Dufner-Beattie J, Langmade SJ, Wang F, Eide D, Andrews GK (2003) Structure, function, and regulation of a subfamily of mouse zinc transporter genes. J Biol Chem 278:50142-50150. Epub 52003 Oct 50142

Eide DJ (2004) The SLC39 family of metal ion transporters. Pflugers Arch 447:796-800. Epub 2003 May 2014

El-Tawil AM (2003) Zinc deficiency in men with Crohn's disease may contribute to poor sperm function and male infertility. Andrologia 35:337-341

Evans GW (1986) Zinc and its deficiency diseases. Clin Physiol Biochem 4:94-98

Favier AE (1992) The role of zinc in reproduction. Hormonal mechanisms. Biol Trace Elem Res 32:363-382

Feng P, Li TL, Guan ZX, Franklin RB, Costello LC (2002) Direct effect of zinc on mitochondrial apoptogenesis in prostate cells. Prostate 52:311-318

Fraker PJ, King LE, Laakko T, Vollmer TL (2000) The dynamic link between the integrity of the immune system and zinc status. J Nutr 1301399:S-1406S

Franklin RB, Ma J, Zou J, Guan Z, Kukoyi BI, Feng P, Costello LC (2003) Human ZIP1 is a major zinc uptake transporter for the accumulation of zinc in prostate cells. J Inorg Biochem 96:435-442

Franklin RB, Milon B, Feng P, Costello LC, Tan M (2005) Zinc and zinc transporters in normal prostate and the pathogenesis of prostate cancer. Front Biosci 10:2230-2239

Role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve. Front Biosci 10:2230-2239

Frederickson CJ (2003) Synaptically released zinc: neuromodulator and toxin. J Neurochem 85:10

Frederickson CJ, Hernandez MD, Goik SA, Morton JD, McGinty JF (1988) Loss of zinc staining from hippocampal mossy fibers during kainic acid induced seizures: a histofluorescence study. Brain Res 446386-383:

Frederickson CJ, Hernandez MD, McGinty JF (1989) Translocation of zinc may contribute to seizure-induced death of neurons. Brain Res 480:317-321

Frederickson CJ, Perez Clausell J, Danscher G (1987) Zinc-containing 7S-NGF complex. Evidence from zinc histochemistry for localization in salivary secretory granules. J Histochem Cytochem 35:579-583 issn: 0022-1554

Gaither LA, Eide DJ (2000) Functional expression of the human hZIP2 zinc transporter. J Biol Chem 275:5560-5564

Gaither LA, Eide DJ (2001a (Eukaryotic zinc transporters and their regulation. Biometals 14:251-270

Gaither LA, Eide DJ (2001b) The human ZIP1 transporter mediates zinc uptake in human K562 erythroleukemia cells. J Biol Chem 276:22258-22264

Gee KR, Zhou ZL, Qian WJ, Kennedy R (200 (2Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator. J Am Chem Soc 124:776-778

Gore A, Moran A, Hershfinkel M, Sekler I (2004) Inhibitory mechanism of store-operated Ca2+ channels by zinc. J Biol Chem 279:11106-11111. Epub 12004 Jan 11108

Hambidge KM (1992) Zinc and diarrhea. Acta Paediatr Suppl 381:82-86

Hambidge M (2000) Human zinc deficiency. J Nutr 130:1344S-1349S

Hambidge M, Krebs N (1999) Zinc, diarrhea, and pneumonia [editorial; comment]. J Pediatr 135:661-664

Han Y, Wu SM (1999) Modulation of glycine receptors in retinal ganglion cells by zinc. Proc Natl Acad Sci USA 96:3234-3238

Hershfinkel M, Moran A, Grossman N, Sekler I (2001) A zinc-sensing receptor triggers the release of intracellular Ca2+ and regulates ion transport. Proc Natl Acad Sci USA 98:11749-11754

Ho E, Ames BN (2002) Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. Proc Natl Acad Sci USA 99:16770-16775. Epub 12002 Dec 16712

Ho LH, Ruffin RE, Murgia C, Li L, Krilis SA, Zalewski PD (2004) Labile zinc and zinc transporter ZnT4 in mast cell granules: role in regulation of caspase activation and NF-kappaB translocation. J Immunol 172:7750-7760

Hosie AM, Dunne EL, Harvey RJ, Smart TG (2003) Zinc-mediated inhibition of GABA(A) receptors: discrete binding sites underlie subtype specificity. Nat Neurosci 6:362-369

Huang L, Gitschier J (1997) A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat Genet 17:292-297

Hwang JJ, Park MH, Choi SY, Koh JY (2005) Activation of the Trk signaling pathway by extracellular zinc: ROLE OF METALLOPROTEINASES. J Biol Chem 280:11995-12001. Epub 12005 Jan 11919

Inoue K, Matsuda K, Itoh M, Kawaguchi H, Tomoike H, Aoyagi T, Nagai R, Hori M, Nakamura Y, Tanaka T (2002) Osteopenia and male-specific sudden cardiac death in mice lacking a zinc transporter gene, Znt5. Hum Mol Genet 11:1775-1784

Jia Y, Jeng JM, Sensi SL, Weiss JH (2002) Zn2+ currents are mediated by calcium-permeable AMPA/kainate channels in cultured murine hippocampal neurones. J Physiol 543:35-48

Jiang D, Sullivan PG, Sensi SL, Steward O, Weiss JH (2001) Zn(2+) induces permeability transition pore opening and release of pro-apoptotic peptides from neuronal mitochondria. J Biol Chem 276:47524-47529

Jirakulaporn T, Muslin AJ (2004) Cation diffusion facilitator proteins modulate Raf-1 activity. J Biol Chem 279:27807-27815. Epub 22004 Apr 27819

Kambe T, Narita H, Yamaguchi-Iwai Y, Hirose J, Amano T, Sugiura N, Sasaki R, Mori K, Iwanaga T, Nagao M (2002) Cloning and characterization of a novel mammalian zinc transporter, zinc transporter 5, abundantly expressed in pancreatic beta cells. J Biol Chem 277:190Epub .19055-49 12002 Mar 19019

Kato K, Isaji S, Kawarada Y, Hibasami H, Nakashima K (1997) Effect of zinc administration on pancreatic regeneration after 80% pancreatectomy. Pancreas 14:158-165

Kim AH, Sheline CT, Tian M, Higashi T, McMahon RJ, Cousins RJ, Choi DW (2000a) L-type Ca(2+) channel-mediated Zn(2+) toxicity and modulation by ZnT-1 in PC12 cells(1). Brain Res 886:99-107

Kim BE, Wang F, Dufner-Beattie J, Andrews GK, Eide DJ, Petris MJ (2004) Zn2+-stimulated endocytosis of the mZIP4 zinc transporter regulates its location at the plasma membrane. J Biol Chem 279:4523-4530. Epub 2003 Nov 4511

Kim BJ, Kim YH, Kim S, Kim JW, Koh JY, Oh SH, Lee MK, Kim KW, Lee MS (2000b) Zinc as a paracrine effector in pancreatic islet cell death. Diabetes 49:367-372

Kirschke CP, Huang L (2003) ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. J Biol Chem 278:4096-4102. Epub 2002 Nov 4021

Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 272:1013-1016

Kolenko VM, Uzzo RG, Dulin N, Hauzman E, Bukowski R, Finke JH (2001) Mechanism of apoptosis induced by zinc deficiency in peripheral blood T lymphocytes. Apoptosis 6:419-429

Komai M, Goto T, Suzuki H, Takeda T, Furukawa Y (2000) Zinc deficiency and taste dysfunction; contribution of carbonic anhydrase, a zinc-metalloenzyme, to normal taste sensation. Biofactors 12:65-70

Krebs NF (2000) Dietary zinc and iron sources, physical growth and cognitive development of breastfed infants. J Nutr 130:358S-360S

Kristiansen LH, Rungby J, Sondergaard LG, Stoltenberg M, Danscher G (2001) Autometallography allows ultrastructural monitoring of zinc in the endocrine pancreas. Histochem Cell Biol 115:125129-

Kroncke KD (2001) Zinc finger proteins as molecular targets for nitric oxide-mediated gene regulation. Antioxid Redox Signal 3:565-575

Kudravi SA, Reed MJ (2000) Aging, cancer, and wound healing. In Vivo 14:83-92

Kury S, Dreno B, Bezieau S, Giraudet S, Kharfi M, Kamoun R, Moisan JP (2002) Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. Nat Genet 31:239-240. Epub 2002 Jun 2017

Lee JM, Zipfel GJ, Park KH, He YY, Hsu CY, Choi DW (2002a) Zinc translocation accelerates infarction after mild transient focal ischemia. Neuroscience 115:871-878

Lee JY, Cole TB, Palmiter RD, Koh JY (2000) Accumulation of zinc in degenerating hippocampal neurons of ZnT3-null mice after seizures: evidence against synaptic vesicle origin. J Neurosci (Online) 20:RC79

Lee JY, Cole TB, Palmiter RD, Suh SW, Koh JY (2002b) Contribution by synaptic zinc to the gender-disparate plaque formation in human Swedish mutant APP transgenic mice. Proc Natl Acad Sci USA 99:7705-7710

Lee JY, Kim JH, Palmiter RD ,Koh JY (2003) Zinc released from metallothionein-iii may contribute to hippocampal CA1 and thalamic neuronal death following acute brain injury. Exp Neurol 184:337-347

Leitzmann MF, Stampfer MJ, Wu K, Colditz GA, Willett WC, Giovannucci EL (2003) Zinc supplement use and risk of prostate cancer. J Natl Cancer Inst 95:1004-1007

Liang JY, Liu YY, Zou J, Franklin RB, Costello LC, Feng P (1999) Inhibitory effect of zinc on human prostatic carcinoma cell growth. Prostate 40:200-207

Lyons TJ, Gasch AP, Gaither LA, Botstein D, Brown PO, Eide DJ (2000) Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast. Proc Natl Acad Sci USA 97:7957-7962

MacDonald RS (2000) The role of zinc in growth and cell proliferation. J Nutr 130:1500S-1508S

MacDonald RS, Wollard-Biddle LC, Browning JD, Thornton WH Jr, O'Dell BL (1998) Zinc deprivation of murine 3T3 cells by use of diethylenetrinitrilopentaacetate impairs DNA synthesis upon stimulation with insulin-like growth factor-1 (IGF-1). J Nutr 128:161605-00

Mackenzie GG, Zago MP, Keen CL, Oteiza PI (2002) Low intracellular zinc impairs the translocation of activated NF-kappa B to the nuclei in human neuroblastoma IMR-32 cells. J Biol Chem 277:34610-34617. Epub 32002 Jun 34627

Maret W (1995) Metallothionein/disulfide interactions, oxidative stress, and the mobilization of cellular zinc. Neurochem Int 27:111-117

Maret W (2001) From the Cover: Crosstalk of the group IIa and IIb metals calcium and zinc in cellular signaling. Proc Natl Acad Sci USA 9812327-12325:

Maret W (2003) Cellular zinc and redox states converge in the metallothionein/thionein pair. J Nutr 133:1460S-1462S

Maynard CJ, Bush AI, Masters CL, Cappai R, Li QX (2005) Metals and amyloid-beta in Alzheimer's disease. Int J Exp Pathol 86159-147:

McMahon RJ, Cousins RJ (1998) Regulation of the zinc transporter ZnT-1 by dietary zinc. Proc Natl Acad Sci USA 95:4841-4846

Michalczyk A, Varigos G, Catto-Smith A, Blomeley RC, Ackland ML (2003) Analysis of zinc transporter, hZnT4 (Slc30A4), gene expression in a mammary gland disorder leading to reduced zinc secretion into milk. Hum Genet 113:202-210. Epub 2003 May 2013

Mocchegiani E, Muzzioli M (2000) Therapeutic application of zinc in human immunodeficiency virus against opportunistic infections. J Nutr 130:1424S-1431S

Nagatomo I, Akasaki Y, Uchida M, Kuchiiwa S, Nakagawa S, Takigawa M (1998) Influence of dietary zinc on convulsive seizures and hippocampal NADPH diaphorase-positive neurons in seizure susceptible EL mouse. Brain Res 789:213220-

Nitzan YB, Sekler I, Frederickson CJ, Coulter DA, Balaji RV, Liang SL, Margulis A, Hershfinkel M, Silverman WF (2003) Clioquinol effects on tissue chelatable zinc in mice. J Mol Med 81:637-644

Nitzan YB, Sekler I, Hershfinkel M, Moran A, Silverman WF (2002) Postnatal regulation of ZnT-1 expression in the mouse brain. Brain Res Dev Brain Res 137:149-157

Noh KM, Kim YH, Koh JY (1999) Mediation by membrane protein kinase C of zinc-induced oxidative neuronal injury in mouse cortical cultures. J Neurochem 72:1609-1616

Nolte C, Gore A, Sekler I, Kresse W, Hershfinkel M, Hoffmann A, Kettenmann H, Moran A (2004) ZnT-1 expression in astroglial cells protects against zinc toxicity and slows the accumulation of intracellular zinc. Glia 48:145-155

Ohana E, Segal D, Palty R, Ton-That D, Moran A, Sensi SL, Weiss JH, Hershfinkel M, Sekler I (2004) A sodium zinc exchange mechanism is mediating extrusion of zinc in mammalian cells. J Biol Chem 279:4278-4284

Ohara I, Tabuchi R, Kimura M, Itokawa Y (1995) Decline of taste sensitivity in protein deficient adult rats. Physiol Behav 57:921-926

Olmez I, Gulovali MC, Gordon GE, Henkin RI (1988) Trace elements in human parotid saliva. Biol Trace Elem Res 17:259-270

Om AS, Chung KW (1996) Dietary zinc deficiency alters 5 alpha-reduction and aromatization of testosterone and androgen and estrogen receptors in rat liver. J Nutr 126:842-848

Ostrakhovitch EA, Cherian MG (2005) Role of p53 and reactive oxygen species in apoptotic response to copper and zinc in epithelial breast cancer cells. Apoptosis 10:111-121

Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488-2492

Palmiter RD (2004) Protection against zinc toxicity by metallothionein and zinc transporter 1. Proc Natl Acad Sci USA 101:4918-4923. Epub 2004 Mar 4923

Palmiter RD, Cole TB, Quaife CJ, Findley SD (1996) ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci USA 93:14934-14939

Palmiter RD, Findley SD (1 (995Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J 14:639-649

Palmiter RD, Huang L (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch 447:744-751. Epub 2003 May 2014

Paoletti P, Ascher P, Neyton J (1997) High-affinity zinc inhibition of NMDA NR1-NR2A receptors. J Neurosci 17:5711-5725

Park KS, Ahn Y, Kim JA, Yun MS, Seong BL, Choi KY (2002) Extracellular zinc stimulates ERK-dependent activation of p21(Cip/WAF1) and inhibits proliferation of colorectal cancer cells. Br J Pharmacol 137:597-607

Park KS, Lee NG, Lee KH, Seo JT, Choi KY (2003) The ERK pathway involves positive and negative regulations of HT29 colorectal cancer cell growth by extracellular zinc. Am J Physiol Gastrointest Liver Physiol 19:19

Pearce LL, Gandley RE, Han W, Wasserloos K, Stitt M, Kanai AJ, McLaughlin MK, Pitt BR, Levitan ES (2000) Role of metallothionein in nitric oxide signaling as revealed by a green fluorescent fusion protein. Proc Natl Acad Sci USA 97:477-482

Perafan-Riveros C, Franca LF, Alves AC, Sanches JA Jr (2002) Acrodermatitis enteropathica: case report and review of the literature. Pediatr Dermatol 19:426-431

Prasad AS (1998) Zinc and immunity. Mol Cell Biochem 188:63-69

Rolfs A, Hediger MA (2001) Intestinal metal ion absorption: an update. Curr Opin Gastroenterol 17:177-183

Sakabe I, Paul S, Dansithong W, Shinozawa T (1998) Induction of apoptosis in Neuro-2A cells by Zn2+ chelating .Cell Struct Funct 23:95-99

Samet JM, Dewar BJ, Wu W, Graves LM (2003) Mechanisms of Zn(2+)-induced signal initiation through the epidermal growth factor receptor. Toxicol Appl Pharmacol 191:86-93

Sandstead HH (2000) Causes of iron and zinc deficiencies and their effects on brain. J Nutr 130:347S-349S

Sandstead HH, Frederickson CJ, Penland JG (2000) History of zinc as related to brain function. J Nutr 130:496S-502S

Sandstead HH, Penland JG, Alcock NW, Dayal HH, Chen XC, Li JS, Zhao F, Yang JJ (1998) Effects of repletion with zinc and other micronutrients on neuropsychologic performance and growth of Chinese children. Am J Clin Nutr 68:470s-475s

Sazawal S, Black RE, Bhan MK, Bhandari N, Sinha A, Jalla S (1995) Zinc supplementation in young children with acute diarrhea in India. N Engl J Med 333:839-844

Schott-Ohly P, Lgssiar A, Partke HJ, Hassan M, Friesen N, Gleichmann H (2004) Prevention of spontaneous and experimentally induced diabetes in mice with zinc sulfate-enriched drinking water is associated with activation and reduction of NF-kappa B and AP-1 in islets, respectively. Exp Biol Med (Maywood) 229:1177-1185

Scott ME, Koski KG (2000) Zinc deficiency impairs immune responses against parasitic nematode infections at intestinal and systemic sites .J Nutr 130:1412S-1420S

Segal D, Ohana E, Besser L, Hershfinkel M, Moran A, Sekler I (2004) A role for ZnT-1 in regulating cellular cation influx. Biochem Biophys Res Commun 323:1145-1150

Sekler I, Moran A, Hershfinkel M, Dori A, Margulis A, Birenzweig N ,Nitzan Y, Silverman WF (2002) Distribution of the zinc transporter ZnT-1 in comparison with chelatable zinc in the mouse brain. J Comp Neurol 447:201-209

Sensi SL, Canzoniero LM, Yu SP, Ying HS, Koh JY, Kerchner GA, Choi DW (1997) Measurement of intracellular free zinc in living cortical neurons: routes of entry. J Neurosci 17:9554-9564

Sensi SL, Jeng JM (2004) Rethinking the excitotoxic ionic milieu: the emerging role of Zn(2+) in ischemic neuronal injury. Curr Mol Med 4:87-111

Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH (2003) Modulation of mitochondrial function by endogenous Zn2+ pools. Proc Natl Acad Sci USA 100:6157-6162

Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH (1999) Preferential Zn2+ influx through Ca2-+permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. Proc Natl Acad Sci USA 96:2414-2419

Sheline CT, Behrens MM, Choi DW (2000) Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD(+) and inhibition of glycolysis. J Neurosci 20:3139-3146

Sloviter RS (1985) A selective loss of hippocampal mossy fiber Timm stain accompanies granule cell seizure activity induced by perforant path stimulation. Brain Res 330:150-153

Snider BJ, Choi J, Turetsky DM, Canzoniero LM, Sensi SL, Sheline CT, Wang X, Yu SP, Choi DW (2000) Nitric oxide reduces Ca(2+) and Zn(2+) influx through voltage-gated Ca(2+) channels and reduces Zn(2+) neurotoxicity. Neuroscience 100:651-661

Spahl DU, Berendji-Grun D, Suschek CV, Kolb-Bachofen V, Kroncke KD (2003) Regulation of zinc homeostasis by inducible NO synthase-derived NO: nuclear metallothionein translocation and intranuclear Zn2+ release. Proc Natl Acad Sci USA 100:13952-13957. Epub 12003 Nov 13914

St Croix CM, Wasserloos KJ, Dineley KE, Reynolds IJ, Levitan ES, Pitt BR (2002) Nitric oxide-induced changes in intracellular zinc homeostasis are mediated by metallothionein/thionein. Am J Physiol Lung Cell Mol Physiol 282:L185-192

Suh SW, Chen JW, Motamedi M, Bell B, Listiak K, Pons NF, Danscher G, Frederickson CJ (2000) Evidence that synaptically-released zinc contributes to neuronal injury after traumatic brain injury. Brain Res 852:268-273

Suh SW, Listiack K, Bell B, Chen J, Motamedi M, Silva D, Danscher G, Whetsell W, Thompson R, Frederickson C (1999) Detection of pathological zinc accumulation in neurons: methods for autopsy, biopsy, and cultured tissue. J Histochem Cytochem 47:969-972

Suzuki T, Ishihara K, Migaki H, Matsuura W, Kohda A, Okumura K, Nagao M, Yamaguchi-Iwai Y, Kambe T (2005) Zinc transporters, ZnT5 and ZnT7, are required for the activation of alkaline phosphatases, zinc-requiring enzymes that are glycosylphosphatidylinositol-anchored to the cytoplasmic membrane. J Biol Chem 280:637-643 . Epub 2004 Nov 2002

Takeda A (2001) Zinc homeostasis and functions of zinc in the brain. Biometals 14:343-351

Takeda A, Tamano H, Ibuki Y (2004) Change of zinc uptake under growth arrest and apoptosis. Anticancer Res 24:3869-3874

Tanaka M (2002) Secretory function of the salivary gland in patients with taste disorders or xerostomia: correlation with zinc deficiency. Acta Otolaryngol Suppl:134-141

Taylor KM, Morgan HE, Johnson A, Hadley LJ, Nicholson RI (2003) Structure-function analysis of LIV-1, the breast cancer-associated protein that belongs to a new subfamily of zinc transporters. Biochem J 375:51-59

Taylor KM, Nicholson RI (2003) The LZT proteins; the LIV-1 subfamily of zinc transporters. Biochim Biophys Acta 1611:16-30

Tenaud I, Leroy S, Chebassier N, Dreno B (2000) Zinc, copper and manganese enhanced keratinocyte migration through a functional modulation of keratinocyte integrins. Exp Dermatol 2000:407-416

Thornton WH Jr, MacDonald RS, Wollard-Biddle LC, Browning JD, O'Dell BL (1998) Chelation of extracellular zinc inhibits proliferation in 3T3 cells independent of insulin-like growth factor-I receptor expression. Proc Soc Exp Biol Med 219:64-68

Truong-Tran AQ, Ruffin RE, Foster PS, Koskinen AM, Coyle P, Philcox JC, Rofe AM, Zalewski PD (2 (002Altered zinc homeostasis and caspase-3 activity in murine allergic airway inflammation. Am J Respir Cell Mol Biol 27:286-296

Vallee BL, Falchuk KH (1993) The biochemical basis of zinc physiology. Physiol Rev 73:79-118

Verstraeten SV, Zago MP, MacKenzie GG, Keen CL, Oteiza PI (2004) Influence of zinc deficiency on cell-membrane fluidity in Jurkat, 3T3 and IMR-32 cells. Biochem J 378:579-587

Wang F, Dufner-Beattie J, Kim BE, Petris MJ, Andrews G, Eide DJ (2004a) Zinc-stimulated endocytosis controls activity of the mouse ZIP1 and ZIP3 zinc uptake transporters. J Biol Chem 279:24631-24639. Epub 22004 Mar 24630

Wang F, Kim BE, Dufner-Beattie J, Petris MJ, Andrews G, Eide DJ (2004b) Acrodermatitis enteropathica mutations affect transport activity, localization and zinc-responsive trafficking of the mouse ZIP4 zinc transporter. Hum Mol Genet 13:563-571. Epub 2004 Jan 2006

Wang K, Zhou B, Kuo YM, Zemansky J, Gitschier J (2002) A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. Am J Hum Genet 71:66-73. Epub 2002 May 2024

Wapnir RA (2000) Zinc deficiency, malnutrition and the gastrointestinal tract. J Nutr 130:1388S-1392S

Wei G, Hough CJ, Li Y, Sarvey JM (2004) Characterization of extracellular accumulation of Zn2+ during ischemia and reperfusion of hippocampus slices in rat. Neuroscience 125:867-877

Wei Q, Wang J, Wang MH, Yu F, Dong Z (2004) Inhibition of apoptosis by Zn2+ in renal tubular cells following ATP depletion. Am J Physiol Renal Physiol 287:F492-F500

Weiss JH, Sensi SL (2000) Ca2+-Zn2+ permeable AMPA or kainate receptors: possible key factors in selective neurodegeneration. Trends Neurosci 23:365-371

Weiss JH, Sensi SL, Koh JY (2000) Zn(2+): a novel ionic mediator of neural injury in brain disease. Trends Pharmacol Sci 21:395-401

Wu W, Graves LM, Gill GN, Parsons SJ, Samet JM (2002) Src-dependent phosphorylation of the epidermal growth factor receptor on tyrosine 845 is required for zinc-induced Ras activation. J Biol Chem 277:24252-24257

Wu W, Graves LM, Jaspers I, Devlin RB, Reed W, Samet JM (1999) Activation of the EGF receptor signaling pathway in human airway epithelial cells exposed to metals. Am J Physiol:277 L924-931

Wu W, Samet JM, Silbajoris R, Dailey LA, Sheppard D, Bromberg PA, Graves LM (2004) Heparin-binding epidermal growth factor cleavage mediates zinc-induced epidermal growth factor receptor phosphorylation. Am J Respir Cell Mol Biol 30:540-547 .Epub 2003 Sep 2011

Yin HZ, Sensi SL, Ogoshi F, Weiss JH (2002) Blockade of Ca2+-permeable AMPA/kainate channels decreases oxygen-glucose deprivation-induced Zn2+ accumulation and neuronal loss in hippocampal pyramidal neurons. J Neurosci 22:1273-1279

Zhang Y, Wang H, Li J, Jimenez DA, Levitan ES, Aizenman E, Rosenberg PA (2004) Peroxynitrite-induced neuronal apoptosis is mediated by intracellular zinc release and 12-lipoxygenase activation. J Neurosci 24:10616-10627

List of abbreviations:

ZnT: Zinc transporter (family of proteins)
Zip: Zrt- and Irt-like proteins
Slc: solute-linked carrier (family of proteins)
MT: Metallothioneins
NO: nitric oxide
DMT: divalent metal transporter
CDF: cation diffusion facilitator
ROS: reactive oxygen species
PKC: protein kinase C
MAP- mitogen activated kinase
ERK: extracellular regulated kinase
Trk: tyrosine receptor kinase
EGF: epidermal growth factor
BNDF: brain-derived neurotrophic factor
NFkB: nuclear factor-kappa B
TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine
LZT: LIV-1 subfamily of ZIP zinc transporters
NHE: Na+/H+ exchanger (family of proteins)

Hershfinkel, Michal
Department of Morphology and the Zlotowski Center for Neuroscience, Faculty of Health Science, Ben Gurion University of the Negev, Beer-Sheva, Israel
hmichal@bgu.ac.il

Iron in mammals: pathophysiological mechanisms of overload and deficiency in relation to disease

Martha B. Johnson, Caroline A. Enns, and An-Sheng Zhang

Abstract

The uptake of iron into the body is tightly regulated in humans and in other mammals. Mutations in key proteins that transport, sense, metabolize, and facilitate the utilization of iron cause perturbations in iron homeostasis that result in iron deficiency or overload diseases. This review focuses on what is currently known about these diseases and the normal function of the proteins that are mutated in the disease-state. The proteins causing hereditary hemochromatosis and anemia are discussed in detail.

1 Overview of iron transport and homeostasis

Iron enters the body principally through enterocyte cells in the duodenum of the intestine (Fig. 1). The iron absorbed by these cells may be in two forms, heme and non-heme. The mechanism by which heme iron enters the body is not established. For non-heme iron, duodenal cytochrome b (DcytB) or another ferrireductase, on the apical membrane of the enterocyte facing the intestinal lumen, first reduces Fe^{3+} from food to the more soluble Fe^{2+} (McKie et al. 2001). The divalent metal ion transporter, DMT1, then transports Fe^{2+} across the apical surface of the intestinal cell (Fleming et al. 1997; Gunshin et al. 1997). Once inside, either iron remains within the cell, stored in ferritin and unabsorbed by the body, until it is lost when, after several days, the cell dies (Kaplan 2002); or iron crosses to the basolateral side where ferroportin1 then transports Fe^{2+} out of the cell (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). After iron exits the enterocyte, a multicopper ferroxidase on the cell surface, hephaestin (Vulpe et al. 1999), or its soluble homolog in the circulation, ceruloplasmin (Cp) (Mukhopadhyay et al. 1998; Harris et al. 1999), re-oxidizes iron to the Fe^{3+} form. The serum protein transferrin (Tf) binds iron and transports it to cells throughout the body.

In most tissues, iron enters cells by receptor-mediated endocytosis. Iron bound to transferrin (Fe_2-Tf) binds to transferrin receptor 1 (TfR1) on the surface of cells. Endocytosis delivers the Fe_2-Tf-TfR1 complex to the early endosome where the acidified environment promotes the release of iron, which is reduced to Fe^{2+} by an undetermined mechanism. The TfR1-Tf complex then recycles to the cell surface

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_99 / Published online: 11 August 2005

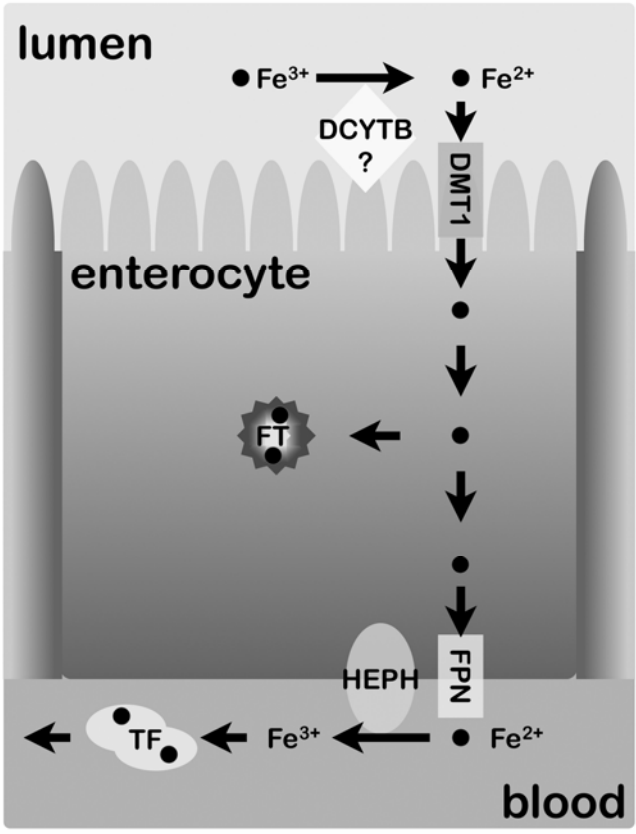


Fig. 1. Absorption of non-heme iron across the enterocyte. On the apical surface of enterocytes facing the lumen of the intestine, non-heme iron from the diet is reduced to Fe^{2+} by DcytB or another ferrireductase and transported into the cell by divalent metal-ion transporter 1 (DMT1). Iron may be stored within the cell bound to ferritin (Ft) or transported across the basolateral surface of the cell by ferroportin (Fpn) into the blood, where iron is oxidized to Fe^{3+} by hephaestin (Heph), bound by transferrin (Tf), and circulated throughout the body.

and dissociates at the neutral pH, releasing Tf into the circulation. On the endosomal membrane, DMT1 transports iron into the cytosol (Fleming et al. 1998), where it is incorporated into newly synthesized proteins or stored in ferritin (Ft) (Kaplan 2002).

Differentiating erythrocytes in the bone marrow utilize the majority of iron in the body for heme biosynthesis. Macrophages phagocytose senescent erythrocytes, degrade their heme, and return the iron to the circulation where it is bound by Tf (Fletcher and Halliday 2002). Efficient recycling of 20-30 mg of iron per day reduces the dietary iron requirement to 1-2 mg, a fraction of the 3-5 g found in

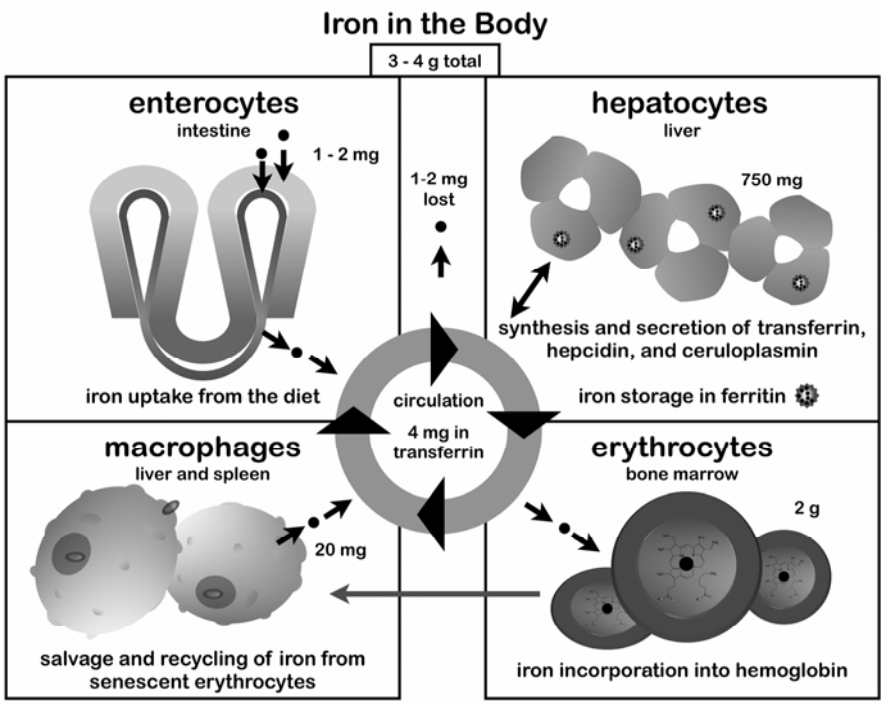


Fig. 2. Iron in the body. The healthy human body maintains a total iron level of 3-5 g by absorbing iron from the diet and recycling iron from red blood cells. Enterocytes in the duodenum of the intestine absorb 1-2 mg of iron from the diet per day to replace iron that is lost in sloughed cells. Cells throughout the body utilize and store iron, but developing erythrocytes incorporate the majority of the body's iron into heme to facilitate oxygen transport, while hepatocytes in the liver serve as the principal repositories of iron. The iron within heme is recycled to the circulation by macrophages that phagocytose senescent erythrocytes.

healthy adult humans (Andrews 1999; Townsend and Drakesmith 2002). To maintain a supply of iron available for erythropoiesis, hepatocytes store iron in Ft that can be released when needed (Fig. 2) (Fletcher and Halliday 2002; Pietrangelo 2002).

The body monitors and regulates iron at the cellular and systemic levels. At the cellular level, iron regulatory proteins (IRP) bind stem-loop structures called iron responsive elements (IRE) found in the untranslated regions (UTR) of the transcripts encoding several iron related genes (Fig. 3). Binding of the IRP either blocks translation of the transcript or protects the transcript from degradation, depending on the location of the IRE. The binding of IRPs to IREs when intracellular iron levels are low increases TfR1 and decreases Ft levels, thereby facilitating iron uptake and minimizing iron storage. The inhibition of IRP binding by high intracellular iron levels decreases TfR1 and increases Ft levels, thereby limiting iron

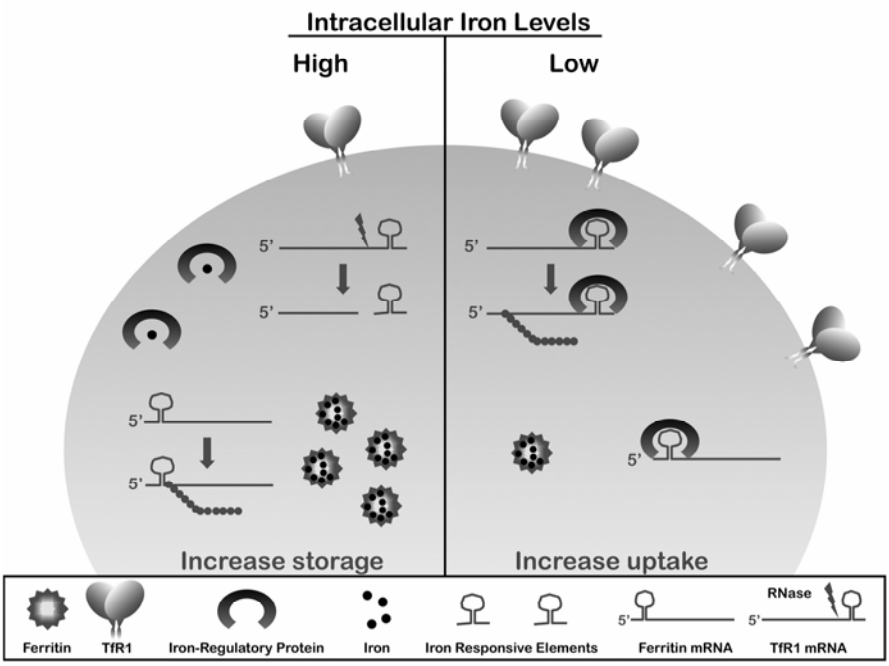


Fig. 3. Cellular iron homeostasis. Cells regulate iron uptake and storage through post-transcriptional control of transferrin receptor 1 (TfR1) and ferritin (Ft). The 3' untranslated region (UTR) of TfR1 mRNA and the 5' UTR of Ft mRNA contain stem-loop structures called iron-responsive elements (IRE). When intracellular iron levels are high, iron regulatory proteins (IRPs) are unable to bind IREs. Unbound TfR1 transcript is degraded, whereas unbound Ft transcript is translated into protein, thus reducing iron uptake and increasing the capacity for iron storage. Conversely, when intracellular iron levels are low, IRPs bind IREs. Binding of IRPs to IREs protects TfR1 transcript from degradation and blocks translation of Ft transcript, thereby increasing iron uptake and decreasing the capacity for iron storage.

uptake and increasing storage. At the systemic level, the body maintains iron at appropriate levels by controlling the absorption of dietary iron. When iron levels are high, hepatocytes secrete higher amounts of a small, soluble peptide called hepcidin that decreases the amount of iron absorbed into the body from the intestine. Conversely, when iron levels are low, hepatocytes suppress hepcidin synthesis, promoting iron absorption.

When mutations disrupt the function of proteins that transport, monitor, regulate, and metabolize iron, diseases of iron overload or deficiency result. We review herein hereditary hemochromatosis, β-thalassemia, and several iron deficiency disorders, including anemia of inflammation, sideroblastic anemia, and anemia due to mutation of DMT1.

2 Misregulation of iron: hereditary hemochromatosis

The most common iron overload disease is hereditary hemochromatosis (HH). Mutations in five molecules, HFE, hemojuvelin, hepcidin, transferrin receptor 2, and ferroportin1, cause HH. Of these, the function of two, hepcidin and ferroportin, are known. The others in unknown ways are essential for hepcidin's regulation. In this section, we describe the variants and animal models of HH; review the properties, expression, regulation, and function of the molecules that cause HH; and discuss mechanisms of systemic iron homeostasis misregulated in HH.

2.1 Hereditary hemochromatosis type 1

Hereditary hemochromatosis type 1 (HFE1) is an autosomal recessive disorder caused by mutations in the hemochromatosis gene, *HFE*, on chromosome 6p21.3 (Feder et al. 1996). The most common form of hemochromatosis, HFE1 accounts for approximately 80% of hemochromatosis cases (Feder et al. 1996). Among these, a missense mutation in HFE converting cysteine to tyrosine at residue 282 (C282Y) occurs most frequently, and a second missense mutation converting histidine to aspartate at residue 63 (H63D) occurs more rarely (Feder et al. 1996; Borot et al. 1997; Carella et al. 1997). The frequency of the H63D allele in the population is high, but its association with HFE1 is low because disease develops only in individuals also carrying the C282Y allele, not in individuals homozygous for H63D. Thus, HFE1 can arise from compound heterozygosity for C282 and H63D, but is usually due to homozygosity for C282Y.

Mutations in *HFE* are common in individuals of Northern European descent, with a heterozygote frequency of 1 in 10 and a homozygote frequency of 1 in 200 (Edwards et al. 1988), but disease penetrance is debated and may be much lower (Ajioka and Kushner 2003; Beutler 2003). Individuals who develop HFE1 regularly absorb excess dietary iron. Iron slowly accumulates throughout life, depositing foremost in hepatocytes within the liver, then in other parenchymal tissues of the heart, pancreas, and thymus. Iron does not accumulate in macrophages of the liver or spleen until later stages of the disease. Serum transferrin saturation and serum ferritin levels increase and serve as diagnostic indicators. If iron levels are not reduced by frequent phlebotomy, liver cirrhosis, hepatoma, heart abnormalities, diabetes, and arthritis develop, generally beginning in the fourth decade of life.

Several mouse strains serve as models for HFE1. $Hfe^{-/-}$ mice are null for the HFE gene (Zhou et al. 1998; Levy et al. 1999b). Hfe^{C282Y} mice are homozygous for the C282Y mutation (Levy et al. 1999b). $Hfe^{\Delta\alpha1\,\alpha2}$ mice are homozygous for a mutated gene that does not encode the $\alpha1$ and $\alpha2$ domains of the protein. These domains contain residues that interact with TfR1 (Bahram et al. 1999). $\beta_2m^{-/-}$ mice are null for β_2-microglobulin, a protein required for efficient transport of HFE and MHC class I molecules to the cell surface (Rothenberg and Voland 1996; Santos et al. 1996). In all the models, the mutant mice have phenotypes similar to individuals with HFE1: elevated liver iron content and transferrin saturation but nor-

mal splenic iron content. Iron deposits appear in hepatocytes, in increasing concentrations from the pericentral to periportal zones (Zhou et al. 1998). Expression of TfR1 in the liver decreases dramatically. However, *Hfe*$^{-/-}$ mice accumulate more liver iron than *Hfe*^{C282Y} mice at 4 weeks of age, suggesting that C282Y mutation in *HFE* does not entirely abolish protein expression, transport, and function (Levy et al. 1999b). Similarly, *β*$_2$*m*$^{-/-}$ mice accumulate less liver iron than *Hfe*$^{-/-}$ mice, probably due to residual expression of HFE at the cell surface. *Hfe*$^{-/-}$ and *Hfe*^{C282Y} mice express lower levels of hepcidin in their livers than do wild type mice (Ahmad et al. 2002; Muckenthaler et al. 2003). When fed an iron rich diet, *Hfe*$^{-/-}$ and *Hfe*^{C282Y} mice differ from wild type mice in their transcriptional regulation of hepcidin. Whereas wild type mice upregulate hepcidin, *HFE*$^{-/-}$ and *Hfe*^{C282Y} mice downregulate hepcidin (Muckenthaler et al. 2003). This suggests that proper hepcidin regulation requires functional HFE. The phenotype of *Thep/Hfe*$^{-/-}$ mice strongly supports this. *Thep* mice overexpress hepcidin and are anemic (Nicolas et al. 2002a). When bred to *Hfe*$^{-/-}$ mice, anemia persists, but iron overload does not develop despite the absence of functional HFE (Nicolas et al. 2003). Thus, HFE is upstream of hepcidin in a regulatory pathway that controls iron levels.

HFE is a major histocompatability (MHC) class I-like molecule cloned and identified as the hemochromatosis protein in 1996 (Feder et al. 1996). The *HFE* gene encodes a protein of 343 amino acids with an immunoglobulin-like domain, transmembrane region, and short cytoplasmic tail. Like other MHC molecules, HFE has three external domains. The α-1 and α-2 domains form a peptide-binding pocket. The groove of the peptide-binding pocket is narrower in HFE than in other MHC molecules that function in antigen presentation, and consequently is unable to accommodate a peptide. Importantly, HFE also contains two disulfide bonds within the α-2 and α-3 domains that stabilize the α-3 domain in a conformation competent to heterodimerize with β_2-microglobulin (β_2m). The C282Y mutation in HFE that results in hemochromatosis eliminates a cysteine residue and a disulfide bond, consequently disrupting the structure of HFE, destabilizing its interaction with β_2m, and impeding its transit to the cell surface (Feder et al. 1997; Waheed et al. 1997).

HFE transcript is present in most tissues, but most abundantly in the liver and intestine (Feder et al. 1996). In the intestine, crypt cells in the ileum express high levels of HFE protein on their basolateral membranes (Parkkila et al. 1997b). In the liver, HFE mRNA and protein expression occurs predominantly in hepatocytes and to a lesser extent in Kupffer cells (Holmstrom et al. 2003; Zhang et al. 2004).

In addition to interacting with β_2m, HFE interacts with TfR1 in cells and tissues (Parkkila et al. 1997a; Feder et al. 1998). Soluble forms of the proteins bind each other with nanomolar affinity (Lebron et al. 1998). In transfected HeLa cells, HFE associates with TfR1 along the biosynthetic and endocytic pathways (Gross et al. 1998). The interaction decreases the affinity of TfR1 for Fe_2-Tf *in vitro*, consistent with the fact that the binding site for HFE on TfR1 overlaps the binding site for Fe_2-Tf (Lebron et al. 1999; West et al. 2001). This data suggests that HFE might function normally to reduce iron uptake into cells by competing with Fe_2-Tf for binding to TfR1. However, at physiological Fe_2-Tf concentrations of ~3 μM, HFE

is unlikely to affect receptor occupancy. Instead, HFE alters iron homeostasis in a TfR1-independent manner (Zhang et al. 2003).

Despite considerable effort devoted to the study of HFE in the years since its discovery, the mechanism by which HFE controls iron homeostasis within the body remains elusive. Numerous studies have addressed the effect of HFE and HFE C282Y expression on intracellular iron levels and iron transport. The results vary depending on the cell system employed. Several studies suggest that HFE functions to reduce iron uptake. In HeLa cells, expression of wild type HFE reduces iron uptake from Tf without altering the cycling kinetics of TfR1 (Roy et al. 1999). Ferritin levels are lower in these cells, but not in HeLa cells expressing HFE C282Y. TfR1 levels are higher in cells expressing HFE, consistent with reduced intracellular iron levels (Roy et al. 2000). However, another study of HeLa cells expressing HFE attributes the decrease in iron uptake from Tf to a reduction in TfR1 number and internalization rate (Salter-Cid et al. 1999). In hepatocytes, transfection of HFE-GFP decreases the rate of Fe_2-Tf influx and the rate of Tf recycling (Ikuta et al. 2000). HFE expression reduces iron uptake from Tf in U937 cells (Drakesmith et al. 2002).

Other studies suggest that HFE functions to increase intracellular iron levels. Macrophages from HFE^{C282Y} hemochromatotic individuals accumulate less iron from Tf than macrophages from individuals with wild type HFE. Expression of wild type HFE in hemochromatotic macrophages increases iron uptake from Tf (Montosi et al. 2000). Expression of HFE inhibits iron efflux and decreases iron uptake from Tf in the THP-1 macrophage cell line. Iron levels increase, indicating that the effect on iron efflux dominates (Drakesmith et al. 2002). In HT-29 cells, expression of HFE increases intracellular iron levels through a TfR1-independent mechanism by inhibiting iron efflux. The increase in iron levels correlates with a decrease in expression of hephaestin, a ferroxidase that facilitates iron export (Davies et al. 2003).

Taken together, the studies suggest that HFE may influence iron homeostasis by more than one mechanism depending on the proteins expressed in a particular cell type. Notably, macrophages and HT-29 cells in which HFE raises iron levels express Fpn1, whereas HeLa and U937 cells in which HFE lowers iron uptake from Tf do not. Consistent with the idea that HFE may interact with TfR1 and iron export machinery, various mutations in HFE differentially affect its abilities to alter iron export and iron uptake from Tf (Drakesmith et al. 2002).

2.2 Hereditary hemochromatosis type 2

Hereditary hemochromatosis type 2 (HFE2), also referred to as juvenile hemochromatosis (JH), is an autosomal recessive disorder caused by mutations in either hemojuvelin (*HJV*) on chromosome 1q21 (Papanikolaou et al. 2004) or in hepcidin antimicrobial peptide (*HAMP*) on chromosome 19q13s (Krause et al. 2000; Park et al. 2001; Pigeon et al. 2001; Roetto et al. 2003). The pathology of HFE2 is similar to that of HFE1, but individuals with HFE2 accumulate higher levels of iron earlier in life, generally within the first two decades. Serum transferrin satura-

tion and serum ferritin levels are elevated. Iron accumulates in parenchymal tissues, notably the heart and liver. If not treated, death results from heart failure.

2.2.1 HFE2A

Numerous mutations in hemojuvelin (Hjv) that cause HFE2A have been identified. These include thirteen missense mutations (C80R, S85P, G99V, L101P, I122N, I128T, A168D, F170S, W191C, R288W, G320V, C321W, R385X), two frame shift mutations (D149fsX245, C361fsX366), and two nonsense mutations (C321X, R326X) (Huang et al. 2004; Lanzara et al. 2004; Lee et al. 2004b, 2004c; Papanikolaou et al. 2004).

Currently, there is not a mouse model for HFE2A. However in individuals with HFE2A, hepcidin levels are abnormally low (Papanikolaou et al. 2004). This implies that disruption of Hjv causes misregulation of hepcidin.

Hemojuvelin (Hjv, also HFE2, RGMc) is a member of the repulsive guidance molecule (RGM) family. Members of this family have roles in the developing nervous system and muscle. Hjv and human RGMa are 48% identical, while human, rat, and mouse Hjv orthologues are 85% identical. Five alternatively spliced transcripts encode three different proteins of 426, 313, and 200 amino acids. All three proteins contain a putative C-terminal transmembrane domain; the longest two contain a partial von Willebrandt factor domain; and the longest contains an N-terminal signal peptide domain and a RGD sequence. The occurrence of disease causing mutations within the N-terminal region suggests that the longest isoform is relevant to iron homeostasis. Consistent with this, northern blots detect transcript encoding the full-length isoform (Papanikolaou et al. 2004). In addition to a transmembrane domain, the C-terminus of Hjv contains a putative GPI attachment motif. When expressed in at least one cell line, Hjv is processed to a GPI-linked protein (A.S. Zhang, unpublished results 2005).

Hjv transcript is present at high levels in human fetal liver, adult liver, heart, skeletal muscle and esophagus; at lower levels in colon and pancreas (Papanikolaou et al. 2004; Rodriguez Martinez et al. 2004). In mice, antisera raised against Hjv peptides detect a 26 kDa protein in the liver, heart, kidney, brain, and muscle; and a 30 kDa protein in the liver, jejunum, kidney, and testis (Rodriguez Martinez et al. 2004). Expression of Hjv transcript in the liver increases late in embryonic development, decreases in the few days after birth, and then increases moderately to levels sustained into adulthood. Hjv transcript expression in the liver does not respond to iron or erythropoietin, but decreases after LPS injection (Krijt et al. 2004).

2.2.2 HFE2B

Mutations in hepcidin that cause HFE2B are rare, but include two missense mutations, C78T (Delatycki et al. 2004) and C70R (Roetto et al. 2004), that disrupt disulfide bonds; a noncoding mutation in the 5' UTR that abrogates hepcidin expression by generating a new initiation codon (Matthes et al. 2004); a nonsense

mutation, R56X (Roetto et al. 2004); and a frameshift mutation after residue 31 (Roetto et al. 2004).

Knockout of upstream stimulatory factor 2 (Usf2) inadvertently produced a mouse model of HFE2B. In the *Usf2*$^{-/-}$ mouse, knockout of the *Usf2* gene abolished expression of the adjacent *Hamp* genes encoding hepcidin. The mice with age accumulate iron in the parenchymal cells of the liver, pancreas, heart, and kidney and have elevated serum iron and serum transferrin saturation levels. Iron levels in the spleen remain low throughout the animals' lifetimes, in contrast to wild type mice that accumulate iron in splenic macrophages with age. Erythroid parameters (RBC count, hemoglobin concentration, and mean corpuscular volume) are normal. Expression levels of HFE, transferrin receptor 2 (TfR2), ceruloplasmin (Cp), heme oxygenase-1 (HO-1), transferrin receptor 1 (TfR1), and divalent metal transporter 1 (DMT1) transcripts are the same in wild type and *Usf2*$^{-/-}$ mice (Nicolas et al. 2001). Characterization of a second *Usf2*$^{-/-}$ strain in which hepcidin expression is unaffected confirms that hepcidin deficiency in the first *Usf2*$^{-/-}$ strain causes iron misregulation (Nicolas et al. 2002a).

Hepcidin (also LEAP-1 for liver-expressed antimicrobial peptide) is a peptide hormone found in serum and urine (Krause et al. 2000; Park et al. 2001). The *HAMP* gene encodes an 84 amino acid prepropeptide. Cleavage of a signal sequence after Gly^{24} generates a 60 amino acid propeptide that is differentially proteolysed to generate a mature peptide of 20, 22, or 25 amino acids (Krause et al. 2000; Park et al. 2001; Pigeon et al. 2001). The three hepcidin peptides differ in their N-termini, but all include eight cysteine residues. Disulfide bonds between the eight cysteines stabilize an amphipathic β-sheet that curls to form a convex hydrophobic surface and a concave basic surface (Hunter et al. 2002). The structural characteristics of hepcidin are similar to antimicrobial peptides called defensins. Consistently, Hepc20 exhibits antimicrobial and antifungal activity. Hepc25 also exhibits antimicrobial activity, but to a lesser extent than Hepc20 (Park et al. 2001). As a result of gene duplication, mice have two genes for hepcidin, *Hamp1* and *Hamp2*, present in tandem on chromosome 17, that encode 83 amino acid prepropeptides (Pigeon et al. 2001). Hepc1 and Hepc2 are 68% identical in their mature forms and 89% identical in their prepro forms. Mouse Hepc1 and human Hepc show 54% identity in their prepro forms and 76% identity in their mature forms (Pigeon et al. 2001; Ilyin et al. 2003).

The liver is the primary site of hepcidin synthesis and secretion (Krause et al. 2000). Hepcidin mRNA appears late in fetal development, rises at birth, declines after birth, then rises again in adulthood when expression peaks (Park et al. 2001; Pigeon et al. 2001). Hepcidin peptide localizes to the basolateral membrane of hepatocytes located in the periportal zones of the liver from where it is presumably secreted into the serum (Kulaksiz et al. 2004). Hepc25, Hepc20 and prohepcidin are present in the serum, the latter at concentrations of 5 – 15 nM in healthy individuals (Kulaksiz et al. 2004). Adult heart, brain, spinal cord, and kidney express lower levels of hepcidin mRNA (Park et al. 2001; Pigeon et al. 2001; Courselaud et al. 2002; Nicolas et al. 2002a; Kulaksiz et al. 2005). In the kidney hepcidin localizes to epithelial cells in the tubules of the cortex, medulla, and papilla (Kulaksiz et al. 2005). Within cells, hepcidin appears apical or intracellular, sug-

gesting it is released into urine within the renal lumen. Hepcidin in urine from healthy individuals comprises predominantly Hepc20 and Hepc25 in concentrations from 4 – 12 nM (Park et al. 2001). In the mouse, Hepc2 transcript is expressed in a similar pattern and to a similar degree as mouse Hepc1 and human Hepc, but Hepc2 is also present in the pancreas at a high level (Ilyin et al. 2003).

Hepcidin is essential for mediating changes in iron absorption, recycling, and storage in normal and pathological conditions. Mice lacking hepcidin have iron overload (Nicolas et al. 2001), whereas mice overexpressing hepcidin are severely anemic (Nicolas et al. 2002a). Misregulation of iron homeostasis in numerous diseases derives from misregulation of hepcidin. Mice and humans homozygous for mutations in HFE or TfR2 fail to upregulate hepcidin and thus develop iron overload (Ahmad et al. 2002; Kawabata et al. 2005), as do individuals with juvenile hemochromatosis who are homozygous for mutations in Hjv (Papanikolaou et al. 2004). Individuals with chronic inflammation continuously upregulate hepcidin and thus develop anemia (Nemeth et al. 2004a).

Since regulation of hepcidin is a central means by which to modulate iron, and numerous normal and pathological conditions require modulation of iron, it is not surprising that hepcidin expression responds to a variety of stimuli. Hypoxia reduces expression of hepcidin (Nicolas et al. 2002b), presumably leading to an increase in iron to support erythropoiesis and oxygen transport. Hypoxia may downregulate hepcidin directly, through the transcription factor HIF-1, or indirectly through erythropoietin (EPO), which is induced by HIF-1 during hypoxia (Wang and Semenza 1993). Mice injected with EPO downregulate hepcidin (Nicolas et al. 2002c). Inflammation increases hepcidin expression (Nicolas et al. 2002b), reducing the iron available to pathogens. A switch from a standard diet to a high iron diet induces hepatic hepcidin expression in mice (Pigeon et al. 2001). Conversely, a switch from a high iron diet to a low iron diet reduces hepcidin expression (Frazer et al. 2002). Though not all studies in mice differentiate between Hepc1 and Hepc2, their mRNAs respond similarly to changes in dietary iron (Mazur et al. 2003), thus the regulation if not the activity of Hepc1 and Hepc2 appears to be the same. Hepcidin expression also decreases in mice with genetic anemia due to mutations in hephaestin (*sla* mouse), DMT1 (*mk* mouse), or transferrin (*hpx* mouse) (Weinstein et al. 2002) or experimental anemia induced by phenylhydrazine (PHZ) injection (Nicolas et al. 2002b). Similarly, in humans hepcidin increases within 24 hours after iron ingestion (Nemeth et al. 2004a). Thus, iron itself alters hepcidin expression in order to maintain iron levels that are nontoxic, but adequate to support biological functions.

When iron levels rise, hepcidin transcript levels rise. Changes in hepcidin levels probably reflect changes in transcription, though changes in transcript stability could also contribute. The hepcidin promoter region in humans and mice contains binding sites for hepatocyte nuclear factor 4 (HNF4), CCAAT/enhancer-binding proteins (C/EBP), and signal transducers and activators of transcription (STAT). C/EBPα induces strong expression from the human hepcidin promoter, while C/EBPβ induces weakly, and HNF4 attenuates. In mice with liver specific knockout of C/EBPα, expression of hepcidin is reduced. Consistent with this misregulation of hepcidin, C/EBPα knockout mice have iron overload in the liver. In mice

fed a high iron diet C/EBPα protein increases. Developmental expression of C/EBPα correlates with that of hepcidin, appearing late in fetal development, peaking near birth, decreasing, then re-accumulating at adulthood. C/EBPα is lower in hepatoma cells than in adult hepatocytes, again correlating with the pattern of hepcidin expression (Courselaud et al. 2002).

2.3 Hereditary hemochromatosis type 3

Hereditary hemochromatosis type 3 (HFE3) is an autosomal recessive disorder that occurs in individuals homozygous for mutations in transferrin receptor 2 (*TFR2*) on chromosome 7q22 (Kawabata et al. 1999; Camaschella et al. 2000). Among the currently known disease causing mutations, Y250X (Camaschella et al. 2000), E60X (Roetto et al. 2001), R105X (Le Gac et al. 2004), Q317X (Pietrangelo et al. 2005), and V561X (Koyama et al. 2005) introduce premature stop codons; Q690P (Mattman et al. 2002), M172K (Le Gac et al. 2004), and L490P (Koyama et al. 2005) produce single amino acid changes in the extracellular region of the receptor; and AVAQ594-597Δ deletes four amino acids from the TfR2 extracellular domain (Girelli et al. 2002). Though the incidence of HFE3 is low, its pathology suggests that TfR2 plays a critical, but as yet undefined, role in iron regulation.

The *Trfr2*^{Y245X} mouse is a model of hemochromatosis arising from mutation in TfR2. The Y245X nonsense mutation is orthologous to the Y250X mutation that occurs in individuals with HFE3. Mice homozygous for the mutation do not express membrane bound TfR2. Compared with wild type mice, liver iron is fourfold higher and splenic iron is twofold lower in mutant mice. Hematological parameters are unchanged (Fleming et al. 2002). In the duodenum, iron levels are the same in mutant and wild type mice, but DMT1-1A transcript expression is higher in *Trfr2*^{245X} mice. Significantly, relative to wild type mice, *Trfr2*^{Y245X} mice express lower levels of hepcidin transcript. After iron dextran injection, *Trfr2*^{Y245X} mice do not induce hepcidin expression even though the iron levels in their livers are as high as the iron levels in the livers of wild type mice that do upregulate hepcidin (Kawabata et al. 2005). Thus, TfR2 contributes to hepcidin regulation.

Mice in which *Trfr2* has been knocked out will most likely serve as a second model for HFE3 (Wallace et al. 2004b). The phenotype of this mouse has not been described, but the development of hemochromatosis in *Trfr2*$^{-/-}$ mice will probably confirm that HFE3 is due to a loss of function in TfR2.

TfR2 is a homolog of TfR1, the ubiquitously expressed receptor that delivers Fe_2-Tf to cells. The human *TFR2* gene was identified and cloned in 1999 (Kawabata et al. 1999). The principle transcript transcribed from this gene encodes a 100 kDa type II transmembrane protein, TfR2-α or TfR2. The second transcript encodes a truncated protein, TfR2-β, which lacks the intracellular and transmembrane domains at the N-terminus. The amino acid sequence in the extracellular domain of TfR2 shows 45% identity and 66% similarity to the extracellular domain of TfR1. The cytoplasmic domain of TfR2 contains a putative endocytic mo-

tif, YQRV, and shows no homology to the cytoplasmic domain of TfR1. TfR2 molecules form dimers stabilized by disulfide bonds (Kawabata et al. 1999). The mouse *Trfr2* gene on chromosome 5 gives rise to three alternative splice variants (Fleming et al. 2000; Kawabata et al. 2001a). The predominant transcript encodes a protein that is 89% identical to human TfR2 (Kawabata et al. 2001a).

TfR2 mRNA is abundant in liver tissue, but is not detectable in the small intestine, kidney, brain, or heart (Kawabata et al. 1999). This expression pattern contrasts the widespread distribution of TfR1 throughout the body. Expression of TfR2 transcript increases in the liver from embryonic and postnatal development to adulthood, whereas expression of TfR2 transcript decreases during erythrocyte differentiation (Kawabata et al. 2001a, 2001b). Within the liver, transcript level is high in hepatocytes and low in Kupffer cells and stellate cells (Fleming et al. 2000; Zhang et al. 2004). TfR2 transcript is present in HepG2 (human hepatoma) K562 (human erythroleukemia), MEL (murine erythroleukemia), and other erythroid cell lines (Kawabata et al. 1999, 2001a, 2001b). HepG2, K562, and HuH7 (human hepatoma) cells also express TfR2 protein, whereas TfR2 is not detectable in SK1-Hep and Hep3B (human hepatoma) cells (Vogt et al. 2003). The liver expresses TfR2 protein (Fleming et al. 2002).

Consistent with its homology to TfR1, TfR2 interacts with Tf in pH-dependent manner. At pH 7.6 – 7.2, TfR2 binds Fe_2-Tf optimally. Below pH 7.2, binding drops sharply. By contrast, TfR1 does not release Fe_2-Tf until the pH falls below 6.8 (Kawabata et al. 1999). Given that sensitivity to pH is integral to the function of TfR1, the difference in the pH sensitivity of the interaction between TfR2 and Tf may have implication for the function of TfR2 in the cell. Both the full-length and a soluble form of TfR2 bind Tf with an affinity of 27nM, approximately 30-fold lower than the affinity of TfR1 for Tf (Kawabata et al. 1999; West et al. 2000). Similar to TfR1, mutation of an RGD sequence in the extracellular domain of TfR2 reduces Tf binding (Kawabata et al. 2004). In contrast to TfR1, TfR2 does not bind HFE in immunoprecipitation or surface plasmon resonance assays (West et al. 2000).

The promoters of both mouse and human TfR2 contain binding sites for C/EBP and two erythroid specific transcription factors, EKLF and GATA-1 (Kawabata et al. 2001a), but little is known about transcriptional regulation of TfR2. At the post-transcriptional level, intracellular iron levels do not regulate TfR2. Unlike TfR1, TfR2 does not contain an iron response element (IRE) in its 3' untranslated region (UTR). Treatment with iron or an iron chelator does not alter TfR2 mRNA or protein levels in K562 cells (Kawabata et al. 2000), HepG2 cells (Johnson and Enns 2004; Robb and Wessling-Resnick 2004) or MEL cells (Kawabata et al. 2001a). TfR2 transcript levels are the same in livers from mice fed standard, high-iron, and low-iron diets. Transcript levels are also the same in livers from wild type and *Hfe*$^{-/-}$ mice (Fleming et al. 2000). TfR2 protein, though, is higher in livers from mice fed high-iron diets and in livers from *Hfe*$^{-/-}$ mice (Robb and Wessling-Resnick 2004; Kawabata et al. 2005). The discrepancy between transcript and protein is due to post-transcriptional regulation of TfR2 by Fe_2-Tf concentrations. Increasing the concentration of Fe_2-Tf in the culture medium of HepG2 cells stabilizes TfR2, resulting in an increase in TfR2 levels in the cell (Johnson and Enns

2004). The proportion of TfR2 molecules at the cell surface also increases in response to Fe_2-Tf (Deaglio et al. 2002). The effects on TfR2 occur at physiologically relevant concentrations of Fe_2-Tf. Consistently, TfR2 is higher in mice with elevated serum transferrin saturation and lower in mice with reduced serum transferrin saturation (Robb and Wessling-Resnick 2004).

The binding properties, tissue distribution, and inability of TfR2 to support growth in TfR1 knockout mice (Levy et al. 1999a) indicate that the receptor has functions distinct from TfR1. Though TfR2 binds and internalizes Fe_2-Tf, the main role of TfR2 is unlikely to be iron import since *Trfr2*^{Y245X} mice and individuals with HFE3 accumulate iron in their livers (Camaschella et al. 1999, 2000; Fleming et al. 2000). The interaction of TfR2 with Tf may be important for iron regulation, however. The regulation of TfR2 stability by transferrin saturation may be part of a mechanism that senses systemic iron levels (Johnson and Enns 2004). The delivery of Tf by TfR2 to a specific intracellular compartment might modulate a signal from the liver regulating iron uptake by the intestine. Expression of TfR2 but not TfR1 in HeLa cells increases the amount of Tf found in multivesicular bodies (MVB). Tf also appears in MVBs in HepG2 cells endogenously expressing TfR2 (Robb et al. 2004). Thus, further characterization of TfR2 trafficking may elucidate its role in iron homeostasis.

2.4 Hereditary hemochromatosis type 4

Hemochromatosis type 4 (HFE4), in contrast to the other forms of HH, is an autosomal dominant disorder. HFE4 arises from mutations in the gene *SLC40A1* (for solute carrier family 40 member 1) on chromosome 2q32 (Haile 2000; Montosi et al. 2001; Njajou et al. 2001) encoding the iron transporter ferroportin (Fpn1; (Donovan et al. 2000)). The numerous mutations in Fpn1 that cause HFE4 include N144H/T/D (Njajou et al. 2001; Arden et al. 2003; Wallace et al. 2004a), A77D (Montosi et al. 2001), V162Δ (Cazzola et al. 2002; Devalia et al. 2002; Roetto et al. 2002; Wallace et al. 2002), Y64N (Rivard et al. 2003), G490D (Jouanolle et al. 2003), and Q248H (Barton et al. 2003; Beutler et al. 2003; Gordeuk et al. 2003).

The pathology of HFE4 is distinct from that of the other forms of HH. Iron accumulates predominantly within macrophages of the liver and spleen. Serum ferritin levels consistently increase. In only a subset of cases, possibly correlating with particular mutations in Fpn1 or the severity and duration of the disease, iron accumulates in hepatocytes and serum transferrin saturation increases.

Fpn1 functions as an iron exporter. In transfected oocytes, mouse Fpn1 stimulates iron efflux 2.5-fold. Iron efflux activity required the presence of ceruloplasmin in the medium (McKie et al. 2000). Zebrafish Fpn1 also increases iron efflux when expressed in oocytes, but requires the presence of apoTf in the medium (Donovan et al. 2000). Overexpression of Fpn1-GFP in J774 macrophages increases export of iron derived from phagocytosed erythrocytes by 70% (Knutson et al. 2005).

The pathology of HFE4 arising from mutation in Fpn1 is consistent with a defect in iron export from reticuloendothelial cells due to haploinsufficiency for

Fpn1 (Montosi et al. 2001). Loss of transporter function would limit iron export from macrophages and enterocytes, increasing iron levels in those cells, but reducing iron levels in the body. Insufficient iron for erythropoiesis would subsequently stimulate intestinal iron absorption. Finally, hepatocytes would eventually accumulate iron due to increased intestinal absorption and decreased hepatic export (Fleming and Sly 2001a). However, a recent study characterizing the activity of wild type and mutant Fpn1 transporters in transfected 293T cells suggests that the various mutations in Fpn1 may lead to different defects (Schimanski et al. 2005). The study demonstrated that wild type Fpn1 localizes to the cell surface and exports iron from the cell, thereby lowering intracellular iron levels. Four disease causing mutants, G490D, L170F, V162Δ, and A77D, do not localize to the cell surface, export iron, or lower intracellular iron levels. A subset of Fpn1 mutations, therefore, results in loss of transporter function and could cause disease according to the haploinsufficiency model. Five other disease causing mutants, C326Y, Q248H, N144H/D, and Y64N, did localize to the cell surface, export iron, and lower intracellular iron levels, however. Thus, a second subset of Fpn1 mutations does not appear to affect transporter function, but nonetheless causes disease. These mutations may disrupt interactions with regulatory or accessory proteins, but how they cause disease is not clear.

Fpn1 (also Ireg1, MTP1) is a 571 amino acid protein of 63 kDa in humans (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). Human, mouse, and rat orthologues are 90% similar and bear some homology to other metal transporters, including DMT1. Topology prediction programs indicate Fpn1 has nine or ten transmembrane domains (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000; Devalia et al. 2002). Fpn1 contains a putative NADP/adenine-binding site, indicative of possible reductase activity, and a C-terminal PDZ target motif, important for targeting of proteins to the basolateral membrane. Consistently, epitope-tagged Fpn1 transfected into MDCK and CaCo-2 cells appears along the basolateral surface (McKie et al. 2000).

In humans and mice, cells in the intestine, spleen, kidney, liver, heart, and placenta express *Fpn1* (Abboud and Haile 2000; Donovan et al. 2000). During embryogenesis, Fpn1 protein first appears in the uterus, then throughout the embryo, including the brain, spinal cord, myocytes, muscles, and Kupffer cells (Abboud and Haile 2000). Synthesis of Fpn1 transcript increases in the duodenum through development to adulthood (McKie et al. 2000). In the adult, Fpn1 protein is present in hepatocytes, macrophages within the liver, spleen and bone marrow, the tubular cells and glomerulus of the kidney, heart-muscle, and duodenum (Abboud and Haile 2000; Donovan et al. 2000; Yang et al. 2002). In the mouse duodenum, expression of Fpn1 transcript and protein increases from the crypt to the villus (Donovan et al. 2000; McKie et al. 2000). In the human placenta, Fpn1 appears at the basal surface of syncytiotrophoblasts where it contacts the fetal circulation. Similarly, in zebrafish embryos, Fpn1 is expressed in the yolk syncytial later (YSL), where it is positioned to transport iron from the yolk to hematopoietic cells and the blood (Donovan et al. 2000). In the duodenum of mice, the protein is expressed in vesicles or at the basolateral surface (Abboud and Haile 2000; Donovan et al. 2000). Hepatocytes in mice express Fpn1 at their sinusoidal membrane (Ab-

boud and Haile 2000). The localization of Fpn1 within the placenta, intestine, and hepatocytes is consistent with a role in exporting iron into the circulation (Donovan et al. 2000).

Regulation of Fpn1 is complex, and a full understanding of how transcriptional, post-transcriptional, and post-translational mechanisms integrate to regulate Fpn1 expression in response to both cellular and systemic iron levels in different cells is lacking. The IRE in the 5' UTR of Fpn1 inhibits translation when iron levels are low. Nonetheless, the expression of Fpn1 transcript increases in duodenal enterocytes when mice are switched to a low iron diet. Conversely, the expression of Fpn1 transcript increases in Kupffer cells after injection of mice with iron dextran (Abboud and Haile 2000). Treatment with iron or phagocytosis of erythrocytes increases Fpn1 transcript levels in J774 macrophages, whereas iron chelation reduces levels (Knutson et al. 2003). Transcriptional mechanisms, perhaps responding to systemic iron signals, regulate Fpn1 expression in the duodenum, whereas post-transcriptional mechanisms, probably responding to cellular iron signals through the IRE/IRP system, regulate Fpn1 expression in macrophages. Additionally, hepcidin regulates the level and localization of Fpn1 protein (Nemeth et al. 2004b; Knutson et al. 2005), inducing the internalization and degradation of the transporter (Nemeth et al. 2004b).

2.5 Mechanisms of iron homeostasis misregulated in hereditary hemochromatosis

HFE, Hjv, Hepc, TfR2, and Fpn1 are critical components of a homeostatic cycle that maintains iron at appropriate levels. This cycle presumably involves a sensing mechanism that detects iron levels, a signaling mechanism that regulates hepcidin expression, and a regulatory mechanism through which hepcidin alters the availability of iron. The first two mechanisms largely remain mysteries, but recent work has elucidated a mechanism by which hepcidin may regulate the amount of iron absorbed from the intestine, recycled by macrophages, and released from hepatocytes. Nemeth et al. showed that hepcidin controls the cell surface levels of Fpn1. Addition of hepcidin to the medium of cells expressing Fpn1-GFP causes internalization of Fpn1 and subsequent degradation of Fpn1 by the lysosome. An increase in ferritin and intracellular iron levels accompanies the decrease in Fpn1, indicating that hepcidin reduces iron export by reducing Fpn1 localization at the cell surface. A direct interaction between hepcidin and Fpn1 appears to mediate the effect (Nemeth et al. 2004b). In support of these results, treatment of J774 macrophages with hepcidin reduces the level of Fpn1 and decreases non-heme iron export (Knutson et al. 2005).

HFE, Hjv, and TfR2 may relay signals of body iron status that regulate hepcidin expression. Hepcidin levels are inappropriately low relative to body iron levels in individuals and mice with hemochromatosis caused by mutations in HFE, Hjv, or TfR2 (Ahmad et al. 2002; Papanikolaou et al. 2004; Kawabata et al. 2005; Nemeth et al. 2005), suggesting that disruption of HFE, Hjv, or TfR2 weakens or eliminates signals regulating hepcidin. Through a mechanism not yet elucidated,

Hjv contributes more than HFE or TfR2 alone to hepcidin regulation, since mutations in Hjv cause more severe hemochromatosis. Interestingly, a recent case study reports that individuals who are compound heterozygous for HFE H63D/C282Y and homozygous for TfR2 Q317X develop clinical and biochemical features of juvenile, type 4 hemochromatosis. Thus, mutation of both HFE and TfR2 disrupts hepcidin expression to the same extent as mutation of Hjv. Hjv, HFE, and TfR2 may function in an independent yet complimentary fashion to control hepcidin synthesis (Pietrangelo et al. 2005). The finding is also consistent, however, with a regulatory pathway in which TfR2 and HFE converge on Hjv to modulate its function. Since levels of Hjv transcript do not change in response to iron (Krijt et al. 2004), changes in Hjv protein level or localization in response to iron signals, perhaps relayed by TfR2 and HFE, may mediate changes in hepcidin expression.

The lack of cell culture systems that recapitulate the *in vivo* response of hepcidin to iron has hindered efforts to understand the mechanism of hepcidin regulation. Treatment of freshly isolated hepatocytes with ferric ammonium citrate (FAC) or the iron chelator desferrioxamine (DFO) does not elicit a change in hepcidin expression (Pigeon et al. 2001). Neither FAC nor Fe_2-Tf affect hepcidin expression in hepatocytes cultured in serum-free medium, while in medium with serum, Fe-Tf decreases, rather than increases, hepcidin expression (Nemeth et al. 2003). In HepG2 cells, hepcidin decreases in response to iron nitrilotriacetate (FeNTA) and does not respond to Fe_2-Tf (Gehrke et al. 2003). These results have prompted the hypothesis that molecules external to the hepatocyte, perhaps released by Kupffer cell macrophages or transported in the serum, relay iron signals to hepatocytes that regulate hepcidin expression. The role of cytokines IL-6 and IL-1, both released by macrophages, in regulating hepcidin in response to inflammation is well established (Nemeth et al. 2004a; Lee et al. 2005). IL-6 is not essential for iron signaling to hepatocytes, though, since *IL-6*$^{-/-}$ mice increase hepcidin expression when switched to a high-iron diet (Nemeth et al. 2004a). In turn, TfR2 is not essential for inflammatory signaling since *Trfr2*$^{-/-}$ mice injected with endotoxin upregulate hepcidin (Frazer et al. 2004; Lee et al. 2004a). Whether inflammatory signaling to hepcidin requires HFE is controversial. In two studies of *Hfe*$^{-/-}$ mice, induction of hepcidin by LPS or Freund's complete adjuvant did not require HFE (Frazer et al. 2004; Lee et al. 2004a); whereas in a study of *Hfe*$^{-/-}$ and *Hfe*^{C282Y} mice it did (Roy et al. 2004). Hepcidin may not appropriately respond to iron in cell culture because culture conditions do not accurately reproduce physiological conditions. Culture conditions may alter the function, interactions, or signaling of proteins upstream from hepcidin. Alternatively, the absence of proper hepcidin regulation by iron in cell culture might be due to the expression profile of the hepatic cell itself. Hepatocytes dedifferentiate after a few days in culture, and thus may cease to express proteins needed to mediate the effect of iron on hepcidin. HepG2 cells express TfR1, TfR2, Tf, Fpn1, DMT1, Ft, HFE, Hepc, Hjv, and ceruloplasmin but in some cases at markedly different levels than hepatocytes *in vivo* (Zhang et al. 2004; M.B. Johnson, unpublished results 2004). In HepG2, cells TfR2 and TfR1 transcript levels are similar, but in hepatocytes, levels of TfR2 transcript are considerably higher than levels of TfR1 transcript. Also in

HepG2 cells, expression of HFE and Hjv is low (M.B. Johnson and A.S Zhang, unpublished results 2004). Thus, though the proteins that participate in iron regulation of hepcidin are present, the extent and stoichiometry of their expression may be inappropriate.

Systemic iron levels and erythropoietic iron requirements may both regulate hepcidin. The signals indicating the status of either are not established. Possible indicators include serum transferrin saturation, serum ferritin, hepatic iron stores, circulating non-transferrin bound iron (NTBI), circulating soluble TfR1, and erythropoietin. Similarly, the molecules and mechanisms that sense the signals are not understood. Complicated cellular processes involving the interaction of HFE, Hjv and TfR2 with one or more indicators of iron level may sense iron and regulate hepcidin. Hepcidin's regulation by other stimuli, such as inflammation and hypoxia, and the need to determine the temporal sequence of responses to changes in iron complicate studies of the correlation between putative iron indicators and hepcidin levels. In rats switched to an iron-deficient diet, hepcidin levels correlate with serum transferrin saturation (Frazer et al. 2002). Importantly, a decrease in serum transferrin saturation occurs at the same time as a decrease in hepcidin synthesis and an increase in intestinal iron absorption. By contrast, hepatic and splenic iron levels do not decrease until several days after hepcidin decreases. However, in a study of human subjects that displayed no symptoms of hemochromatosis, hepcidin transcript levels correlated with serum ferritin levels but not transferrin saturation (Gehrke et al. 2003). Several outliers, though, perhaps corresponding to individuals whose hepcidin levels were elevated due to inflammation, might have masked a positive correlation between hepcidin transcript levels and serum transferrin saturation. Another study reported a correlation between urinary hepcidin excretion and serum ferritin levels (Nemeth et al. 2003). Indicators other than or in addition to serum ferritin and transferrin saturation appear to signal iron levels to hepcidin, though, since in $Hbb^{th3/+}$ mice that model β-thalassemia (see Section 7.3.1), hepcidin expression is decreased, despite iron overload.

3 Misregulation of iron: anemias

Hemoglobin has three components: iron, protoporphyrin IX, and globin. Most of the iron required by the body is needed for hemoglobin synthesis in erythroid cells. Erythroid cells acquire iron through the receptor-mediated endocytosis of transferrin, the iron transport protein in blood (Ponka 1997). After endocytosis, the acidic environment of the endosome promotes the release of iron from transferrin. Iron then crosses three membranes, the endosomal membrane and the outer and inner mitochondrial membranes, to be incorporated into protoporphyrin IX by ferrochelatase. The divalent metal transporter, DMT1, also known as NRAMP2 and DCT1, transports iron across the endosomal membrane. The mechanism of how iron is transported into the matrix of mitochondria is not known. Once in the matrix, ferrochelatase incorporates iron into protoporphyrin IX to form heme. Ferrochelatase localizes to the inner mitochondrial membrane facing the mito-

chondrial matrix (Ponka 1997). Since protoporphyrin IX is synthesized in mitochondria and globin is synthesized in cytosol, heme is transported out of the mitochondria through an uncharacterized mechanism into the cytosol and subsequently incorporated into globin, forming hemoglobin. Erythroid cells coordinate the acquisition or biosynthesis of iron, protoporphyrin IX, and globin to ensure that each component is present in stoichiometric amounts. Disturbance of any part of this ordered, biosynthetic pathway most commonly results in aberrations in iron homeostasis that manifest as hypochromic, microcytic anemias.

3.1 β-thalassemia

Human adult hemoglobin (Hb) consists of a major component, hemoglobin A (HbA), and a minor component, HbA2 (about 2.5%). Both hemoglobins are tetramers of heme-binding globin chains. HbA has two α globin and two β globin subunits ($\alpha 2\beta 2$); while HbA2 has two α globin and two δ globin subunits ($\alpha 2\delta 2$) (Ponka 1997).

β-thalassemia is characterized by defective β-globin synthesis resulting from β-globin gene mutations. Over 200 different mutations that give rise to the clinical phenotype of β-thalassemia have been identified. Based on the extent of β-globin production, the β-thalassemias are classified into two main varieties, β0 thalassemia, in which no β-globin is produced, and β+ thalassemia, in which some β chains are produced but at a reduced rate. As a result, the severity of anemia in β-thalassemia patients varies widely. The reduced rate of β-globin synthesis causes an imbalance in globin chain synthesis, which leads to the following two main consequences. First, the unpaired α chains are unable to form a viable Hb tetramer and so aggregate to form inclusion bodies in erythroid cell precursors. These inclusion bodies trigger the intramedullary destruction of erythroid precursors resulting in ineffective erythropoiesis. Second, red cells that enter into the circulation containing inclusion bodies are vulnerable to hemolysis when they pass through the microcirculation (Weatherall 2001).

Clinically, the β-thalassemias are also classified into β-thalassemia major, intermediate, and minor based on the degree of anemia. Patients with β-thalassemia major have the most severe anemia and require regular transfusion of red blood cells (RBC) starting from early childhood to enable normal growth and development and prevent congestive heart failure, severe bone deformities, and endocrinopathies. This requirement for RBC transfusion is life-long, and life expectancy is reduced regardless. Patients suffer from iron overload, which is mainly attributed to the blood transfusions required for treatment of the disease. The increased iron absorption by the intestine induced by anemia also contributes to iron overload. Patients with β-thalassemia intermediate have a milder anemia and usually do not require transfusions. However, this group of patients develops iron-overload in the absence of transfusion. β-thalassemia minor usually does not need clinical intervention (Weatherall 2001; Schrier and Angelucci 2005).

The mechanisms regulating iron absorption by the intestine are not completely understood. Recent studies have documented that hepcidin levels in the circulation principally control the rate of iron absorption. Synthesized and secreted by hepatocytes, hepcidin inhibits cellular iron efflux by binding to the iron exporter ferroportin and inducing its internalization and degradation (Nemeth et al. 2004b). A variety of stimuli regulate hepcidin mRNA levels. High iron levels and hypoxia induce hepcidin expression, whereas low iron levels and inflammation reduce hepcidin expression (Pigeon et al. 2001; Frazer et al. 2002; Nicolas et al. 2002b). Notably, injection of mice with erythropoietin (EPO) also reduces hepcidin expression (Nicolas et al. 2002c). Clinical studies have shown significantly elevated serum erythropoietin levels in both thalassemias major and intermedia (Dore et al. 1993; Chaisiripoomkere et al. 1999). Thus, in β-thalassemia two signals regulating hepcidin, high iron levels and high EPO levels, conflict. To determine if the increased intestinal iron absorption in β-thalassemia correlates with reduced hepcidin expression, urinary hepcidin levels in seven patients with β-thalassemia intermediate and eight patients with β-thalassemia major were measured. Results showed that hepcidin levels in both groups were low relative to serum ferritin levels (Papanikolaou 2005). These results are consistent with the finding that thalassemic mice have reduced levels of hepcidin mRNA (Adamsky et al. 2004). Taken together, these results indicate that the erythropoietic signal dominates over the iron stores signal in the regulation of hepcidin expression in β-thalassemias.

HFE mutations may modify the severity of iron loading in patients with β-thalassemias. The results are controversial. Two studies suggest that association of the β-thalassemic trait with the HFE H63D mutation may exacerbate iron absorption (Melis 2002; Martins et al. 2004), while other studies do not support this view (Borgna-Pignatti 1998; Piperno et al. 2000). In HFE deficient mice, iron overload is attributed to the downregulation of hepcidin gene expression (Muckenthaler et al. 2003). Constitutive overexpression of hepcidin prevents iron overload in HFE deficient mice (Nicolas et al. 2003). Therefore, we speculate that if the HFE gene is a modifier of iron overload in β-thalassemias, it will function through the regulation of hepcidin expression in the hepatocytes.

3.2 Anemia of inflammation

The anemia of inflammation (AI, also called the anemia of chronic disease) is a condition that afflicts patients with a wide variety of diseases, including infections, malignancies, and rheumatologic disorders. AI is characterized by a blunted erythropoietin response by erythroid precursors, decreased red blood cell survival, decreased iron absorption by the intestine, and increased macrophage iron retention. The latter interrupts iron delivery to erythroid precursor cells (Rivera et al. 2005).

In the past two years, a great deal of progress has been made toward understanding the mechanisms underlying AI. Several lines of evidence indicate that an increased level of hepcidin in the circulation is the key mediator of AI. The first

evidence came from a clinical study of patients with large hepatic adenomas (Weinstein et al. 2002). These patients had severe iron refractory anemia similar to that observed in AI. Surprisingly, the anemia disappeared spontaneously after adenoma resection or liver transplantation. Further analysis of the adenoma tissue by northern blot and *in situ* hybridization showed that the adenoma expressed inappropriately high levels of hepcidin mRNA, about 10 to 30 times that of unaffected liver tissues. On the basis of this finding and the role of hepcidin in iron homeostasis, the authors speculated that hepcidin is important in the pathogenesis of AI. A study using an animal model substantiated this (Rivera et al. 2005). Tumor xenografts engineered to overexpress human hepcidin or control tumor xenografts were transplanted into non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice. The mice with hepcidin producing tumors had more severe anemia, lower serum iron, and increased hepatic iron stores than mice with control tumors (Rivera et al. 2005).

Additional evidence supporting the importance of hepcidin in AI comes from a patient study (Nemeth et al. 2003). Urinary hepcidin excretion, assumed to be indicative of hepcidin production, by healthy controls or patients with transfusion-induced iron overload, compensated hereditary hemochromatosis, or iron deficiency anemia was measured and compared (Nemeth et al. 2003). Results showed that urinary excretion of hepcidin was greatly increased in patients with AI, to levels similar to those in patients with iron overload. Three separate studies using mice made similar observations. In one study, mice were injected with LPS, a classic inducer of acute phase proteins involved in the response to infection or inflammation. Hepcidin mRNA expression increased as early as 1.5 hours after injection (Roy et al. 2004). In other studies, turpentine was used to induce inflammation. After a single injection, hepcidin mRNA levels increased 6 to 12-fold and serum iron levels decreased two-fold (Nicolas et al. 2002b; Nemeth et al. 2004a). These findings support the hypothesis that hepcidin contributes to iron deficiency in AI.

Studies seeking to elucidate how inflammation induces hepcidin expression have established that interleukin-6 (IL-6) is an important mediator. First, *in vitro* studies in both human hepatocytes and Hep3B (human hepatoma) cells showed that IL-6, LPS, and conditioned medium from LPS-stimulated macrophages or Kupffer cells strongly induce hepcidin mRNA expression. Importantly, the addition of anti-IL-6 antibody to the medium blocks hepcidin induction by all these stimuli (Nemeth et al. 2003, 2004a). Second, studies in mice showed that hepcidin induction and hypoferremia during inflammation require IL-6 (Nemeth et al. 2004a). In normal mice, injection of turpentine increases the median hepcidin-1 expression in the liver by 12-fold. In *IL-6*$^{-/-}$ mice, however, hepcidin expression falls below the baseline. In the same study, the authors also demonstrated that IL-6 increases hepcidin and induces hypoferremia in humans. Two hours after infusion of IL-6 into healthy human subjects urinary hepcidin levels had increased 7.5-fold. Serum iron decreased by 34% and transferrin saturation decreased by 33% (Nemeth et al. 2004a).

One study suggests that a second interleukin, IL-1, may also regulate hepcidin in AI. IL-1 induces hepcidin transcription in murine hepatocytes to the same ex-

tent as IL-6. The hepcidin stimulatory activity of macrophages from *IL-6*$^{-/-}$ mice is attributable to IL-1. Furthermore, hepatocytes from *IL-6*$^{-/-}$ mice, *Hfe*$^{-/-}$ mice, and *TfR2*^{Y250X} mice upregulate hepcidin transcript when stimulated with either IL-6 or IL-1 (Lee et al. 2005).

Taken together, the above findings are consistent with the model proposed by Fleming and Sly (Fleming and Sly 2001b) in which hepcidin is an iron-regulatory hormone whose misregulation underlies the anemia of chronic disease.

3.3 Inherited sideroblastic anemias

Sideroblastic anemias are a heterogeneous group of acquired and inherited bone marrow disorders characterized by the presence of pathologic iron deposits in erythroblast mitochondria. The acquired sideroblastic anemias account for the majority of cases and are classified clinically as myelodysplastic syndromes (MDS) (Fleming 2002). MDS are a heterogeneous spectrum of stem cell malignancies whose molecular pathogenesis is unknown. The majority of patients succumb to complications of bone marrow failure (List 2004). The inherited sideroblastic anemias are relatively rare. They include X-linked sideroblastic anemia (XLSA), XLSA with ataxia (XLSA/A), mitochondrial myopathy and sideroblastic anemia (MSA), erythropoietic protoporphyria (EPP), and Pearson marrow/pancreas syndrome (PMPS) (List et al. 2004). The molecular bases of this group of disorders are relatively well characterized.

3.3.1 X-linked sideroblastic anemia

Heme biosynthesis occurs in all cells, whereas hemoglobin synthesis occurs exclusively in developing erythroid cells. Since as much as three quarters of total body iron is in hemoglobin, the process of heme synthesis is extremely important in developing erythroid cells (Fleming 2002). Heme biosynthesis involves eight steps, each catalyzed by a distinct enzyme. The first two steps and the last two steps occur within mitochondria, whereas the other four steps occur in the cytosol. The first seven steps generate protoporphyrin IX. The final step inserts one atom of Fe^{2+} into protoporphyrin IX through the activity of the inner mitochondrial membrane-associated enzyme, ferrochelatase. XLSA results from mutations of δ-aminolevulinic acid synthase 2 (ALAS2), the first enzyme in the heme biosynthetic pathway in erythroid cells (Ponka 1997). ALAS has two isoforms, ALAS1 and ALAS2, encoded by two distinct genes. ALAS1 is encoded on chromosome 3p21and is the housekeeping gene, expressed in all tissues. ALAS2 is encoded on Xp11.21 and is solely expressed in erythroid precursors (Ponka 1997). ALAS is located in the mitochondrial inner membrane facing the mitochrondrial matrix. It functions to catalyze the condensation of glycine and succinyl coenzyme A to form δ-aminolevulinic acid (ALA) (Ponka 1997).

Currently, there are more than twenty mutations in the coding region and at least one mutation in the promoter region of *ALAS2* that cause XLSA. The former mutations tend to cluster within exons 5 and 9 of the *ALAS2* gene (Fleming 2002;

Bekri et al. 2003; Furuyama 2003; Collins 2004). The essential features of XLSA include: a hypochromic microcytic anemia, often with Pappenheimer bodies (iron-positive erythrocytic inclusions) and two discrete populations of red blood cells, one microcytic and the other normocytic (erythrocyte dimorphism); an X-linked pattern of inheritance; ringed sideroblasts in the bone marrow that are particularly prominent in the late erythroid precursors; a variable hematological response to pharmacologic doses of pyridoxine; and systemic iron overload secondary to chronic ineffective erythropoiesis (Fleming 2002). Excess iron deposits in mitochondria in the form of mitochondrial ferritin (Levi 2001). Mitochondrial ferritin is highly expressed in sideroblasts of patients with XLSA but not in normal erythroblasts (Levi 2001; Cazzola et al. 2003), indicating that the expression of mitochondrial ferritin is induced by iron loading into mitochondria.

Heme biosynthesis is strictly dependent upon iron supply. TfR1-mediated iron uptake is the major physiological pathway by which erythroid cells acquire iron (Ponka 1997). After iron dissociates from transferrin in the acidic endosomal lumen, the ferric iron is reduced to ferrous iron by an uncharacterized mechanism and then transported out of endosome through the iron transporter, DMT1 (Canonne-Hergaux et al. 1999). The mechanism by which iron reaches mitochondria after export from the endosome has not been established. Recent data are consistent with the hypothesis that the endosomal membrane contacts the mitochondrial outer membrane allowing iron to pass directly from DMT1 to the mitochondrion (Ponka 1997; Zhang 2005). How iron translocates across two mitochondrial membranes to reach ferrochelatase is still a mystery. The fact that mitochondrial iron loading occurs in erythroid cells in patients with XLSA suggests that iron continues to enter mitochondria despite the lack of heme precursor protoporphyrin IX. This assumption is supported by *in vitro* studies in reticulocytes. When inhibitors of ALAS or ALA dehydratase, the second enzyme in the heme biosynthetic pathway, are used to block heme biosynthesis, iron continues to accumulate in mitochondria at a constant rate (Ponka 1982; Richardson 1996; Zhang 2005). This indicates that in erythroid cells mitochondrial iron uptake is not coupled to the rate of protoporphyrin IX synthesis.

Interestingly, systemic iron overload is a common complication of XLSA, even in the absence of transfusion therapy. Although hepcidin levels in patients with XLSA have not been reported, we speculate that iron overload is a result of the anemia, which suppresses hepcidin expression in hepatocytes and consequently increases iron absorption from intestine. This is consistent with other anemias that are not caused by dietary iron deficiency.

3.3.2 X-linked sideroblastic anemia with ataxia

XLSA/A is a rare, recessive form of inherited sideroblastic anemia characterized by infantile to early onset of non-progressive cerebellar ataxia and mild anemia with hypochromia and microcytosis. The peripheral blood and bone marrow findings resemble mild XLSA. Bone marrow analysis shows ringed sideroblasts (Allikmets et al. 1999; Fleming 2002). In contrast to ALAS2-related XLSA, the XLSA/A syndrome has elevated free erythrocyte protoporphyrin levels and lacks

excessive parenchymal iron deposition. We speculate that the lack of systemic iron overload is due to the relatively mild nature of the anemia and its presentation early in life. The serum transferrin saturation and serum ferritin levels are always nearly normal. The anemia is not responsive to pyridoxine treatment (Allikmets et al. 1999; Fleming 2002). Importantly, XLSA/A has no defect in the *ALAS2* gene. Instead, XLSA/A is caused by mutation of a gene on the long arm of the X chromosome (Xq13) (Raskind 1991). The gene was cloned and named *ABCB7* (ATP-binding cassette, sub-family B, member 7) (Csere 1998; Allikmets et al. 1999). Subsequent mutation analysis in XLSA/A patients identified missense mutations in ABC7 (Fleming 2002).

The *ABC7* gene is an orthologue of the yeast *ATM1* (ataxia telangiectasia mutated) gene, whose product localizes to the mitochondrial inner membrane and is involved in iron homeostasis (Csere 1998; Allikmets et al. 1999). In yeast, deletion of *ATM1* (Δatm1) results in mitochondrial iron overload and a selective deficiency in cytoplasmic, but not mitochondrial, enzymes containing iron sulfur (Fe-S) clusters (Kispal 1997, 1999). Expression of wild type ABC7 almost fully complements the defect in the maturation of cytosolic Fe-S proteins in Δatm1 yeast. In contrast, introduction of mutated ABC7 or Atm1p proteins in Δatm1 yeast leads to a low efficiency of cytosolic Fe-S protein maturation (Csere 1998; Allikmets et al. 1999; Bekri et al. 2000). Thus, ABC7 is a functional orthologue of Atm1p and plays a critical role in the maturation of Fe-S proteins.

The mild hypochromic microcytic anemia in patients with XLSA/A suggests a defect in heme synthesis in developing erythroid cells. Obviously, ABC7 plays an important role in this process. However, the presence of ringed sideroblasts excludes a possible defect in iron transport into mitochondria, and the elevated free protoporphyrin in bone marrow erythrocytes excludes a possible defect in the generation of protoporphyrin. Therefore, it is reasonable to speculate that ABC7 protein is involved in the last step of heme biosynthesis in mitochondria, the insertion of ferrous ion into protoporphyrin IX by ferrochelatase. This step couples iron uptake and protoporphyrin synthesis. The activity of ferrochelatase in XLSA/A patients has not been reported. Δatm1 yeast express ferrochelatase normally. Yeast ferrochelatase, however, does not contain a [2Fe-2S] cluster at its carboxy-terminus as the mammalian ferrochelatase does (Furukawa 1995). Moreover, in yeast, Atm1p is only involved in the maturation of cytoplasmic, not mitochondrial, enzymes containing Fe-S clusters (Kispal 1997, 1999). Therefore, defects in Atm1p or ABC7 may not affect ferrochelatase similarly in yeast and humans. Interestingly, a more recent study using mouse erythroleukemia (MEL) cells demonstrated the involvement of ABC7 in the biosynthesis of heme via interaction with ferrochelatase (Taketani 2003). In that study, the authors showed that when MEL cells are induced to erythroid differentiation by DMSO, ABC7 mRNA and ferrochelatase mRNA levels increase markedly in parallel. An *in vitro* pull down assay using anti-ferrochelatase antibody revealed that ABC7 protein interacts with the carboxy-terminal region containing the Fe-S cluster of ferrochelatase. Introduction of antisense oligonucleotides targeting mouse ABC7 mRNA decreases heme production in DMSO treated MEL cells. Conversely, overexpression of ABC7 increases ferrochelatase activity threefold and increases heme levels 2.5-fold over

control cells. This indicates that ABC7 positively regulates ferrochelatase activity to promote the production of heme during the differentiation of erythroid cells (Taketani 2003). This study, therefore, suggests that mutations of the ABC7 gene in XLSA/A disrupt its interaction with ferrochelatase and thereby impede the process of heme biosynthesis. These findings need to be confirmed in humans.

3.4 Anemia due to mutation of DMT1

DMT1 is a transmembrane protein with twelve predicted transmembrane domains. It is critical for duodenal iron absorption and erythroid iron transport. DMT1 is expressed at the brush border of enterocytes in the proximal duodenum where it is presumed to mediate pH-dependent uptake of ferrous iron from the intestinal lumen. In the erythroblast, DMT1 is localized to the endosomal membrane where it is presumed to transport the iron released from transferrin out of the endosome for subsequent transport via an undefined pathway into the mitochondria for heme biosynthesis (Gunshin et al. 1997; Canonne-Hergaux et al. 1999; Canonne-Hergaux et al. 2001). There are four major mammalian DMT1 isoforms that result from alternative splicing at the 5' and 3' ends of the pre-mRNA (Hubert and Hentze 2002). Two isoforms have iron responsive elements (IRE) in their 3' untranslated regions, and the other two isoforms lack IREs (Lee et al. 1998; Hubert and Hentze 2002). The duodenum predominantly expresses the IRE forms, whereas erythroblast mainly expresses the non-IRE forms (Canonne-Hergaux et al. 1999, 2001).

There are two animal models, the microcytic anemia (*mk*) mouse and the Belgrade rat, that carry the same natural missense mutation, G185R, in DMT1 (Fleming et al. 1997, 1998). Homozygous mutant animals have diminished intestinal iron absorption and severe anemia. This is at least partially due to defective targeting of the mutated DMT1 protein (Touret et al. 2004). *In vitro* studies demonstrated significantly decreased rates of iron uptake from transferrin and of heme biosynthesis in reticulocytes from both homozygous animals. These decreases could be corrected by addition of iron-saturated salicylaldehyde isonicotinoyl hydrazone (Fe-SIH) (Garrick et al. 1991; Canonne-Hergaux et al. 2001). Fe-SIH is able to bypass the TfR/DMT1 iron transport pathway to deliver iron to cells. These findings indicate that DMT1 plays a crucial role in erythroid iron uptake.

Recently, the first case of an anemic patient with a mutation in DMT1 was reported (Priwitzerova et al. 2004; Mims et al. 2005). The patient first presented at the age of 3 months with severe hypochromic, microcytic anemia with erythroid hyperplasia and high serum iron levels. The patient received, on average, one transfusion per year. At the age of 19, the liver biopsy showed significantly increased iron deposition in both Kupffer cells and hepatocytes. Thalassemia, TfR1 mutation, and ferroportin mutation were excluded. Analysis of the DMT1 gene sequences revealed that the patient is homozygous for a G1285C mutation. The mutation affects the final nucleotide of exon 12 (DMT1) and leads to a conservative E399D amino acid substitution in the protein. During pre-mRNA processing, the mutation causes preferential skipping of exon 12. Intestinal cells and erythroid

precursors consequently lack full length DMT1 mRNA. DMT1 protein is present in the patient's duodenum at a level equal to or higher than in wild type individuals (Mims et al. 2005).

The severe hypochromic microcytic anemia observed in the patient with a mutation in DMT1 is consistent with the phenotypes of the Belgrade rat and *mk* mouse. Though information on the targeting and processing of the mutated DMT1 in the patient is lacking, it is easily envisioned how the absence of full-length DMT1 could cause severe hypochromic microcytic anemia. The iron overload in the liver of the patient, occurring prior to transfusion, is puzzling, though, since this phenotype is absent from the Belgrade rat and *mk* mouse. The authors propose that iron overload develops as a consequence of either: (1) decreased hepcidin levels; (2) absorption of heme iron; or (3) increased iron absorption across the basolateral surface of the enterocytes (Mims et al. 2005). The recent elucidation of the mechanism by which hepcidin regulates intestinal iron absorption (Nemeth et al. 2004b) allows the first and the third possibilities to be combined. One explanation for the phenotypic discrepancy between the patient and the animal models incorporates the contributions of both heme and hepcidin. Rats and mice absorb heme iron poorly. The absence of functional DMT1 severely diminishes their ability to take up dietary iron. The reduced expression of hepcidin is irrelevant since there is little iron in the intestine available to transport into the circulation through Fpn1. By contrast, in humans who eat red meat an estimated two-thirds of the iron absorbed from the diet derives from heme. In the absence of functional DMT1, the patient is able to take up iron through heme. Since the anemia suppresses the synthesis of hepcidin and its downregulation of Fpn1, the body absorbs high levels of this iron. Given that in many cases of anemia the underlying cause is not characterized, we speculate that there might be many more patients whose anemia is due to mutations in DMT1.

References

Abboud S, Haile DJ (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Biol Chem 275:19906-19912

Adamsky K, Weizer O, Amariglio N, Breda L, Harmelin A, Rivella S, Rachmilewitz E, Rechavi G (2004) Decreased hepcidin mRNA expression in thalassemic mice. Br J Haematol 124:123-124

Ahmad KA, Ahmann JR, Migas MC, Waheed A, Britton RS, Bacon BR, Sly WS, Fleming RE (2002) Decreased liver hepcidin expression in the hfe knockout mouse. Blood Cells Mol Dis 29:361-366

Ajioka RS, Kushner JP (2003) Clinical consequences of iron overload in hemochromatosis homozygotes. Blood 101:3351-3358

Allikmets R, Raskind W, Hutchinson A, Schueck N, Dean M, Koeller D (1999) Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). Hum Mol Genet 8:743-749

Andrews NC (1999) Disorders of iron metabolism. N Engl J Med 341:1986-1995

Arden KE, Wallace DF, Dixon JL, Summerville L, Searle JW, Anderson GJ, Ramm GA, Powell LW, Subramaniam VN (2003) A novel mutation in ferroportin1 is associated with haemochromatosis in a Solomon Islands patient. Gut 52:1215-1217

Bahram S, Gilfillan S, Kuhn LC, Moret R, Schulze JB, Lebeau A, Schumann K (1999) Experimental hemochromatosis due to MHC class I HFE deficiency: immune status and iron metabolism. Proc Natl Acad Sci USA 96:13312-13317

Barton JC, Acton RT, Rivers CA, Bertoli LF, Gelbart T, West C, Beutler E (2003) Genotypic and phenotypic heterogeneity of African Americans with primary iron overload. Blood Cells Mol Dis 31:310-319

Bekri S, Kispal G, Lange H, Fitzsimons E, Tolmie J, Lill R, Bishop DF (2000) Human ABC7 transporter: gene structure and mutation causing X-linked sideroblastic anemia with ataxia with disruption of cytosolic iron-sulfur protein maturation. Blood 96:3256-3264

Bekri S, May A, Cotter PD, Al-Sabah AI, Guo X, Masters GS, Bishop DF (2003) A promoter mutation in the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene causes X-linked sideroblastic anemia. Blood 102:698-704

Beutler E (2003) The HFE Cys282Tyr mutation as a necessary but not sufficient cause of clinical hereditary hemochromatosis. Blood 101:3347-3350

Beutler E, Barton JC, Felitti VJ, Gelbart T, West C, Lee PL, Waalen J, Vulpe C (2003) Ferroportin 1 (SCL40A1) variant associated with iron overload in African-Americans. Blood Cells Mol Dis 31:305-309

Borgna-Pignatti C, Solinas A, Bombieri C, Micciolo R, Gamberini MR, De Stefano P, De Menis E, Pignatti PF (1998) The haemochromatosis mutations do not modify the clinical picture of thalassaemia major in patients regularly transfused and chelated. Br J Haematol 103:813-816

Borot N, Roth M, Malfroy L, Demangel C, Vinel JP, Pascal JP, Coppin H (1997) Mutations in the MHC class I-like candidate gene for hemochromatosis in French patients. Immunogenetics 45:320-324

Camaschella C, Fargion S, Sampietro M, Roetto A, Bosio S, Garozzo G, Arosio C, Piperno A (1999) Inherited HFE-unrelated hemochromatosis in Italian families. Hepatology 29:1563-1564

Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, Majorano N, Totaro A, Gasparini P (2000) The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. Nat Genet 25:14-15

Canonne-Hergaux F, Gruenheid S, Ponka P, Gros P (1999) Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. Blood 93:4406-4417

Canonne-Hergaux F, Zhang AS, Ponka P, Gros P (2001) Characterization of the iron transporter DMT1 (NRAMP2/DCT1) in red blood cells of normal and anemic mk/mk mice. Blood 98:3823-3830

Carella M, D'Ambrosio L, Totaro A, Grifa A, Valentino MA, Piperno A, Girelli D, Roetto A, Franco B, Gasparini P, Camaschella C (1997) Mutation analysis of the HLA-H gene in Italian hemochromatosis patients. Am J Hum Genet 60:828-832

Cazzola M, Cremonesi L, Papaioannou M, Soriani N, Kioumi A, Charalambidou A, Paroni R, Romtsou K, Levi S, Ferrari M, Arosio P, Christakis J (2002) Genetic hyperferritinaemia and reticuloendothelial iron overload associated with a three base pair deletion in the coding region of the ferroportin gene (SLC11A3). Br J Haematol 119:539-546

Cazzola M, Invernizzi R, Bergamaschi G, Levi S, Corsi B, Travaglino E, Rolandi V, Biasiotto G, Drysdale J, Arosio P (2003) Mitochondrial ferritin expression in erythroid cells from patients with sideroblastic anemia. Blood 101:1996-2000

Chaisiripoomkere W, Jootar S, Chanjarunee S, Ungkanont A (1999) Serum erythropoietin levels in thalassemia major and intermedia. Southeast Asian J Trop Med Public Health 30:786-788

Collins TS, Arcasoy MO (2004) Iron overload due to X-linked sideroblastic anemia in an African American man. Am J Med Genet 116:501-502

Courselaud B, Pigeon C, Inoue Y, Inoue J, Gonzalez FJ, Leroyer P, Gilot D, Boudjema K, Guguen-Guillouzo C, Brissot P, Loreal O, Ilyin G (2002) C/EBPalpha regulates hepatic transcription of hepcidin, an antimicrobial peptide and regulator of iron metabolism. Cross-talk between C/EBP pathway and iron metabolism. J Biol Chem 277:41163-41170

Csere P, Lill R, Kispal, G (1998) Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast Atm1p. FEBS Lett 441:266-270

Davies PS, Zhang AS, Anderson EL, Roy CN, Lampson MA, McGraw TE, Enns CA (2003) Evidence for the interaction of the hereditary haemochromatosis protein, HFE, with the transferrin receptor in endocytic compartments. Biochem J 373:145-153

Deaglio S, Capobianco A, Cali A, Bellora F, Alberti F, Righi L, Sapino A, Camaschella C, Malavasi F (2002) Structural, functional, and tissue distribution analysis of human transferrin receptor-2 by murine monoclonal antibodies and a polyclonal antiserum. Blood 100:3782-3789

Delatycki MB, Allen KJ, Gow P, MacFarlane J, Radomski C, Thompson J, Hayden MR, Goldberg YP, Samuels ME (2004) A homozygous HAMP mutation in a multiply consanguineous family with pseudo-dominant juvenile hemochromatosis. Clin Genet 65:378-383

Devalia V, Carter K, Walker AP, Perkins SJ, Worwood M, May A, Dooley JS (2002) Autosomal dominant reticuloendothelial iron overload associated with a 3-base pair deletion in the ferroportin 1 gene (SLC11A3). Blood 100:695-697

Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, Law TC, Brugnara C, Lux SE, Pinkus GS, Pinkus JL, Kingsley PD, Palis J, Fleming MD, Andrews NC, Zon LI (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature 403:776-781

Dore F, Bonfigli S, Gaviano E, Pardini S, Cianciulli P, Papa G, Longinotti M (1993) Serum erythropoietin levels in thalassemia intermedia. Ann Hematol 67:183-186

Drakesmith H, Sweetland E, Schimanski L, Edwards J, Cowley D, Ashraf M, Bastin J, Townsend AR (2002) The hemochromatosis protein HFE inhibits iron export from macrophages. Proc Natl Acad Sci U S A 99:15602-15607

Edwards CQ, Griffen LM, Goldgar D, Drummond C, Skolnick MH, Kushner JP (1988) Prevalence of hemochromatosis among 11,065 presumably healthy blood donors. N Engl J Med 318:1355-1362

Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo RJ, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prasss CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 13:399-408

Feder JN, Penny DM, Irrinki A, Lee VK, Lebron JA, Watson N, Tsuchihashi Z, Sigal E, Bjorkman PJ, Schatzman RC (1998) The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. Proc Natl Acad Sci USA 95:1472-1477

Feder JN, Tsuchihashi Z, Irrinki A, Lee VK, Mapa FA, Morikang E, Prass CE, Starnes SM, Wolff RK, Parkkila S, Sly WS, Schatzman RC (1997) The hemochromatosis founder mutation in HLA-H disrupts β2-microglobulin interaction and cell surface expression. J Biol Chem 272:14025-14028

Fleming MD (2002) The genetics of inherited sideroblastic anemias. Semin Hematol 39:270-281

Fleming MD, Romano MA, Su MA, Garrick LM, Garrick MD, Andrews NC (1998) Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. Proc Natl Acad Sci USA 95:1148-1153

Fleming MD, Trenor CC, Su MA, Foernzler D, Beier DR, Dietrich WF, Andrews NC (1997) Microcytic anaemia mice have a mutation in nramp2, a candidate iron transporter gene. Nat Genet 16:383-386

Fleming RE, Ahmann JR, Migas MC, Waheed A, Koeffler HP, Kawabata H, Britton RS, Bacon BR, Sly WS (2002) Targeted mutagenesis of the murine transferrin receptor-2 gene produces hemochromatosis. Proc Natl Acad Sci USA 99:10653-10658

Fleming RE, Migas MC, Holden CC, Waheed A, Britton RS, Tomatsu S, Bacon BR, Sly WS (2000) Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. Proc Natl Acad Sci USA 97:2214-2219

Fleming RE, Sly WS (2001a) Ferroportin mutation in autosomal dominant hemochromatosis: loss of function, gain in understanding. J Clin Invest 108:521-522

Fleming RE, Sly WS (2001b) Hepcidin: A putative iron-regulatory hormone relevant to hereditary hemochromatosis and the anemia of chronic disease. Proc Natl Acad Sci USA 98:8160-8162

Fletcher LM, Halliday JW (2002) Haemochromatosis: Understanding the mechanism of disease and implications for diagnosis and patient management following the recent cloning of novel genes involved in iron metabolism. J Intern Med 251:181-192

Frazer DM, Wilkins SJ, Becker EM, Vulpe CD, McKie AT, Trinder D, Anderson GJ (2002) Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats. Gastroenterology 123:835-844

Frazer DM, Wilkins SJ, Millard KN, McKie AT, Vulpe CD, Anderson GJ (2004) Increased hepcidin expression and hypoferraemia associated with an acute phase response are not affected by inactivation of HFE. Br J Haematol 126:434-436

Furukawa T, Kohno H, Tokunaga R, Taketani S (1995) Nitric oxide-mediated inactivation of mammalian ferrochelatase *in vivo* and *in vitro*: possible involvement of the iron-sulphur cluster of the enzyme. Biochem J 310:533-538

Furuyama K, Harigae H, Kinoshita C, Shimada T, Miyaoka K, Kanda C, Maruyama Y, Shibahara S, Sassa S (2003) Late-onset X-linked sideroblastic anemia following hemodialysis. Blood 101:4623-4624

Garrick LM, Gniecko K, Hoke JE, al-Nakeeb A, Ponka P, Garrick MD (1991) Ferric-salicylaldehyde isonicotinoyl hydrazone, a synthetic iron chelate, alleviates defective iron utilization by reticulocytes of the Belgrade rat. J Cell Physiol 146:460-465

Gehrke SG, Kulaksiz H, Herrmann T, Riedel HD, Bents K, Veltkamp C, Stremmel W (2003) Expression of hepcidin in hereditary hemochromatosis: evidence for a regula-

tion in response to the serum transferrin saturation and to non-transferrin-bound iron. Blood 102:371-376

Girelli D, Bozzini C, Roetto A, Alberti F, Daraio F, Colombari R, Olivieri O, Corrocher R, Camaschella C (2002) Clinical and pathologic findings in hemochromatosis type 3 due to a novel mutation in transferrin receptor 2 gene. Gastroenterology 122:1295-1302

Gordeuk VR, Caleffi A, Corradini E, Ferrara F, Jones RA, Castro O, Onyekwere O, Kittles R, Pignatti E, Montosi G, Garuti C, Gangaidzo IT, Gomo ZA, Moyo VM, Rouault TA, MacPhail P, Pietrangelo A (2003) Iron overload in Africans and African-Americans and a common mutation in the SCL40A1 (ferroportin 1) gene. Blood Cells Mol Dis 31:299-304

Gross CN, Irrinki A, Feder JN, Enns CA (1998) Co-trafficking of HFE, a nonclassical major histocompatibility complex class I protein, with the transferrin receptor implies a role in intracellular iron regulation. J Biol Chem 273:22068-22074

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388:482-488

Haile DJ (2000) Assignment of Slc11a3 to mouse chromosome 1 band 1B and SLC11A3 to human chromosome 2q32 by *in situ* hybridization. Cytogenet Cell Genet 88:328-329

Harris ZL, Durley AP, Man TK, Gitlin JD (1999) Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci USA 96:10812-10817

Holmstrom P, Dzikaite V, Hultcrantz R, Melefors O, Eckes K, Stal P, Kinnman N, Smedsrod B, Gafvels M, Eggertsen G (2003) Structure and liver cell expression pattern of the HFE gene in the rat. J Hepatol 39:308-314

Huang FW, Rubio-Aliaga I, Kushner JP, Andrews NC, Fleming MD (2004) Identification of a novel mutation (C321X) in HJV. Blood 104:2176-2177

Hubert N, Hentze MW (2002) Previously uncharacterized isoforms of divalent metal transporter (DMT)- 1: implications for regulation and cellular function. Proc Natl Acad Sci USA 99:12345-12350

Hunter HN, Fulton DB, Ganz T, Vogel HJ (2002) The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. J Biol Chem 277:37597-37603

Ikuta K, Fujimoto Y, Suzuki Y, Tanaka K, Saito H, Ohhira M, Sasaki K, Kohgo Y (2000) Overexpression of hemochromatosis protein, HFE, alters transferrin recycling process in human hepatoma cells. Biochimica Biophysica Acta 1496:221-231

Ilyin G, Courselaud B, Troadec MB, Pigeon C, Alizadeh M, Leroyer P, Brissot P, Loreal O (2003) Comparative analysis of mouse hepcidin 1 and 2 genes: evidence for different patterns of expression and co-inducibility during iron overload. FEBS Lett 542:22-26

Johnson MB, Enns CA (2004) Diferric transferrin regulates transferrin receptor 2 protein stability. Blood 104:4287-4293

Jouanolle AM, Douabin-Gicquel V, Halimi C, Loreal O, Fergelot P, Delacour T, de Lajarte-Thirouard AS, Turlin B, Le Gall JY, Cadet E, Rochette J, David V, Brissot P (2003) Novel mutation in ferroportin 1 gene is associated with autosomal dominant iron overload. J Hepatol 39:286-289

Kaplan J (2002) Mechanisms of cellular iron acquisition: another iron in the fire. Cell 111:603-606

Kawabata H, Fleming RE, Gui D, Moon SY, Saitoh T, O'Kelly J, Umehara Y, Wano Y, Said JW, Koeffler HP (2005) Expression of hepcidin is downregulated in TfR2 mutant mice manifesting a phenotype of hereditary hemochromatosis. Blood 105:376-381

Kawabata H, Germain RS, Ikezoe T, Tong X, Green EM, Gombart AF, Koeffler HP (2001a) Regulation of expression of murine transferrin receptor 2. Blood 98:1949-1954

Kawabata H, Germain RS, Vuong PT, Nakamaki T, Said JW, Koeffler HP (2000) Transferrin receptor 2-alpha supports cell growth both in iron-chelated cultured cells and *in vivo*. J Biol Chem 275:16618-16625

Kawabata H, Nakamaki T, Ikonomi P, Smith RD, Germain RS, Koeffler HP (2001b) Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells. Blood 98:2714-2719

Kawabata H, Tong X, Kawanami T, Wano Y, Hirose Y, Sugai S, Koeffler HP (2004) Analyses for binding of the transferrin family of proteins to the transferrin receptor 2. Br J Haematol 127:464-473

Kawabata H, Yang R, Hirama T, Vuong PT, Kawano S, Gombart AF, Koeffler HP (1999) Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. J Biol Chem 274:20826-20832

Kispal G, Csere P, Guiard B, Lill R (1997) The ABC transporter Atm1p is required for mitochondrial iron homeostasis. FEBS Lett 418:346-350

Kispal G, Csere P, Prohl C, Lill R (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. EMBO J 18:3981-3989

Knutson MD, Oukka M, Koss LM, Aydemir F, Wessling-Resnick M (2005) Iron release from macrophages after erythrophagocytosis is upregulated by ferroportin 1 overexpression and down-regulated by hepcidin. Proc Natl Acad Sci USA 102:1324-1328

Knutson MD, Vafa MR, Haile DJ, Wessling-Resnick M (2003) Iron loading and erythrophagocytosis increase ferroportin 1 (FPN1) expression in J774 macrophages. Blood 102:4191-4197

Koyama C, Wakusawa S, Hayashi H, Suzuki R, Yano M, Yoshioka K, Kozuru M, Takayamam Y, Okada T, Mabuchi H (2005) Two novel mutations, L490R and V561X, of the transferrin receptor 2 gene in Japanese patients with hemochromatosis. Haematologica 90:302-307

Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K (2000) LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 480:147-150

Krijt J, Vokurka M, Chang KT, Necas E (2004) Expression of Rgmc, the murine ortholog of hemojuvelin gene, is modulated by development and inflammation, but not by iron status or erythropoietin. Blood 104:4308-4310

Kulaksiz H, Gehrke SG, Janetzko A, Rost D, Bruckner T, Kallinowski B, Stremmel W (2004) Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut 53:735-743

Kulaksiz H, Theilig F, Bachmann S, Gehrke SG, Rost D, Janetzko A, Cetin Y, Stremmel W (2005) The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. J Endocrinol 184:361-370

Lanzara C, Roetto A, Daraio F, Rivard S, Ficarella R, Simard H, Cox TM, Cazzola M, Piperno A, Gimenez-Roqueplo AP, Grammatico P, Volinia S, Gasparini P, Cam-

aschella C (2004) Spectrum of hemojuvelin gene mutations in 1q-linked juvenile hemochromatosis. Blood 103:4317-4321

Le Gac G, Mons F, Jacolot S, Scotet V, Ferec C, Frebourg T (2004) Early onset hereditary hemochromatosis resulting from a novel TFR2 gene nonsense mutation (R105X) in two siblings of north French descent. Br J Haematol 125:674-678

Lebron JA, Bennett MJ, Vaughn DE, Chirino AJ, Snow PM, Mintier GA, Feder JN, Bjorkman PJ (1998) Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. Cell 93:111-123

Lebron JA, West AP Jr, Bjorkman PJ (1999) The hemochromatosis protein HFE competes with transferrin for binding to the transferrin receptor. J Mol Biol 294:239-245

Lee P, Peng H, Gelbart T, Beutler E (2004a) The IL-6- and lipopolysaccharide-induced transcription of hepcidin in HFE-, transferrin receptor 2-, and beta 2-microglobulin-deficient hepatocytes. Proc Natl Acad Sci USA 101:9263-9265

Lee P, Peng H, Gelbart T, Wang L, Beutler E (2005) Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proc Natl Acad Sci USA 102:1906-1910

Lee PL, Barton JC, Brandhagen D, Beutler E (2004b) Hemojuvelin (HJV) mutations in persons of European, African-American, and Asian ancestry with adult onset haemochromatosis. Br J Haematol 127:224-229

Lee PL, Beutler E, Rao SV, Barton JC (2004c) Genetic abnormalities and juvenile hemochromatosis: mutations of the HJV gene encoding hemojuvelin. Blood 103:4669-4671

Lee PL, Gelbart T, West C, Halloran C, Beutler E (1998) The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. Blood Cells Mol Dis 24:199-215

Levi S, Corsi B, Bosisio M, Invernizzi R, Volz A, Sanford D, Arosio P, Drysdale J (2001) A human mitochondrial ferritin encoded by an intronless gene. J Biol Chem 276:24437-24440

Levy JE, Jin O, Fujiwara Y, Kuo F, Andrews NC (1999a) Transferrin receptor is necessary for development of erythrocytes and the nervous system. Nat Genet 21:396-399

Levy JE, Montross LK, Cohen DE, Fleming MD, Andrews NC (1999b) The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. Blood 94:9-11

List A, Vardiman J, Issa JP, Dewitte TM (2004) Myelodysplastic syndromes. Hematology (Am Soc Hematol Educ Program):297-317

List AF, Vardiman J, Issa JP, Dewitte TM (2004) Myelodysplastic syndromes. Hematology (Am Soc Hematol Educ Program):297-317

Martins R, Picanco I, Fonseca A, Ferreira L, Rodrigues O, Coelho M, Seixas T, Miranda A, Nunes B, Costa L, Romao L, Faustino P (2004) The role of HFE mutations on iron metabolism in beta-thalassemia carriers. J Hum Genet 49:651-655

Matthes T, Aguilar-Martinez P, Pizzi-Bosman L, Darbellay R, Rubbia-Brandt L, Giostra E, Michel M, Ganz T, Beris P (2004) Severe hemochromatosis in a Portuguese family associated with a new mutation in the 5'-UTR of the HAMP gene. Blood 104:2181-2183

Mattman A, Huntsman D, Lockitch G, Langlois S, Buskard N, Ralston D, Butterfield Y, Rodrigues P, Jones S, Porto G, Marra M, De Sousa M, Vatcher G (2002) Transferrin receptor 2 (TfR2) and HFE mutational analysis in non-C282Y iron overload: identification of a novel TfR2 mutation. Blood 100:1075-1077

Mazur A, Feillet-Coudray C, Romier B, Bayle D, Gueux E, Ruivard M, Coudray C, Rayssiguier Y (2003) Dietary iron regulates hepatic hepcidin 1 and 2 mRNAs in mice. Metabolism 52:1229-1231

McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F, Simpson RJ (2001) An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 291:1755-1759

McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 5:299-309

Melis M, Cau M, Deidda F, Barella S, Cao A, Galanello R (2002) H63D mutation in the HFE gene increases iron overload in beta-thalassemia carriers. Haematologica 87:242-245

Mims MP, Guan Y, Pospisilova D, Priwitzerova M, Indrak K, Ponka P, Divoky V, Prchal JT (2005) Identification of a human mutation of DMT1 in a patient with microcytic anemia and iron overload. Blood 105:1337-1342

Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Pietrangelo A (2001) Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. J Clin Invest 108:619-623

Montosi G, Paglia P, Garuti C, Guzman CA, Bastin JM, Colombo MP, Pietrangelo A (2000) Wild-type HFE protein normalizes transferrin iron accumulation in macrophages from subjects with hereditary hemochromatosis. Blood 96:1125-1129

Muckenthaler M, Roy CN, Custodio AO, Minana B, DeGraaf J, Montross LK, Andrews NC, Hentze MW (2003) Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. Nat Genet 34:102-107

Mukhopadhyay CK, Attieh ZK, Fox PL (1998) Role of ceruloplasmin in cellular iron uptake. Science 279:714-717

Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T (2004a) IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 113:1271-1276

Nemeth E, Roetto A, Garozzo G, Ganz T, Camaschella C (2005) Hepcidin is decreased in TFR2 hemochromatosis. Blood 105:1803-1806

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J (2004b) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306:2090-2093

Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T (2003) Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 101:2461-2463

Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, Vaulont S (2001) Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc Natl Acad Sci USA 98:8780-8785

Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, Sirito M, Sawadogo M, Kahn A, Vaulont S (2002a) Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc Natl Acad Sci USA 99:4596-4601

Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S (2002b) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 110:1037-1044

Nicolas G, Viatte L, Bennoun M, Beaumont C, Kahn A, Vaulont S (2002c) Hepcidin, a new iron regulatory peptide. Blood Cells Mol Dis 29:327-335

Nicolas G, Viatte L, Lou DQ, Bennoun M, Beaumont C, Kahn A, Andrews NC, Vaulont S (2003) Constitutive hepcidin expression prevents iron overload in a mouse model of hemochromatosis. Nat Genet 34:97-101

Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JW, Breuning MH, Snijders PJ, Rutten WP, Sandkuijl LA, Oostra BA, van Duijn CM, Heutink P (2001) A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. Nat Genet 28:213-214

Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, Andres L, MacFarlane J, Sakellaropoulos N, Politou M, Nemeth E, Thompson J, Risler JK, Zaborowska C, Babakaiff R, Radomski CC, Pape TD, Davidas O, Christakis J, Brissot P, Lockitch G, Ganz T, Hayden MR, Goldberg YP (2004) Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 36:77-82

Papanikolaou G, Tzilianos M, Christakis JI, Bogdanos D, Tsimirika K, Macfarlane J, Goldberg YP, Sakellaropoulos N, Ganz T, Nemeth E (2005) Hepcidin in iron overload disorders. Blood 105:4103-4105

Park CH, Valore EV, Waring AJ, Ganz T (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276:7806-7810

Parkkila S, Waheed A, Britton RS, Bacon BR, Zhou XY, Tomatsu S, Fleming RE, Sly WS (1997a) Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. Proc Natl Acad Sci USA 94:13198-13202

Parkkila S, Waheed A, Britton RS, Feder JN, Tsuchihashi Z, Schatzman RC, Bacon BR, Sly WS (1997b) Immunohistochemistry of HLA-H, the protein defective in patients with hereditary hemochromatosis, reveals unique pattern of expression in gastrointestinal tract. Proc Natl Acad Sci USA 94:2534-2539

Pietrangelo A (2002) Physiology of iron transport and the hemochromatosis gene. Am J Physiol Gastrointest Liver Physiol 282:G403-414

Pietrangelo A, Caleffi A, Henrion J, Ferrara F, Corradini E, Kulaksiz H, Stremmel W, Andreone P, Garuti C (2005) Juvenile hemochromatosis associated with pathogenic mutations of adult hemochromatosis genes. Gastroenterology 128:470-479

Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 276:7811-7819

Piperno A, Mariani R, Arosio C, Vergani A, Bosio S, Fargion S, Sampietro M, Girelli D, Fraquelli M, Conte D, Fiorelli G, Camaschella C (2000) Haemochromatosis in patients with beta-thalassaemia trait. Br J Haematol 111:908-914

Ponka P (1997) Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. Blood 89:1-25

Ponka P, Wilczynska A, Schulman HM (1982) Iron utilization in rabbit reticulocytes. A study using succinylacetone as an inhibitor or heme synthesis. Biochim Biophys Acta 720:96-105

Priwitzerova M, Pospisilova D, Prchal JT, Indrak K, Hlobilkova A, Mihal V, Ponka P, Divoky V (2004) Severe hypochromic microcytic anemia caused by a congenital defect of the iron transport pathway in erythroid cells. Blood 103:3991-3992

Raskind W, Wijsman E, Pagon RA, Cox TC, Bawden MJ, May BK, Bird TD (1991) X-linked sideroblastic anemia and ataxia: linkage to phosphoglycerate kinase at Xq13. Am J Hum Genet 48:335-341

Richardson D, Ponka P, Vyoral D (1996) Distribution of iron in reticulocytes after inhibition of heme synthesis with succinylacetone: examination of the intermediates involved in iron metabolism. Blood 87:3477-3488

Rivard SR, Lanzara C, Grimard D, Carella M, Simard H, Ficarella R, Simard R, D'Adamo AP, De Braekeleer M, Gasparini P (2003) Autosomal dominant reticuloendothelial iron overload (HFE type 4) due to a new missense mutation in the FERROPORTIN 1 gene (SLC11A3) in a large French-Canadian family. Haematologica 88:824-826

Rivera S, Liu L, Nemeth E, Gabayan V, Sorensen OE, Ganz T (2005) Hepcidin excess induces the sequestration of iron and exacerbates tumor-associated anemia. Blood 105:1797-1802

Robb A, Wessling-Resnick M (2004) Regulation of transferrin receptor 2 protein levels by transferrin. Blood 104:4294-4299

Robb AD, Ericsson M, Wessling-Resnick M (2004) Transferrin receptor-2 mediates a biphasic pattern of transferrin uptake associated with ligand delivery to multivesicular bodies. Am J Physiol Cell Physiol 287:C1769-C1775

Rodriguez Martinez A, Niemela O, Parkkila S (2004) Hepatic and extrahepatic expression of the new iron regulatory protein hemojuvelin. Haematologica 89:1441-1445

Roetto A, Daraio F, Porporato P, Caruso R, Cox TM, Cazzola M, Gasparini P, Piperno A, Camaschella C (2004) Screening hepcidin for mutations in juvenile hemochromatosis: identification of a new mutation (C70R). Blood 103:2407-2409

Roetto A, Merryweather-Clarke AT, Daraio F, Livesey K, Pointon JJ, Barbabietola G, Piga A, Mackie PH, Robson KJ, Camaschella C (2002) A valine deletion of ferroportin 1: a common mutation in hemochromastosis type 4. Blood 100:733-734

Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C (2003) Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 33:21-22

Roetto A, Totaro A, Piperno A, Piga A, Longo F, Garozzo G, Cali A, De Gobbi M, Gasparini P, Camaschella C (2001) New mutations inactivating transferrin receptor 2 in hemochromatosis type 3. Blood 97:2555-2560

Rothenberg BE, Voland JR (1996) beta2 knockout mice develop parenchymal iron overload: A putative role for class I genes of the major histocompatibility complex in iron metabolism. Proc Natl Acad Sci USA 93:1529-1534

Roy CN, Carlson EJ, Anderson EL, Basava A, Starnes SM, Feder JN, Enns CA (2000) Interactions of the ectodomain of HFE with the transferrin receptor are critical for iron homeostasis in cells. FEBS Lett 484:271-274

Roy CN, Custodio AO, de Graaf J, Schneider S, Akpan I, Montross LK, Sanchez M, Gaudino A, Hentze MW, Andrews NC, Muckenthaler MU (2004) An Hfe-dependent pathway mediates hyposideremia in response to lipopolysaccharide-induced inflammation in mice. Nat Genet 36:481-485

Roy CN, Penny DM, Feder JN, Enns CA (1999) The hereditary hemochromatosis protein, HFE, specifically regulates transferrin-mediated iron uptake in HeLa cells. J Biol Chem 274:9022-9028

Salter-Cid L, Brunmark A, Li Y, Leturcq D, Peterson PA, Jackson MR, Yang Y (1999) Transferrin receptor is negatively modulated by the hemochromatosis protein HFE: implications for cellular iron homeostasis. Proc Natl Acad Sci USA 96:5434-5439

Santos M, Schilham MW, Rademakers LH, Marx JJ, de SM, Clevers H (1996) Defective iron homeostasis in beta 2-microglobulin knockout mice recapitulates hereditary hemochromatosis in man. J Exp Med 184:1975-1985

Schimanski LM, Drakesmith H, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, Robson KJ, Townsend AR (2005) *In vitro* functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations. Blood 105:4096-4102

Schrier S, Angelucci E (2005) New strategies in the treatment of the thalassemias. Annu Rev Med 56:157-171

Taketani S, Kakimoto K, Ueta H, Masaki R, Furukawa T (2003) Involvement of ABC7 in the biosynthesis of heme in erythroid cells: interaction of ABC7 with ferrochelatase. Blood 101:3274-3280

Touret N, Martin-Orozco N, Paroutis P, Furuya W, Lam-Yuk-Tseung S, Forbes J, Gros P, Grinstein S (2004) Molecular and cellular mechanisms underlying iron transport deficiency in microcytic anemia. Blood 104:1526-1533

Townsend A, Drakesmith H (2002) Role of HFE in iron metabolism, hereditary haemochromatosis, anaemia of chronic disease, and secondary iron overload. Lancet 359:786-790

Vogt TM, Blackwell AD, Giannetti AM, Bjorkman PJ, Enns CA (2003) Heterotypic interactions between transferrin receptor and transferrin receptor 2. Blood 101:2008-2014

Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat Genet 21:195-199

Waheed A, Parkkila S, Zhou XY, Tomatsu S, Tsuchihashi Z, Feder JN, Schatzman RC, Britton RS, Bacon BR, Sly WS (1997) Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with beta2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. Proc Natl Acad Sci USA 94:12384-12389

Wallace DF, Clark RM, Harley HA, Subramaniam VN (2004a) Autosomal dominant iron overload due to a novel mutation of ferroportin1 associated with parenchymal iron loading and cirrhosis. J Hepatol 40:710-713

Wallace DF, Pedersen P, Dixon JL, Stephenson P, Searle JW, Powell LW, Subramaniam VN (2002) Novel mutation in ferroportin1 is associated with autosomal dominant hemochromatosis. Blood 100:692-694

Wallace DF, Tonks ID, Zournazi A, Kay GF, Subramaniam VN (2004b) Inactivation of the murine Transferrin Receptor 2 gene using the Cre recombinase: loxP system. Genesis 39:38-41

Wang GL, Semenza GL (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci USA 90:4304-4308

Weatherall D (2001) Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat Rev Genet 2:245-255

Weinstein DA, Roy CN, Fleming MD, Loda MF, Wolfsdorf JI, Andrews NC (2002) Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. Blood 100:3776-3781

West AP Jr, Bennett MJ, Sellers VM, Andrews NC, Enns CA, Bjorkman PJ (2000) Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE. J Biol Chem 275:38135-38138

West AP Jr, Giannetti AM, Herr AB, Bennett MJ, Nangiana JS, Pierce JR, Weiner LP, Snow PM, Bjorkman PJ (2001) Mutational analysis of the transferrin receptor reveals overlapping HFE and transferrin binding sites. J Mol Biol 313:385-397

Yang F, Liu XB, Quinones M, Melby PC, Ghio A, Haile DJ (2002) Regulation of reticuloendothelial iron transporter MTP1 (Slc11a3) by inflammation. J Biol Chem 277:39786-39791

Zhang A, Sheftel D, Ponka P (2005) Intracellular kinetics of iron in reticulocytes: evidence for endosome involvement in iron targeting to mitochondria. Blood 105:368-375

Zhang AS, Davies PS, Carlson HL, Enns CA (2003) Mechanisms of HFE-induced regulation of iron homeostasis: Insights from the W81A HFE mutation. Proc Natl Acad Sci USA 100:9500-9505

Zhang AS, Xiong S, Tsukamoto H, Enns CA (2004) Localization of iron metabolism-related mRNAs in rat liver indicate that HFE is expressed predominantly in hepatocytes. Blood 103:1509-1514

Zhou XY, Tomatsu S, Fleming RE, Parkkila S, Waheed A, Jiang J, Fei Y, Brunt EM, Ruddy DA, Prass CE, Schatzman RC, O'Neill R, Britton RS, Bacon BR, Sly WS (1998) HFE gene knockout produces mouse model of hereditary hemochromatosis. Proc Natl Acad Sci USA 95:2492-2497

Note added in press: The generation of a *Trfr-2* knock out mouse has been reported (Wallace DF, Summerville L, Lusby PE, and Subramaniam VN, First phenotypic description of transferrin receptor 2 knockout mouse, and the role of hepcidin Gut 54:980-6 (2005)). The pattern and extent of iron overload is similar to the *Trfr2* $^{Y245X/Y245X}$ mouse indicating the mutations in TfR2 resulting in iron overload are due to lack of function of TfR2.

Abbreviations

ABC: ATP-binding cassette
AI: anemia of inflammation
ALAS: δ-aminolevulinic acid synthase
ATM: ataxia telangiectasia mutated
β_2m: beta-2-microglobulin
C/EBP: CCAAT: enhancer binding protein
DFO: desferrioxamine
DMT1: divalent metal transporter 1
EPO: erythropoietin
FAC: ferric ammonium citrate
Fe_2-Tf: diferric transferrin
Fpn1: ferroportin 1
Ft: ferritin
Hamp: hepcidin antimicrobial peptide
Hepc: hepcidin
HH: hereditary hemochromatosis
Hjv: hemojuvelin
IL-1: interleukin-1
IL-6: interleukin-6
IRE: iron responsive element

IRP: iron regulatory protein
LPS: lipopolysaccharide
MHC: major histocompatability complex
RBC: red blood cell
Tf: transferrin
TfR1: transferrin receptor 1
TfR2: transferrin receptor 2
USF2: upstream stimulatory factor 2
UTR: untranslated region
XLSA: X-linked sideroblastic anemia
XLSA/A: X-linked sideroblastic anemia with ataxia

Enns, Caroline A.
Department of Cell & Developmental Biology, L-125, 3181 SW Sam Jackson Park Road, Oregon Health & Science University, Portland, OR 97239 USA

Johnson, Martha B.
Department of Cell & Developmental Biology, L-125, 3181 SW Sam Jackson Park Road, Oregon Health & Science University, Portland, OR 97239 USA

Zhang, An-Sheng
Department of Cell & Developmental Biology, L-125, 3181 SW Sam Jackson Park Road, Oregon Health & Science University, Portland, OR 97239 USA
zhanga@ohsu.edu

Cellular and whole organism aspects of iron transport and storage in plants

Jean-François Briat

Abstract

Plants depend upon iron for their growth and development. However, availability of this metal is low in soils, because of its insolubility at basic pH in presence of oxygen. Plants have, therefore, evolved various mechanisms to actively acquire iron from the soil, based either on reducing or chelating strategies. The molecular characterization of these uptake systems and the regulation of their synthesis have been widely documented the last few years. Distribution of iron to the various parts of a plant, and its compartmentation in various subcellular organelles is also described, but the molecular determinants required for these functions are yet poorly documented. Beside transport activities to establish iron homeostasis in plants, storage is also an important parameter. Part of this function is achieved by ferritins. These iron storage proteins are located within the plastids in plants, and regulated by iron at a transcriptional level.

1 Introduction

Plants are an essential component of the food chain because they are responsible for mineral acquisition from the soil and for carbon, sulfur and nitrogen assimilation leading to amino- acid and vitamin synthesis. They bring, therefore, essential nutrients to the animal and human diets. Among essential mineral elements, iron is important because of its physicochemical properties. Coordinated at metalloprotein active sites, it participates in most of the basic redox reactions required in both the production (photosynthesis) and the consumption (respiration) of oxygen. Iron is also involved in many enzymatic reactions required for nitrogen fixation, DNA, and hormone synthesis, for example. However, iron physicochemical properties make this element uneasy to use by aerobic living organisms. In aqueous phase, at physiological pH, iron tends to precipitate under insoluble (oxidized) form. Furthermore, its redox cycling contributes to activation of reduced forms of oxygen through Fenton chemistry, leading to lipid peroxidation, protein oxidation, and DNA mutations, and consequently to cellular damage and possible cell death. As a consequence, plants have evolved mechanisms to control iron uptake, transport in various organs, and storage to ensure an optimal development by preventing both iron deficiency and toxicity. Various transporters are required to achieve

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_93 / Published online: 26 July 2005

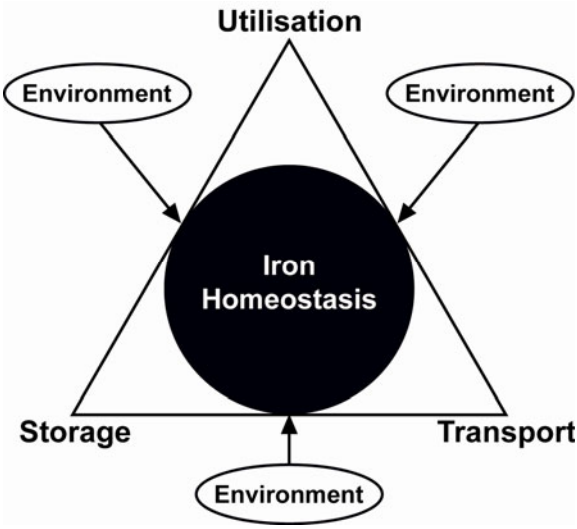


Fig. 1. Schematic representation of the determinants of iron homeostasis in plants. Normal plant growth and development are possible within a sharp range of iron concentrations avoiding deficiency or toxicity. This homeostasis is genetically determined at the level of iron transport, storage, and utilization. These three functions are deeply influenced by environmental factors.

these iron fluxes (Curie and Briat 2003). Iron storage takes place in the apoplasmic space, between the plasma membrane and the cell wall of plant cells, and also likely in the vacuoles, where low pH and high organic acid concentrations represent optimal conditions for iron deposit (Briat and Lobréaux 1998). Ferritins are iron storage proteins located in the plastids of plant cells. They are also part of these mechanisms by their capacity to store up to 4500 iron atoms in their cavity in a soluble and bioavailable form (Harrison and Arosio 1996).

Iron homeostasis in the various plant tissues during growth and development, throughout the life cycle, is a dynamic process resulting from an integrated regulation of the expression of the various genes encoding proteins acting in the transport, storage and utilization of iron. It requires coordinated action of cellular and systemic mechanisms. These processes depend upon the plant species and genotypes considered and are deeply influenced by environmental cues (Fig. 1).

2 Iron acquisition and trafficking

The dynamic fate of iron accumulation in various organs and tissues of a plant during the course of its growth and development is an integrated process, which results from the coordinated regulation of iron uptake from the rhizosphere, iron

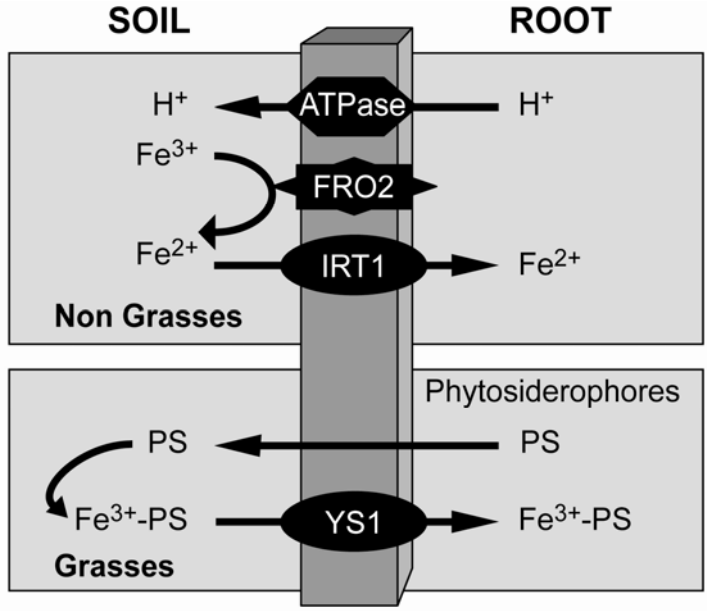


Fig. 2. Iron uptake from the soil by grass and non-grass families of plants. Non-grass plants acidify their rhizosphere in response to iron deficiency by activating a $^{+}$H-ATPase. This enables a better solubilization of ferric iron (Fe^{3+})-chelates, which are reduced by a ferric chelate reductase (FRO2 in *Arabidopsis*) belonging to the NADPH oxidase family. The resulting ferrous iron (Fe^{2+}) is transported inside the root by a transporter (IRT1 in *Arabidopsis*) belonging to the ZIP family (ZRT, IRT-like Protein; IRT: Iron-Regulated Transporter; ZRT: Zinc-Regulated Transporter). Grass plants use a chelation strategy instead of a reducing strategy. Under iron deficiency conditions, phytosiderophores (PS) belonging to the mugineic acids family are synthesized from S-adenosylmethionine, and secreted into the rhizosphere where they chelate ferric iron (Fe^{3+}). The Fe^{3+}-PS complex is then after taken up by the Yellow Stripe 1 (YS1) root transporter.

fluxes between various plant organs and tissues, and iron subcellular compartmentation. To achieve these goals iron must cross various membranes and the molecular characterization of the transporters involved has made huge progress these last five years.

2.1 Iron uptake by the roots and its control

Grasses and non-grasses use different strategies to acquire iron from the soil in response to deficiency conditions (Fig. 2). In *Arabidopsis*, a non-grass model plant, iron deficiency induces synthesis of FRO2 (Robinson et al. 1999), a ferric-chelate reductase leading to Fe(II) generation. The *Arabidopsis FRO2* gene was cloned based on a PCR approach using degenerated oligonucleotides derived from the yeast *FRE* reductase sequences. This gene is allelic to *frd1-1*, one of three *Arabi-*

dopsis mutants (*frd1-1*, *frd1-2*, *frd1-3*) that do not show induction of Fe^{3+}-chelate reductase under iron deficient conditions. It confirms that iron must be reduced prior to its transport and that Fe^{3+}-reduction can be uncoupled from proton release (Yi and Guerinot 1996). It restores Fe^{3+}-reductase activity when expressed in the *frd1-1* mutant line, and is upregulated in roots under iron deficiency conditions. *FRO2* encodes a 725 amino acid protein with eight putative transmembrane domains, and shares similarities with human phagocytic NADPH $gp91^{phox}$ oxydoreductase and with the yeast ferric chelate reductases. Like $gp91^{phox}$ and yeast FREp, FRO2 contains binding sites for heme and for nucleotide cofactors, consistent with its function in electron transfer from cytosolic NADPH to extracellular Fe^{3+}. The FRO2-generated Fe^{2+} is then taken up by IRT1, which is the major root Fe(II) uptake system under iron deficient conditions. IRT1, an eight transmembrane domain protein located at the plasmalemma of root epidermal cells, is the founder of a new eukaryotic transporter family denominated ZIP (Guerinot 2000). It was shown by the characterization of *Arabidopsis* IRT1-KO lines to be essential for plant growth and development in *Arabidopsis* (Eide et al. 1996; Vert et al. 2002). IRT1 has a large substrate specificity, and in addition to iron it is able to transport Zn, Cd, Co, and Mn, but not Cu. In contrast to yeast, copper is not required for iron uptake through the activity of the FRO2/IRT1 system. It indicates that in non-grass plants no copper oxidase activity, such as the one of FET3p in yeast, is necessary for iron uptake. However, zinc, as iron, is involved in the regulation of expression of the IRT1/FRO2 system in *Arabidopsis* (Connolly et al. 2003). IRT2, is an *Arabidopsis* gene highly related to IRT1 that also encodes an iron transporter expressed in root epidermal cells (Vert et al. 2001), but it is not redundant to the IRT1 function, enabling to conclude that the IRT1/FRO2 system constitutes the high affinity iron uptake system which is induced in response to iron deficiency in *Arabidopsis*. Regulation of this root high affinity iron uptake system by whole plant signals was investigated at the molecular level, through monitoring *FRO2* and *IRT1* gene expression (Vert et al. 2003). Recovery from iron deficient conditions, and modulation of apoplastic iron pools indicated that iron itself plays a major role in the regulation of root iron deficiency responses at the mRNA and protein levels. Split-root experiments showed that the expression of *IRT1* and *FRO2* is controlled both by a local induction from the root iron pool, and through a systemic pathway involving a shoot-borne signal, both signals being integrated in order to tightly control production of the root iron uptake proteins. *IRT1* and *FRO2* are expressed during the day and downregulated at night, and this additional control is overruled by iron starvation, indicating that the nutritional status prevails on the diurnal regulation. The tomato chlorotic *fer* mutant fails to activate iron deficiency responses and the tomato *IRT1* ortholog, *LeIRT1*, is downregulated in the *fer* genetic background. The *FER* gene was identified by map based cloning. It encodes a basic helix-loop-helix (bHLH), transcription factor, making FER the first identified regulator for iron nutrition in plants (Ling et al. 2002). Plants from the legume family also take up iron through Fe(III) reduction and Fe(II) transporters. For example, in pea, the *FR01*gene encodes a protein 55% identical to *Arabidopsis* FRO2 (Waters et al. 2002), which displays a ferric chelate reductase activity when expressed in yeast, and which is thought to represent the pea reductase involved in

root iron acquisition. The PsFRO1-generated Fe(II) is likely then taken up by pea roots through a tranporter encoded by the *RIT1* gene, which is upregulated in iron deficiency and encodes a protein 63% identical to IRT1. PsRIT1 complements both the *fet3fet4* and *zrt1zrt2* yeast mutants, thus, potentially mediating high affinity Fe and Zn uptake. In contrast to other dicotyledonous plants, legume plants specifically develop a symbiosis with some soil bacteria enabling nitrogen fixation. This symbiotic process takes place in specific root structures, the nodules, within the cortical cells of which bacteria evolved into bacteroids able to reduce atmospheric nitrogen into ammonia. This process requires some essential iron containing proteins such as nitrogenase and leghemoglobin (Tang et al. 1990). Iron uptake by the bacteroids within the nodule requires three activities: (i) Fe(III) chelated to organic acids like citrate is transported across the peribacteroidal membrane prior to accumulate within the peribacteroidal space (Levier et al. 1996; Moreau et al. 1995) where it will bind to bacterial-type siderophores (Wittenberg et al. 1996); (ii) Fe(III)-chelate reductases are active at the peribacteroidal membrane and uptake of Fe(III) in isolated symbiosomes is stimulated by NADH (Levier et al. 1996); and (iii) Fe(II) is also transported across the peribacteroidal membrane (Moreau et al. 1998) likely through the GmDMT1 transporter (Kaiser et al. 2003), which belongs to the NRAMP family, a ubiquitous class of metal transporters also involved in iron and other metals transport in yeast, plants and mammals (Portnoy et al 2000; Curie et al. 2000; Thomine et al. 2000; Forbes and Gros 2001).

In contrast to non-grasses plants, iron deficiency in grasses induces the secretion by the roots of mugineic acids (MA), which are synthesized from nicotianamine (NA), a structurally related precursor, found in all plants, and resulting of the condensation of three S-adenosyl methionine molecules (von Wiren et al. 1999). Then, MA binds to soil Fe(III) into the rhizosphere. The resulting complex is recognized and transported across the root plasma membrane by an Fe(III)-MA uptake system. The Fe(III)-MA transport system, specific of the iron deficiency response in grasses, has been characterized by using maize as a model grass organism. The maize *ys1* mutant has been extensively studied. It carries a monogenic recessive mutation, responsible for a defect in the transport of the Fe(III)-mugineic acid through the root plasma membrane. In this mutant, mugineic acid synthesis and secretion is normal. The maize *ys1* gene has been cloned (Curie et al. 2001). It has been expressed in the *fet3fet4* yeast mutant strain, which is deficient in low and high affinity iron transport activities, as well as in *Xenopus* oocytes, in order to demonstrate its transport activity and to investigate its properties (Curie et al. 2001; Roberts et al. 2004; Schaff et al. 2004). An intriguing output of this work was the discovery by sequence database mining that eight *Arabidopsis* genes share important sequence similarities with maize *ys1*, although *Arabidopsis* does not produce mugineic acids. However, all plants, grasses and non-grasses, synthesized NA, the MAs precursor. NA and MAs have very closely related structures, and NA, as MAs, is also a strong metal chelator. This molecule has been described as potentially involved in long distance metal transport (von Wiren et al. 1999), including iron, and in iron subcellular compartmentation into the vacuoles (Pich et al. 2001). It has been, therefore, postulated that *Arabidopsis* YSL genes

could code for metal-NA transporters involved in metal trafficking within plants, once uptake from the soil has been achieved (Curie et al. 2001).

2.2 Iron fluxes within the plant

Iron, once taken up by roots, is then loaded in the xylem sap and translocated to the plant aerial parts through the transpiration stream. Organic acids, and especially citrate, are the main metal chelators in the xylem, and this has been well documented (Cataldo et al. 1988). This mechanism implies that active root transporters must load iron from the root cortex cells to the xylem. However, efflux iron transporters are still uncharacterized at the molecular level in plants (Fig. 3). Once in the leaves, Fe(III)-citrate is likely to be the substrate of leaf ferric chelate reductase, since such an enzymatic activity has been described in leaf mesophyll cells (Bruggemann et al. 1993). Whether some of the *FRO* genes could be involved in this process in *Arabidopsis* remains to be established (Robinson et al. 1999). In pea, however, the fact that *FRO1* is also expressed in leaves (Waters et al. 2002) makes it a good candidate for the function of ferric reduction in leaf. Recently, AtYSL2, one of the maize YS1 homologues in *Arabidopsis* (see above) has been characterized (DiDonato et al. 2004). It is able to complement the *fet3fet4* yeast mutant strain which is affected in low and high affinity iron transport, but only when Fe(II) and not Fe(III) is chelated to nicotianamine (NA) the precursor found in all plants, of the grass specific mugineic acids (MA). AtYSL2 is, therefore, likely a Fe(II)-NA transporter in plants. It is expressed in the vasculature of both roots and leaves, at the level of xylem-associated cells, and its transcript amount decreases in response to iron deficiency. Based on imaging of AtYSL2::GFP in transgenic *Arabidopsis* plants, this transporter is located at the plasmalemma of xylem parenchyma cells, exclusively at the edges of these cells, and not at their apical or basal ends. Such a localization implies that AtYSL2 could move Fe(II)-NA complex laterally within the veins of both leaves and roots. This set of data enable the authors (DiDonato et al. 2004) to hypothesize that the major physiological role for AtYSL2 is to take up iron that has arrived in tissues *via* xylem transport, thus, moving it away from the xylem vessels (Fig. 3).

Mobility of iron from source to sink tissues *via* the phloem sap is poorly documented. It is, nevertheless, well established that the phloem sap contains Fe (Stephan et al. 1994) coming from its mobilization in source organs (Grusak 1995). One of the molecules identified as a potential phloem metal-transporter is the nicotianamine (Stephan and Scholz 1993). The rice genome contains 18 putative *OsYSL* genes, and it has been very recently reported that *OsYSL2* expression was induced in leaves in response to iron deficiency, at the level of the phloem vessels (Koike et al. 2004). *OsYSL2* was also shown to be expressed in the reproductive organs, such as the grain, during their development. OSYSL2 fused to the GFP localizes at the plasmalemma, and when expressed in *Xenopus* oocytes it is able to transport Fe(II)-NA but not Fe(III)-NA nor Fe(III) deoxyMA. It is, therefore, hypothesized that OSYSL2 is required in the long distance transport of Fe(II)-NA in the phloem (Fig. 3), and for Fe accumulation in rice grain. Iron has

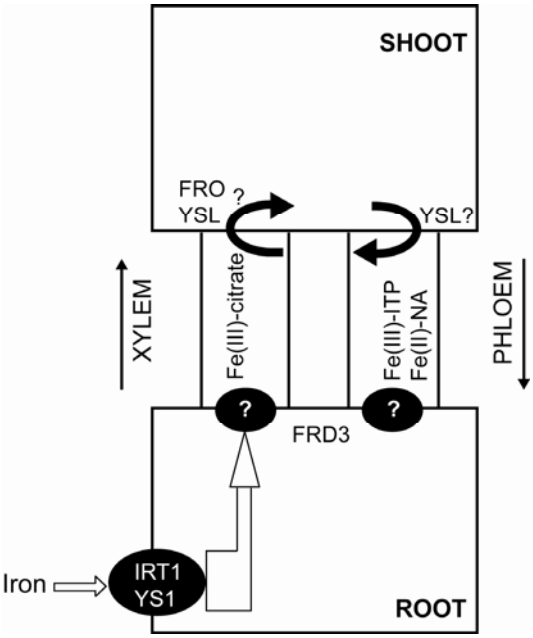


Fig. 3. Long distance iron trafficking between plant organs and tissues. Once taken up within root epidermal and cortex cells, iron is loaded within the xylem sap through not yet molecularly characterized efflux transporters. Within the xylem sap, Fe(III) is chelated to organic acids, among which citrate is the major one, and is transported to the shoot part of the plant by the transpiration stream. Unloading of the xylem has not been yet fully described at a molecular level, but could involve reductases of the Ferric Reductase Oxidase (FRO) family and members of the YSL (Yellow Stripe Like) family. Loading of the phloem, which contains Iron Transport Peptides (ITP) chelating Fe(III) and nicotianamine (NA) chelating Fe(II) is an important process for iron delivery to sink tissues (roots, seeds), and likely for long distance signaling and control of the root uptake system. *YSL* genes are good candidates to code for transporters involved in phloem loading, but no molecular candidates have been identified for its unloading. These long distance iron transport activities are under the control of various genes, among which the *Ferric Reductase Defective 3* (*FRD3*) gene encoding a root transmembrane protein of the MATE (Multidrug Associated and Toxin Efflux) family.

also been suggested to travel in the phloem of *Ricinus communis* in a ferric complex with a molecular weight of 2.4 kDa (Maas et al. 1988). Indeed, ITP, a phloem protein of *Ricinus communis* has recently been purified and shown to complex Fe(III) *in vivo,* but not Fe(II). A cDNA corresponding to ITP has been cloned and encodes a 96 amino acid protein belonging to the Late Embryogenesis Abundant (LEA) family (Krueger et al. 2002). The preference of ITP for ferric iron is in agreement with the observation that only 4% of the total iron in the phloem exudate of *Ricinus communis* seedlings is in the ferrous form (Schmidke et al. 1999). Although the affinity constant of nicotianamine for Fe(III) is 20.6, and only 12.8

for Fe(II), the Fe(II)-nicotianamine complex possesses an unusual kinetic stability, explaining why NA is found complexed to Fe(II), and not to Fe(III), in the phloem sap (von Wiren et al. 1999). Since there is a low but significant steady-state concentration of ferrous iron in the phloem (Maas et al. 1988), and since the bulk of iron in the phloem is chelated in the Fe(III) form by ITP (Krueger et al. 2002), it is tempting to speculate that nicotianamine could play a role of shuttle by chelating Fe(II) from ITP-bound Fe(III) during loading and unloading of the phloem (Fig. 3). Such an hypothesis would imply the existence of a redox system within the phloem for ensuring Fe(III)/Fe(II) cycling.

2.3 Iron subcellular compartmentation

Very little information is presently available concerning intracellular iron movement in plant cells. Among the *ZIP*, *NRAMP*, *YSL* (Curie and Briat 2003) and still uncharacterized gene families, it is likely that some members will encode proteins involved in iron transport into and/or out of the various plant cell organelles (Fig. 4).

Plant vacuoles are likely to play a major role in handling iron excess. The best evidence for such a vacuole function comes from the finding that upon iron overload, or in pea mutants overaccumulating iron, the nicotianamine concentration increases and the bulk of this iron chelator is relocated into the vacuoles, whereas NA is observed in the cytoplasm under normal or deficient iron conditions (Pich et al. 2001). This indicates that transporters of iron-nicotianamine complexes must be present at the tonoplast. In this context, it will be of great interest to scrutinize the subcellular localization of the transporters encoded by the various members of the *Arabidopsis* YSL gene family, which are potential Fe-nicotianamine transporters (Curie et al. 2001). Remobilization of vacuolar iron stores in order to meet cellular needs has been shown to occur in yeast and to be partly mediated by SMF3, a member of the NRAMP metal transporter family. Interestingly, Thomine and collaborators (2003) recently reported that in *Arabidopsis,* AtNRAMP3 localizes to the vacuolar membrane, and could serve to remobilize vacuolar iron in case of iron deficiency.

Mitochondria also contain iron proteins, thus iron needs to enter this organelle. So far no data has been reported to document this point. Concerning iron efflux from mitochondria, it has been reported that the *STA1* gene from *Arabidopsis* encodes an homologue of the yeast ATM1p (Kushnir et al. 2001), an ABC-transporter located at the mitochondrial inner membrane, and involved in the export of Fe-S clusters from the mitochondrial matrix to the cytoplasm (Kispal et al. 1999; Lill and Kispal 2000).

The bulk of iron in leaves is found within the chloroplasts where it is engaged in the photosynthetic process. Plastids contain ferritin, an iron storage protein (Briat et al. 1999), and iron-ferritin represents more than 90% of the iron found in a pea embryo axis (Marentes and Grusak 1998). Iron transport into the plastids is, therefore, of primary importance in plant physiology, and paradoxically this subcellular iron transport activity is poorly documented. Light was shown to be

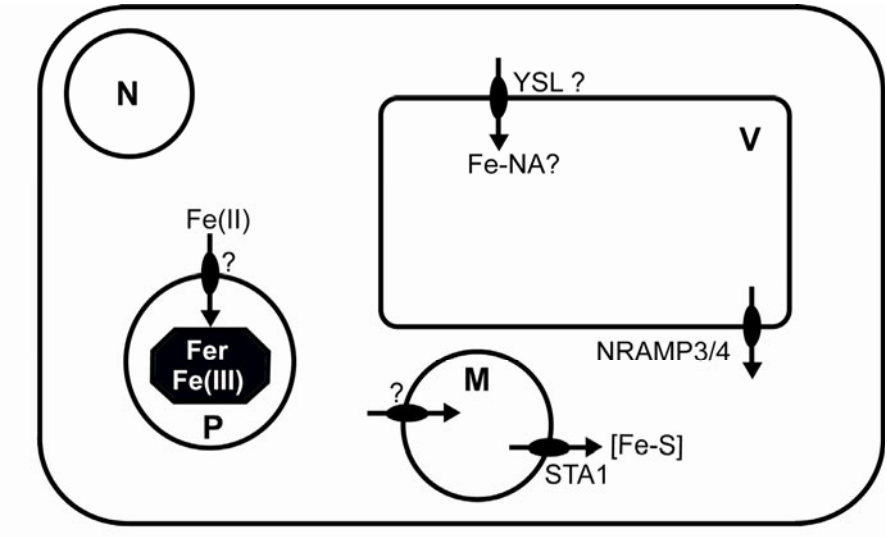


Fig. 4. Intracellular compartmentation of iron into plant cells. Nicotianamine (NA) has been observed within vacuoles (V) of plant cells after iron overload treatments. It indicates that iron-NA transporters of the YSL family could be located at the tonoplast. Iron stored within the vacuole could be remobilized by Nramp3 and 4 transporters belonging to the Natural resistance associated macrophage protein (Nramp) family. It is unknown how iron is taken up by mitochondria (M). However, it is established that the STA1 ABC transporter, homologous to the yeast Atm1p transporter required for [Fe-S] cluster export from mitochondria to cytoplasm, is located at the plant mitochondria membrane. The plastids (P), among which chloroplasts support photosynthesis activity, are iron rich. This plastid iron is acquired through a Fe(II) transporter, which has not been yet characterized at a molecular level. The iron storage ferritin (Fer) is located within plastids in plants, and plays an important role to buffer iron in its ferric form, avoiding its toxicity. N: nucleus.

necessary for efficient iron transport from the leaf veins to the mesophyll cells. Iron uptake studies with isolated barley chloroplasts indicated that this process is also light dependent, and requires a Fe(III)-chelate reductase activity (Bughio et al. 1997). In agreement with this result, an inward-directed Fe(II) transport across the chloroplast inner membrane occurs by a potential-stimulated uniport mechanism, as shown by stopped flow spectrofluorometry using inner membrane vesicles (Shingles et al. 2002).

3 Iron storage by plant ferritins

Ferritins are a class of high molecular weight 24-mer proteins able to accommodate a few thousand iron atoms in their central cavity in a safe bioavailable form. No ferritin sequence is present in the yeast genome, iron storage, and detoxification being mainly achieved by the vacuole in this unicellular organism. Animal

and plant ferritins are highly conserved both at the amino acid sequence level and at the 3-D structure level (Harrison and Arosio 1996). However, there are two major differences between plant and animal ferritins. First, animal ferritins are localized in the cytoplasm whereas plant ferritins are found in a plant specific organelle family, the plastids, among which the chloroplast is the site where photosynthesis takes place (Fig. 4; Briat et al. 1999). Second, ferritin expression in response to iron excess is mainly regulated at the translational level through the IRE/IRPs system in animals, and at the transcriptional level in plants (Lescure et al. 1991; Hentze et al. 2004).

3.1 Leaf ferritin

Iron accumulation in leaves is important and chloroplasts contain 80% of the metal present in this organ. It is consistent with the major function of leaves, photosynthesis, a reaction requiring iron in many of the proteins of the electron transfer chain of thylakoid membranes. The iron storage protein ferritin accumulates mainly in non-green plastids, like etioplasts or amyloplasts, whereas low level of this protein is found in mature chloroplasts where the photosynthetic process is active. However, this distribution of iron in leaves changes according to their developmental stage. The developmental control of ferritin synthesis in leaves is evidenced by an increase of ferritin abundance in developing and in senescent leaves (Briat and Lobréaux 1997). These results have been obtained by immunodetection of ferritin subunits in protein extracts during the life cycle of pea plants (Lobréaux and Briat 1991). Ferritins were only detected in leaves of young plantlets, and remained undetectable in the corresponding organs of adult plants at later developmental stages. These data have been further documented by using the determinate nature of maize leaf development (Theil and Hase 1993; Seckback 1982). In this plant, the leaf grows from the basal part, creating a cell age gradient from the base to the tip of the leaf. By an immunodetection approach, high ferritin levels were detected in the young basal section of the leaf and in the tip, the older part of the leaf. On the opposite, a very low level of ferritin was present in the central section of this organ. This part has the higher chlorophyll content, and the detection of the phosphoenolpyruvate carboxylase enzyme in this region, a marker of photosynthetic activity in C4 plants like maize, proves that the photosynthetic process is active in these cells. These results demonstrate the developmental regulation of ferritin synthesis in leaves. Such data are in complete agreement with previous electron microscopy observations showing that ferritins were synthesized in meristematic zones and apices, in leaf primordia, and disappeared during bud development (Marinos 1967; Seckback 1982). All these observations suggest that ferritin would be an iron source at early stages of development for the synthesis of iron containing proteins involved in photosynthesis. This hypothesis is consistent with the fact that ferritins are present in young leaves or etiolated leaves containing non photosynthetic etioplasts, and then become undetectable in photosynthetic or de-etiolated leaves (Lobréaux and Briat 1991; Seckback 1982). The high ferritin concentration in maize leaf tips could be linked to cell senescence.

Ultrastructural studies have clearly established that ferritin accumulates in senescing tissues (Seckback 1982). Furthermore, a *Brassica napus* cDNA corresponding to a mRNA encoding a ferritin subunit has been cloned as a senescence induced mRNA (Buchanan-Wollaston and Ainsworth 1997). The neo-synthesis of plant ferritins at later stages of development, during leaf senescence has also been reported. The regulatory mechanisms controlling ferritin synthesis during leaf development are still unknown, but it has been established in soybean and maize that there is no direct correlation between the levels of ferritin subunits and mRNA (Theil and Hase 1993; Ragland et al. 1990). Ferritin mRNA was detected in mature leaves where ferritin subunits were not detected by western blot experiments, demonstrating that post-trancriptional controls are involved in the regulation of ferritin synthesis in this organ. More recently, however, it has been reported in *Arabidopsis* that *cis*-regulatory element(s) involved in the *AtFer1* ferritin gene activation during age-dependent senescence do exist within 1.4 kbp promoter sequence of this gene. These regulatory elements are different of the IDRS box (see below) known to regulate the iron-dependent expression of the *AtFer1* gene (Tarantino et al. 2003). In contrast dark-induced senescence promotes *AtFer1* gene activation through the IDRS box; in that case, iron release during the disorganization of the photosynthetic apparatus, and the requirement to store this metal, could trigger the transcriptional regulation of *AtFer1* expression.

3.2 Ferritins in roots and nodules

Iron concentration in roots is much lower than in leaves. Nevertheless, ferritin is also present in the non-green plastids of this organ. In *Arabidopsis* the *AtFer1* gene has been observed to be expressed close to the root tip in the endoderm cell layer, and also at the emergence of secondary roots (Tarantino et al. 2003). It is, therefore, plausible that non-green plastids and their ferritin play a buffering role in the endodermal cells after iron uptake at the epidermis and cortex levels. Ferritins would, therefore, be active in modulating the flux of iron to the upper part of the plant, through loading of the xylem sap by still uncharacterized transporters.

In legume species ferritin has been shown to accumulate at early stages of nodule development resulting from the interaction between soybean and a *Bradyrhizobium* strain (Ko et al. 1987). Ferritin levels decrease with the appearance of nitrogenase and leghemoglobin when the nodule becomes mature for nitrogen fixation (Bergersen 1963). In senescing nodules of *Lupinus luteus*, ferritin is resynthesized through the expression of two out of the three lupine ferritin genes (Strozycki et al. 2003). When soybean plants are cultured in the presence of a mutant strain of *Bradyrhizobium* unable to develop functional nodules, ferritin is detected at any stages (Ko et al. 1987). These data suggest again that iron would be transiently stored in ferritins and used for the accumulation of iron containing proteins. Ferritin mRNA levels have also been investigated during nodulation. This study reveals that, as for leaf development, no strict correlation between ferritin subunit and mRNA levels is observed (Ragland and Theil 1993). Then, some post-

transcriptional events would occur in the regulation of ferritin level during nodule development.

3.3 Ferritins in seeds

Study of the fate of iron during the course of vegetative organs growth and development has evidenced the dynamic nature of this process. Iron concentration changes within organs, in a tissue specific manner, during the course of development. The role of the iron storage protein ferritin as a transient iron buffer for important iron-dependent processes like photosynthesis and nitrogen fixation has been well documented in these developmental processes. Ferritins are also key proteins in long term iron storage, as evidenced by studying the process of seed formation. An important amount of iron is stored in pea seeds, and an increase in iron uptake by the roots occurs at early stages of seed development (Lobréaux and Briat 1991). Iron is also remobilized from vegetative organs to the seed; for example, it has been documented that leaf iron can account for 20-30% of the total seed iron content (Hocking and Pate 1978; Grusak 1994). In soybean, however, it has been suggested that 40 to 60% of the seed iron could come from nodules (Burton et al. 1998). Root nodules of legumes have higher concentrations of iron than other vegetative organs. Therefore, an active remobilization of nodule iron to the seed could explain why legume seeds have high concentration of Fe relative to other plants.

Seed iron is used during germination for the plantlet development. Immunodetection experiments revealed that ferritin subunits accumulated in seed during their maturation and remained present in dry seeds (Lobréaux and Briat 1991). This accumulation occurred in the embryo, and no ferritins were detected in the seed coat (Lobréaux and Briat 1991; Marentes and Grusak 1998). The amount of iron stored inside ferritins was estimated to be 92% of the total seed iron content (Marentes and Grusak 1998), suggesting that this protein is the major form of iron storage in seeds. Whether this iron pool is used only for plastid development or is also transferred to the cytosol remains to be determined. During germination, ferritins are degraded and iron used for the growth of the seedling. This process would involve a protein stability control to degrade the protein shell when iron is released from the mineral core. A model has been proposed based on the observation that the immunodetection pattern of pea seedling ferritin is similar to the pattern of ferritin degraded by free radical during *in vitro* iron exchanges (Lobréaux and Briat 1991). When iron release is induced from purified pea seed ferritins by incubation in the presence of ascorbate or light, a progressive degradation of the protein is detected (Laulhère et al. 1989, 1990). This degradation is initiated by iron-dependent free radical cleavages at the aminoterminus of the ferritin subunit (Laulhère et al. 1989, 1990; Lobréaux et al. 1992b). It has been hypothesized that such a mechanism occurs during seedling germination. When iron is released from the ferritin, free radical cleavages could alter the protein shell which would be then degraded by proteases.

3.4 Iron-dependent regulation of ferritin gene expression

Plant and animal ferritins have evolved from a common ancestor gene as suggested by amino acid sequence comparison of ferritin subunits (Andrews et al. 1992). However, in the two kingdoms, ferritins are localized in different compartments; plant ferritins are localized in the plastids (Van der Mark et al. 1983; Lescure et al. 1991), while animal ferritins are cytosolic proteins (Harrison and Arosio 1996). This specific compartmentation, and, as described above, the requirement to adapt the ferritin content to plant development, suggest that some plant specific pathways would be involved in the regulation of the ferritin subunit and mRNA levels in this organism.

Iron starvation of plants leads to the chlorosis symptoms, resulting from chlorophyll deficiency and impaired photosynthesis (Briat et al. 1995). Under such conditions, root iron uptake systems are induced in order to enhance iron acquisition and to maintain a physiological integrity (Briat and Lobréaux 1997). Then, addition of an excess of iron in the culture medium of iron starved plantlets leads to a large iron influx into the plant (Lobréaux et al. 1992a). This iron is translocated within three hours into the leaves to restore the essential photosynthetic process in chloroplasts (Young and Terry 1982; Branton and Jacobson 1962). During this period of regreening, which takes about 24 to 48 hours, ferritins are used as a safe iron buffer and transiently store the iron required for the synthesis of iron containing proteins. During this period of recovery from iron deficiency, ferritin mRNA is already detectable in maize leaf plantlets 3 hours after iron resupply. This accumulation reaches its maximum respectively 6 and 24 hours after the treatment in leaves and roots, and then gradually decreased (Lobréaux et al. 1993). This increase in ferritin mRNA abundance precedes the accumulation of ferritin subunits, with a maximum detected 24 hours after the beginning of the iron treatment. Then, a gradual decrease of ferritin content is observed, consistent with a transient iron buffer function of this protein during the regreening of plants (Lobréaux et al. 1992a). It has been demonstrated that iron resupply to iron starved soybean cell suspension cultures induces a ferritin mRNA accumulation controlled at the transcriptional level (Lescure et al. 1991). In maize, part of this response has been shown to be mediated by an abscissic acid (ABA)-dependent pathway whereas a specific gene (*ZmFer1*) was observed to be iron-regulated in an ABA independent manner. This result was confirmed for *AtFer1*, the *Arabidopsis ZmFer1* gene ortholog (Gaymard et al. 1996). It has been shown that both *AtFer1* and *ZmFer1* iron-dependent gene expression was mediated by a *cis*-regulatory element named IDRS (Iron-dependent Regulatory Sequence) (Petit et al. 2001). The IDRS sequence has also been shown to be required for NO or dark-induced senescence activation of the *Arabidopsis AtFer1* gene expression (Murgia et al. 2002; Tarantino et al. 2003). A different iron-dependent *cis*-regulatory element than the IDRS, named FRE (Fe Responsive Element), has also been characterized in the promoter region of a soybean ferritin gene (Wei J and Theil 2000). So far, no *trans*-acting factors interacting with these *cis*-regulatory sequences have been characterized.

4 Deregulation of iron homeostasis in plants

Iron accumulation in various plant tissues is under genetic control and this is evidenced by alteration of iron homeostasis either in plant mutants altered in Fe signaling or in transgenic plants overexpressing ferritin ectopically.

4.1 Alteration of iron accumulation in Fe-signaling plant mutants

A number of mutants that are altered in Fe status signaling have abnormal iron deficiency response of the roots leading to modified iron accumulation properties. Among these, the pea mutants *brz* (E107) and *dgl* show respectively bronze necrotic spotted leaves and brown degenerative leaves due to hyperaccumulation of iron in this organ. These two non-allelic mutants have a constitutive root ferric reductase activity and are incapable of turning off root iron deficiency responses under iron-replete conditions (Grusak and Pezeshgi 1996; Welch and LaRue 1990). Interestingly, iron-regulated expression seems to follow different pathways in shoots and roots. The pea ferric reductase FRO1 is involved in iron reduction in root epidermal cells and in shoots where its mRNA accumulates under iron deficiency (Waters et al. 2002). But while *FRO1* expression becomes constitutive in the roots of *dgl* and *brz* mutants, its pattern of expression is unchanged in the mutant shoots, indicating that expression of FRO1 is affected by different signals in shoots and roots.

The tomato *chloronerva* (*chln*) mutant has helped elucidating a key regulatory component of non-grasses responses to iron deficiency. *chln* lacks the ability to synthesize nicotianamine (NA) due to a mutation in the gene encoding the enzyme NA synthase that converts S-adenosyl methionine into NA (Ling et al. 1999). Like *brz* and *dgl*, *chln* accumulates high levels of iron in its shoot and root, regardless of the external iron status. But in spite of this, *chln* exhibits morphological and physiological symptoms of iron deficiency. All these characters can be normalized by exogeneous application of NA (Stephan and Grun 1989) or by grafting on wild type rootstock. The pleiotropic phenotype produced by the absence of NA indicates that NA is a key component in iron physiology. NA may play multiple roles including long-distance iron transport *via* phloem loading or unloading as well as iron detoxification *via* compartmentalization into the vacuole. In addition, *chln* is unable to sense its iron status and, like *dgl* and *brz*, cannot turn off the iron deficiency response.

Arabidopsis frd3 mutant was isolated for its inability to turn off the root ferric reductase activity in conditions of iron sufficiency (Yi and Guerinot 1996). *frd3* accumulates a variety of metals including Fe and Mn due to the upregulation of the IRT1 metal transporter (Eide et al. 1996; Delhaize 1996). The *FRD3* gene encodes a transmembrane protein belonging to the Multidrug Associated and Toxin Efflux (MATE) family of efflux transporters (Rogers and Guerinot 2002) and is, therefore, likely to transport small organic molecules. FRD3 seems to be required for the shoot-to-root signaling of the iron status, but because *FRD3* is expressed specifically in roots, it may mediate perception of a shoot-derived signal. FRD3

could repress the root iron deficiency response when activated by a shoot signal released in iron-replete conditions. Alternately, if such a signal is produced in response to iron deficiency, absence of this signal in iron-replete conditions could de-repress *FRD3* activity which would result in inhibition of expression of the iron deficiency response genes. Whether FRD3 acts as a true transporter or whether it is a receptor that binds a signal molecule is not known yet, nor is known the *cis*-regulatory elements controlling expression of the iron deficiency response genes.

4.2 Ferritin overexpression in transgenic plants and its consequences

Our knowledge of the role that ferritins play in plant physiology is still very limited. Their functions have been addressed by evaluating the consequences of their overexpression, either in the plastids (their natural cytological localization) or in the cytoplasm, on plant development and physiology (Deak et al. 1999; Goto et al. 1999; Van Wuytswinkel et al. 1999). An illegitimate ferritin accumulation was obtained in leaves and in seeds. Although no major phenotypic alterations were reported to occur in these transgenic plants, in tobacco leaves grown *in vitro* on a media containing 25 μM Fe(III)-EDTA yellow zones were observed, consistent with a 20% decrease in chlorophyll concentration. Indeed, in these plants, some chloroplasts had an altered sub-structure with diffused thylakoids, and large stromal areas with very weak electron density (Briat et al. 1999).

Iron and oxygen metabolisms can interact to promote oxidative stress. Therefore, iron sequestration in ferritin transformed plants could counteract iron-mediated oxidative stress. Methylviologen acts by promoting an oxidative stress in the chloroplast, leading to proteolysis, lipid peroxidation and ultimately to cell death (Dodge 1994). The toxic effect of methylviologen requires free iron to take place, and can be antagonized by iron chelators such as desferrioxamine (Deak et al. 1999; Goto et al. 1999; Van Wuytswinkel et al. 1999). Indeed plants overexpressing ferritin are more resistant to methylviologen toxicity, confirming that the transgenic ferritins were functional *in vivo, i.e.,* able to sequester iron atoms (Deak et al. 1999; Van Wuytswinkel et al. 1999). However, it has been documented in animal cells, that ferritin can act either as anti- and pro-oxidant (Cairo et al. 1995). Therefore, the increased resistance to methylviologen treatment could have also arisen, at least in part, from a general activation of plant defense against oxidative stress generated in response to illegitimate accumulation of ferritin in leaves. This point was addressed by measuring various enzyme activities involved in oxygen detoxification in leaf discs of control tobacco plants, and tobacco plants overexpressing ferritin. All the enzyme activities measured (catalase, ascorbate peroxidase, gaiacol peroxidase, and glutathione reductase) were indeed increased by 1.5 to 3-fold in the ferritin overexpressors (Briat et al. 1999), suggesting that an oxidative stress occurs in plants overexpressing ferritin.

The major consequence of the ferritin accumulation in transgenic plants was to increase leaf iron concentration by 2 to 3 fold (Goto et al. 1999; Van Wuytswinkel et al. 1999), concomitantly with an increase in root ferric reductase and root H^+-

ATPase activities (Van Wuytswinkel et al. 1999; Vansuyt et al. 2000, 2003), two key determinants of iron uptake by dicotyledonous plants (Curie and Briat 2003). This can be explained by the increased iron storage capacity of the ferritin transformed plants in which excessive iron sequestration disturbs the metabolism, driving leaf physiology towards an iron deficient state. As a consequence, these transgenic plants, sensing an iron deficiency, logically activate their iron uptake systems (Curie and Briat 2003). Such a situation of increased iron uptake in plants which sense their iron status as deficient whereas they paradoxically accumulate too much iron is reminiscent of the phenotype of the *brz* and *dgl* pea mutants (Grusak and Pezeshgi 1996; Marinos 1967), and of the *chloronerva* tomato mutant (Ling et al. 1999).

5 Conclusions

Although physiological responses to iron deficiency or toxicity were known for a long time, it is only recently that a molecular characterization of the primary targets involved, such as transporters and ferritins, were reported. We are now at the beginning of deciphering the regulatory mechanisms that control the expression of these primary targets by sensing the variations of iron availability within the root environment on one hand, and the fluctuations of the plant iron status on the other hand. Undoubtedly, understanding the various signaling pathways controlling iron homeostasis in plants, and how they are integrated to the physiology of the whole plant will bring new exciting findings in a close future.

References

Andrews SC, Arosio P, Bottke W, Briat JF, von Darl M, Harrison P, Laulhere JP, Levi S, Lobreaux S, Yewdall SJ (1992) Structure, function and evolution of ferritins. J Inorg Biochem 1992 47:161-174

Bergersen FJ (1963) Iron in the developing soybean nodules. Aust J Biol Sci 16:916-919

Branton D, Jacobson L (1962) Iron transport in pea plants. Plant Physiol 37:539-545

Briat JF, Fobis-Loisy I, Grignon N, Lobréaux S, Pascal N, Savino G, Thoiron S, von Wiren N, Van Wuytswinkel O (1995) Cellular and molecular aspects of iron metabolism in plants. Biol Cell 84:69-81

Briat JF, Lobréaux S (1997) Iron transport and storage in plants. Trends Plant Sci 2:187-193

Briat JF, Lobréaux S (1998) Iron storage and Ferritin in Plants. In: Sigel A, Sigel H (eds) Iron Transport and Storage in Microorganism, Plants and Animals. Marcel Dekker Inc, New York, pp 563-584

Briat JF, Lobréaux S, Grignon N, Vansuyt G (1999) Regulation of plant ferritin synthesis: how and why. Cell Mol Life Sci 56:155-166

Bruggemann W, Maas-Kantel K, Moog PR (1993) Iron uptake by leaf mesophyll cells: the role of the plasma membrane-bound ferric-chelate reductase. Planta 190:151-155

Buchanan-Wollaston V, Ainsworth C (1997) Leaf senescence in *Brassica napus*: cloning of senescence related genes by substractive hybridisation. Plant Mol Biol 33:821-834

Bughio N, Takahashi M, Yoshimura E, Nishizawa NK, Mori S (1997) Light-dependent iron transport into isolated barley chloroplasts. Plant Cell Physiol 38:101-105

Burton JW, Harlow C, Theil EC (1998) Evidence for reutilization of nodule iron in soybean seed development. J Plant Nutr 21:913-927

Cairo G, Tacchini L, Pogliaghi G, Anzon E, Tomasi A, Bernelli-Zazzera A (1995) Induction of ferritin synthesis by oxidative stress. Transcriptional and post-transcriptional regulation by expansion of the "free" iron pool. J Biol Chem 270:700-703

Cataldo DA, McFadden KM, Garland TR, Wildung RE (1988) Organic constituents and complexation of nickel (II), iron (III), cadmium (II), and plutonium (IV) in soybean xylem exudates. Plant Physiol 86:734-739

Connolly EL, Campbell NH, Grotz N, Prichard CL, Guerinot ML (2003) Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptionalcontrol. Plant Physiol 133:1102-1110

Curie C, Alonso JM, Le Jean M, Ecker JR, Briat JF (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. Biochem J 347:749-755

Curie C, Briat JF (2003) Iron transport and signaling in plants. Ann Rev Plant Biol 54:183-206

Curie C, Panaviene Z, Loulergue C, Dellaporta SL, Briat JF, Walker EL (2001) Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. Nature 18:346-349

Deak M, Horvath GV, Davletova S, Torok K, Sass L, Vass I, Barna B, Kiraly Z, Dudits D (1999) Plants ectopically expressing the iron-binding protein, ferritin, are tolerant to oxidative damage and pathogens. Nat Biotechnol 17:192-196

Delhaize E (1996) A metal-accumulator mutant of *Arabidopsis thaliana*. Plant Physiol 111:849-855

DiDonato RJ Jr, Roberts LA, Sanderson T, Eisley RB, Walker EL (2004) *Arabidopsis* Yellow Stripe-Like2 (YSL2): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine-metalcomplexes. Plant J 39:403-414

Dodge A (1994) Herbicide action and effects on detoxification processes. In: Foyer CH, Mullineaux PM (eds) Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, Florida, pp 219-236

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci USA 28:5624-5648

Forbes JR, Gros P (2001) Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. Trends Microbiol 9:397-403

Gaymard F, Boucherez J, Briat JF (1996) Characterization of a ferritin mRNA from *Arabidopsis thaliana* accumulated in response to iron through an oxidative pathway independent of abscisic acid. Biochem J 318:67-73

Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F (1999) Iron fortification of rice seed by the soybean ferritin gene. Nat Biotechnol 17:282-286

Grusak MA (1994) Iron transport to developing ovules of *Pisum sativum*. Seed: import characteristics and phloem iron-loading capacity of sources regions. Plant Physiol 104:649-655

Grusak MA (1995) Whole-root iron(III)-reductase activity throughout the life cycle of iron-grown *Pisum sativum* L. (Fabaceae): relevance to the iron nutrition of developing seeds. Planta 197:111-117

Grusak MA, Pezeshgi S (1996) Shoot-to-root signal transmission regulates root Fe(III) reductase activity in the *dgl* mutant of pea. Plant Physiol 110:329-334

Guerinot ML (2000) The ZIP family of metal transporters. Biochim Biophys Acta 1465:190-198

Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochem Biophys Acta 1275:161-203

Hentze MW, Muckenthaler MU, Andrews NC (2004) Balancing acts: molecular control of mammalian iron metabolism. Cell 117:285-297

Hocking PJ, Pate JS (1978) Mobilization of minerals to developing seeds of legumes. Ann Bot 41:1259-1278

Kaiser BN, Moreau S, Castelli J, Thomson R, Lambert A, Bogliolo S, Puppo A, Day DA (2003) The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. Plant J 35:295-304

Kispal G, Csere P, Prohl C, Lill R (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. EMBO J 18:3981-3989

Krueger C, Berkowitz O, Stephan UW, Hell R (2002) A metal-binding member of the late embryogenesis abundant protein family transports iron in the phloem of *Ricinus communis* L. J Biol Chem 277:25062-25069

Kushnir S, Babiychuk E, Storozhenko S, Davey MW, Papenbrock J, De Rycke R, Engler G, Stephan UW, Lange H, Kispal G, Lill R, Van Montagu M (2001) A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the *Arabidopsis* mutant starik. Plant Cell 13:89-100

Ko MP, Huang PY, Huang JS, Baker KR (1987) The occurrence of phytoferritin and its relationship to effectiveness of soybean nodules. Plant Physiol 83:299-305

Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant J 39:415-424

Laulhère JP, Labouré AM, Briat JF (1989) Mechanism of the transition from plant ferritin to phytosiderin. J Biol Chem 264:3629-3635

Laulhère JP, Labouré AM, Briat JF (1990) Photoreduction and incorporation of iron into ferritins. Biochem J 269:79-84

Lescure AM, Proudhon D, Pesey H, Ragland M, Theil EC, Briat JF (1991) Ferritin gene transcription is regulated by iron in soybean cell cultures. Proc Natl Acad Sci USA 88:8222-8226

Levier K, Day DA, Guerinot ML (1996) Iron uptake by symbiosomes from soybean root nodules. Plant Physiol 111:893-900

Lill R, Kispal G Maturation of cellular Fe-S proteins: an essential function of mitochondria. Trends Biochem Sci 2000 25:352-356

Ling HQ, Bauer P, Bereczky Z, Keller B, Ganal M (2002) The tomato *fer* gene encoding a bHLH protein controls iron-uptake responses in roots. Proc Natl Acad Sci USA 99:13938-13943

Ling HQ, Koch G, Baumlein H, Ganal MW (1999) Map-based cloning of *chloronerva*, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. Proc Natl Acad Sci USA 96:7098-7103

Lobréaux S, Briat JF (1991) Ferritin accumulation and degradation in different organs of pea (*Pisum sativum*) during development. Biochem J 274:601-606

Lobréaux S, Hardy T, Briat JF (1993) Abscisic acid is involved in the iron-induced synthesis of maize ferritin. EMBO J 12:651-657

Lobréaux S, Massenet O, Briat JF (1992a) Iron induces ferritin synthesis in maize plantlets. Plant Mol Biol 19:563-576

Lobréaux S, Yewdall S, Briat JF, Harrison PM (1992b) Amino-acid sequence and predicted three-dimensional structure of pea seed (*Pisum sativum*) ferritin. Biochem J 288:931-939

Maas FM, Wetering DA, Beusichem ML, Bienfait HF (1988) Characterization of phloem iron and its possible role in the regulation of Fe-efficiency reactions. Plant Physiol 87:167-171

Marentes E, Grusak MA (1998) Iron transport and storage within the seed coat and embryo of developing seeds of pea (*Pisum sativum* L.). Seed Sci Res 8:367-375

Marinos NG (1967) Multifunctionnal plastids in the meristematic region of potato tuber buds. J Ultrastr Res 17:91-113

Moreau S, Day DA, Puppo A (1998) Ferrous iron is transported across the peribacteroid membrane of soybean nodules. Planta 207:83-87

Moreau S, Meyer JM, Puppo A (1995) A. Uptake of iron by symbiosomes and bacteroids from soybean nodules. FEBS Lett 361:225-228

Murgia I, Delledonne M, Soave C (2002) Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. Plant J 30:521-528

Petit JM, Van Wuytswinkel O, Briat JF, Lobreaux S (2001) Characterization of an iron-dependent regulatory sequence (IDRS) involved in the transcriptional control of *AtFer1* and *ZmFer1* plant ferritin genes by iron. J Biol Chem 276:5584-5590

Pich A, Manteuffel R, Hillmer S, Scholz G, Schmidt W (2001) Fe homeostasis in plant cells: does nicotianamine play multiple roles in the regulation of cytoplasmic Fe concentration? Planta 213:967-976

Portnoy ME, Liu XF, Culotta VC (2000) *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the nramp family of metal transporters. Mol Cell Biol 20:7893-7902

Ragland M, Briat JF, Gagnon J, Laulhère JP, Massenet O, Theil EC (1990) Evidence for conservation of ferritin sequences among plants and animals and for a transit peptide in soybean. J Biol Chem 265:18339-18344

Ragland M, Theil EC (1993) Ferritin (mRNA, protein) and iron concentrations during soybean nodule development. Plant Mol Biol 21:555-560

Roberts LA, Pierson AJ, Panaviene Z, Walker EL (2004) Yellow stripe1. Expanded roles for the maize iron-phytosiderophore transporter. Plant Physiol 135:112-120

Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric-chelate reductase for iron uptake from soils. Nature 397:694-6971

Rogers EE, Guerinot ML (2002) FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in *Arabidopsis*. Plant Cell 14:1787-1799

Schaaf G, Ludewig U, Erenoglu BE, Mori S, Kitahara T, von Wiren N (2004) ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. J Biol Chem 279:9091-9096

Schmidke I, Kruger C, Frommichen R, Scholz G, Stephan U (1999) Phloem loading and transport characteristics of iron in interaction with plant-endogenous ligands in castor bean seedlings. Physiol Plant 106:82-89

Shingles R, North M, McCarty RE (2002) Ferrous ion transport across chloroplast inner envelope membranes. Plant Physiol 128:1022-1030

Seckback JJ (1982) Ferreting out the secret of plant ferritin - A review. J Plant Nutr 5:369-394

Stephan UW, Grun M (1989) Physiological disorders of the nicotianamine-auxotroph tomato mutant *chloronerva* at different levels of iron nutrition. II. Iron deficiency response and heavy metal metabolism. Biochem Physiol Pflanzen 185:3-4

Stephan U, Schmidke I, Pich A (1994) Phloem translocation of Fe, Cu, Mn and Zn in *Ricinus* seedlings in relation to the concentration of nicotianamine, an endogenous chelator of divalent metal ions, in different seedling parts. Plant Soil 165:181-188

Stephan UW, Scholz G (1993) Nicotianamine: mediator of transport of iron and heavy metals in the phloem. Physiol Plant 88:522-529

Strozycki PM, Skapska A, Szczesniak K, Sobieszczuk E, Briat JF, Legocki AB (2003) Differential expression and evolutionary analysis of the three ferritin genes in the legume plant *Lupinus luteus*. Physiol Plant 118:380-389

Tang C, Robson AD, Dilworth MJ (1990) The role of iron in nodulation and nitrogen fixation in *Lupinus augustifolius L*. New Phytol 115:61-67

Tarantino D, Petit JM, Lobreaux S, Briat JF, Soave C, Murgia I (2003) Differential involvement of the IDRS *cis*-element in the developmental and environmental regulation of the *AtFer1* ferritin gene from *Arabidopsis*. Planta 217:709-716

Theil EC, Hase T (1993) Plant and microbial ferritins. In: LL. Barton, BC Hemming (eds). Iron chelation in Plants and Soil Microorganisms, Academic Press, San Diego, pp133-156

Thomine S, Lelievre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H (2003) AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. Plant J 34:685-695

Thomine S, Wang R, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. Proc Natl Acad Sci USA 97:4991-4996

Van der Mark F, Van der Briel W, Huisman HG (1983) Phytoferritin is synthesized *in vitro* as a high-molecular-weight precursor. Biochem J 214:943-950

Vansuyt G, Mench M, Briat JF (2000) Soil-dependent variability of leaf iron accumulation in transgenic tobacco overexpressing ferritin. Plant Physiol Biochem 38:499-506

Vansuyt G, Souche G, Straczek A, Briat JF, Jaillard B (2003) Flux of proton released by wild type and ferritin over-expressor tobacco plants: effect of phosphorus and iron nutrition. Plant Physiol Biochem 41:27-33

Van Wuytswinkel O, Vansuyt G, Grignon N, Fourcroy P, Briat JF (1999) Iron homeostasis alteration by ferritin overexpression in transgenic tobacco. Plant J 17:93-98

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briat JF, Curie C (2002) IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. Plant Cell 14:1223-1233

Vert G, Briat JF, Curie C (2001) *Arabidopsis* IRT2 gene encodes a root-periphery iron transporter. Plant J 26:181-189

Vert G, Briat JF, Curie C (2003) Dual regulation of the *Arabidopsis* high-affinity root iron uptake system by local and long-distance signals. Plant Physiol 132:796-804

von Wiren N, Klair S, Bansal S, Briat JF, Khodr H, Shioiri T, Leigh RA, Hider RC (1999) Nicotianamine chelates both FeIII and FeII. Implications for metal transport in plants. Plant Physiol 119:1107-1114

Waters BM, Blevins DG, Eide DJ (2002) Characterization of FRO1, a pea ferric-chelate reductase involved in root iron acquisition. Plant Physiol 129:85-94

Wei J, Theil EC (2000) Identification and characterization of the iron regulatory element in the ferritin gene of a plant (soybean). J Biol Chem 275:17488-17493

Welch RM, LaRue TA (1990) Physiological characteristics of Fe accumulation in the "bronze" mutant of *Pisum sativum* L., cv "Sparkle" E107 (*brz brz*). Plant Physiol 93:723-729

Wittenberg JB, Wittenberg BA, Day DA, Udvardi MK, Appleby CA (1996) Siderophore-bound iron in the peribacteroid space of soybean root nodules. Plant Soil 178:161-169

Yi Y, Guerinot ML (1996) Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. Plant J 10:835-844

Young TF, Terry N (1982) Transport of iron into leaves following iron resupply to iron-stressed sugar beet plants. J Plant Nutr 5:1273-1283

Briat, Jean-François

Biochimie et Physiologie Moléculaire des Plantes. Centre National de la Recherche Scientifique (UMR 5004), Institut National de la Recherche Agronomique, Université Montpellier 2, École Nationale Supérieure d'Agronomie, 2 Place Viala, F-34060 Montpellier cedex 2, France
briat@ensam.inra.fr

Functions and homeostasis of zinc, copper, and nickel in plants

Ute Krämer and Stephan Clemens

Abstract

Nutritional micronutrient deficiencies and exposure to pollutant metals threaten human health globally. Plant crops are at the beginning of a food chain that largely determines food metal contents. In order to survive, all organisms have to supply appropriate amounts of each micronutrient to the correct target apometalloproteins and at the same time avoid adventitious metal binding to non-target metal binding sites or other cellular compounds. This requires the operation of metal homeostasis networks, which orchestrate the mobilization, uptake, distribution, intracellular trafficking, chelation, and sequestration of all metal ions. Presumably as a result of time-dependent and local variations in bioavailable soil metal concentrations, plant metal homeostasis networks exhibit a remarkably high degree of plasticity and natural diversity. This is a review covering the current knowledge of metal-dependent processes and proteins, metal homeostasis and its regulation, and the molecular mechanisms underlying naturally selected metal hypertolerance and metal hyperaccumulation in higher plants.

1 Introduction

At all times in evolution, life forms have been exposed to chemical environments of often fluctuating compositions, from which available inorganic nutrients were eventually selected to perform essential biochemical functions (Fraústo da Silva and Williams 2001). The chemical composition of the biosphere varies locally and can change profoundly over time. The most notable change occurred when the Earth's atmosphere became oxygenic, which led to a radical change in the availability of a number of transition metal ions for life on Earth. This dramatically reduced the bioavailability of some transition metals – primarily iron - and made other transition metals more available – primarily copper. For the latter group, it can be postulated that the evolution of detoxification systems was dominant initially and that the use in biochemical functions evolved later. Indeed, the use of transition metal ions for biochemical functions has been extremely successful: about one third of all structurally characterized proteins are metalloproteins

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_96 / Published online: 2 August 2005

(Finney and O'Halloran 2003). Today several transition metals[1], namely iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), molybdenum (Mo), and nickel (Ni), are known to be essential micronutrients for living higher plants (Marschner 1995). The list of essential transition metals may grow as more and more protein structures are elucidated and analytical techniques as well as the purity of chemicals are being continuously improved. This may apply for cobalt (Co), which is currently considered a beneficial element (Marschner 1995), and cadmium (Cd), which acts as the cofactor of a carbonic anhydrase isoform produced under Zn-deficient conditions in the diatom *Thalassiosira weissflogii* (Lane and Morel 2000).

As an approximate, a search of the *Arabidopsis* Information Resource (TAIR) database protein descriptions retrieved 1230 proteins predicted to contain, bind, or transport Zn(II), including, among others, a large number of Zn-finger containing proteins and transcription factors, oxidoreductases, and hydrolytic enzymes such as metalloproteases. The same search retrieved 105 proteins for Cu and three proteins for Ni. To date, most information is derived from characterized metalloprotein homologues in other organisms, and plant-specific direct experimental evidence of the use of a specific metal ion in a given protein is scarce.

The chemical properties that made transition metal ions indispensable for life were their ability to undergo changes in redox state under biological conditions and to establish and maintain several stable coordinative bonds to electron pair donor atoms of organic ligands in a defined geometry. These properties of transition metal ions, however, pose a serious risk as soon as their interaction and binding partners are not fully controlled. Metal-induced uncontrolled redox reactions or the deactivation or modification of functional groups of organic molecules can endanger the survival and reproduction of an organism. It is, thus, not surprising that all organisms possess a tightly knit metal homeostasis network that serves to maintain concentrations of metal ions within physiological limits. Notably, different transition metal ions possess different chemical properties, i.e., different redox potential, coordination geometry, charge and thermodynamic and kinetic properties of ligand exchange. In a given metalloenzyme, a specific metal ion is thus used for a specific chemical function. However, according to the Irving-Williams series ($Zn^{2+} < Cu^{+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$) metal ions bind to organic ligands, such as those in a metal-binding site of an apometalloprotein, with different affinities (Nieboer and Richardson 1980; Fraústo da Silva and Williams 2001). According to this, Cu ions can bind to metal binding sites of non-Cu metalloproteins, and so can each metal ion replace other metal ions down-

[1] In biology, only the oxidized forms of transition metals (and not the elemental forms) are relevant. In this chapter, the element names, e.g., zinc or Zn refer to the elements in their biologically relevant oxidation states, for example, the oxidation state +II for Zn [i.e. Zn is equivalent to Zn(II)]. However, biologically redox-active metals such as Cu occur in different oxidation states in biological systems. We specify the oxidation state, e.g. Cu(I), only to put a special emphasis on it, or when referring explicitly to one of several biologically relevant oxidation states, as for Cu(I) or Cu(II). The cationic form, e.g., Zn^{2+} is used to denote the free aqueous cation of a transition metal.

stream in the Irving-Williams series. Although binding affinity for a metal ion is also determined by other secondary factors, for example, the size of the metal binding site cavity in a protein, the geometry of ligand atoms and other characteristics, one of the predicted consequences is that Cu ions, for instance, must not be available in cells in free form. Thus, the metal homeostasis system has to control very tightly the availability of metal ions in the cell and has to orchestrate the specific delivery of metal ions to their respective apometalloproteins. A further implication of the chemical principles illustrated by the Irving-Williams series is that metal homeostasis of one transition metal ion should generally not be considered in isolation, but always in the context of all metal cations and their respective concentrations.

Within the metal homeostasis network, metal ions generally undergo three types of processes: (1) transport across biomembranes mediated by metal transport proteins, (2) chelation by low-molecular-weight chelator molecules, and (3) controlled binding to specific proteins. Interestingly, some of the molecular components mediating these processes are highly conserved across organisms both functionally and structurally. In particular, this is the case for metal-binding and metal transporter proteins.

At the cellular level, the plant metal homeostasis network involves the modification of the solubility of extracellular metal ions by electron transfer, chelation or acidification of the apoplast, the uptake of metal ions, chelation and/or the trafficking within the cell, delivery into cellular compartments and organelles, and storage or efflux of metal ions under conditions of excess (Clemens et al. 2002). Metal-specific sensing and regulation mechanisms are likely to be in place to coordinate these processes depending on the metal supply, and with the metabolic state and the life cycle of the cell. Chloroplasts and mitochondria are organelles of high metal ion demand due to the involvement of metallic cofactors in electron transport chains and other proteins. The large central vacuole of plant cells is used as a compartment for metalloenzymes and for metal storage.

In a complex multicellular organism like a higher plant, metal status has to be communicated between cells and organs, according to their requirements, which depend on their specialized functions and the developmental stage of the organism. When the external metal supply changes, metal sinks or priorities are defined among the cell types and organs, and long-distance transport and reallocation of metals between cells and organs is common. This requires long-distance communication and an additional layer of signaling and regulatory components.

In recent years, aided by the completion of sequencing of the first higher plant genomes, complete sets of all members of a number of protein families involved in metal homeostasis have been identified (Mäser et al. 2001). This has been supported by rapid progress in the understanding of metal homeostasis, especially in non-photosynthetic, but also in photosynthetic unicellular model systems. Using mutant strains of unicellular models, like the yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, as expression systems, the functions of a number of newly identified plant proteins in cellular metal homeostasis have been clarified (Grotz et al. 1998; Clemens and Simm 2003). First insights have also been obtained concerning the function of a few of these proteins in metal homeostasis at

the whole-plant level. However, large gaps remain in our understanding of metal homeostasis in plants.

Why is it important and worthwhile to study metal homeostasis in plants? The micronutrient intake of a large proportion of the World's population is insufficient. For example, the WHO estimates that 66 to 80% of the World's population is iron-deficient, with 30% suffering from iron-deficiency-induced anemia. One third of the World's population is thought to be at risk of mild to moderate Zn deficiency (Adamson 2004). Since plants are a major entry point for essential micronutrients into the food chain, the understanding of what controls metal accumulation, localization, and binding forms is essential for devising strategies to improve human micronutrient nutrition. Large areas of agricultural soils are micronutrient-deficient because of low concentrations or low availability of micronutrients. For example, Zn deficiency is common in soils in the Middle East, India, and in parts of Australia, America, and Central Asia (Robson 1993). To obtain good yields, farmers have to fertilize these soils with Zn. Alternatively, scientific knowledge on metal homeostasis could one day be employed to breed more micronutrient-efficient crops.

Since the beginning of the industrial age, human activities have resulted in an accelerating change of the elemental composition of the biosphere through the release of large amounts of potentially toxic trace metals, such as Zn, Cd, and lead (Pb) (Nriagu and Pacyna 1988). Cd and Pb ions are chemically similar to other divalent cations and can enter plants by competing with uptake pathways for macronutrient and micronutrient metal cations. The metal homeostasis networks of most plants are not equipped to be confronted with these transition metal ions at high concentrations so that plants can encounter metal toxicity. The entry of these toxic metal ions into the food chain is a source of great concern in metal-polluted areas. A better understanding of the localization and detoxification pathways for these metals within plants could help to reduce the environmental damage and health risks associated with metal polluted soils. Moreover, since plants have the basic capability of extracting inorganic ions from soils and accumulating them in their biomass, an improved understanding of plant metal homeostasis can aid in the development of cost-effective plant-based technologies for the clean-up of polluted soils (Salt et al. 1998)(Chapter 11).

In addition to these applied aspects, plants are a source of unparalleled biodiversity of metal homeostasis networks among higher eukaryotes. Thus, the investigation of metal homeostasis in plants promises insights into the functioning and regulation of complex metal homeostasis networks. Extreme metal tolerance as well as extraordinary levels of metal accumulation can be found among plant communities on geologically or anthropogenically metal-enriched soils (Salt and Krämer 2000). Nutrient-poor soils host plant taxa possessing highly powerful nutrient acquisition systems. Plant metal homeostasis networks must exhibit a high level of plasticity in order to accommodate enormous fluctuations in metal ion availability encountered during growth of roots in soil, both in space and time. For example, an increase in the pH of a typical soil by one pH unit is accompanied by an approximately 1000-fold decrease in iron availability (Buchanan et al. 2000). Only recently have researchers begun to explore the potential of genetically dis-

secting the vast differences in metal homeostasis that can be found among taxonomically closely related plant varieties and species (Lahner et al. 2003; Dräger et al. 2004; Vreugdenhil et al. 2004).

Here we review our current state of knowledge of plant metal homeostasis, focusing on Cu as a transition metal that can change between the oxidation states Cu(I) and Cu(II) and, thus, participates in biological redox chemistry, and on Zn(II), a metal that only occurs in a single oxidation state in biology. Especially Cu, but to a lesser extent also Zn, have become more available upon the transition from an oxygen-free to an oxygen-containing atmosphere during the evolution of metal biochemistry and metal homeostasis networks. Both Cu and Zn are required in all organelles of a plant cell. We also consider the essential element Ni, the biochemical importance of which is thought to have decreased as a result of the increased availability of other transition metal ions for biological chemistry.

2 Requirement, acquisition, and trafficking of Zn, Cu, and Ni in plants

2.1 Requirement of Zn, Cu, and Ni in plants

Zinc is predominant in the transcriptional and translational machinery, where it has been estimated to account for 12 to 50% of all cellular Zn (Finney and O'Halloran 2003). Critical Zn deficiency concentrations in leaves are given as 15 to 20 $\mu g\ g^{-1}$ dry biomass by Marschner (1995). Zinc is toxic at concentrations above between 100 and 300 $\mu g\ g^{-1}$. These values are only general guidelines. Different species and even varieties of the same species differ in their Zn efficiency, i.e., the ability to maintain growth and yield under Zn-limiting conditions (Graham and Rengel 1993). In comparisons among selected bean and wheat cultivars, respectively, Zn efficiency was found to reside primarily in the ability of the leaves to maintain expression and activity of Zn-requiring enzymes at low total leaf Zn concentrations (Hacisalihoglu et al. 2003, 2004). The rates and affinities of high and low-affinity Zn uptake systems in the root were similar (0.6 to 2 nM and 2 to 5 $\mu M\ Zn^{2+}$, respectively) in two wheat varieties irrespective of differing Zn efficiencies (Hacisalihoglu et al. 2001).

Visible Zn deficiency symptoms range from initial early senescence of old leaves or slight yellowing of younger leaves to the formation of yellow chlorotic or even necrotic areas on leaves. Severely Zn-deficient plants appear stunted and exhibit reduced elongation and tip growth (see Marschner 1995). In wheat, the stem and the growing zones of the plant, i.e., the root tips and the meristematic region at the base of the leaves, are the predominant sinks for $^{65}Zn^{2+}$ applied to the cut surface of a leaf blade (Haslett et al. 2001). The published accounts are consistent with a high requirement for Zn in dividing and elongating plant cells (see also Marschner 1995). Biochemically, 100 $\mu g\ g^{-1}$ and 70 $\mu g\ g^{-1}$ Zn in dry biomass are necessary to prevent the disintegration of 80S ribosomes in rice meristems and tobacco cells, respectively. At lower Zn concentrations, biomass production may be

reduced or arrested because a reduction in protein concentrations has been observed only at lower tissue Zn concentrations of about 25 μg g^{-1} in rice meristems, 50 μg g^{-1} in tobacco cells and approximately 13 μg g^{-1} in the shoot apex of bean plants (Marschner 1995). The *A. thaliana hma2-2hma4-1* double mutant, which has a defect in Zn translocation from the root to the shoot (Hussain et al. 2004), is a bushy plant with short stunted inflorescence bolts producing shortened internodes. Reproductive organs have an absolute requirement for Zn. Flowers contain higher concentrations of Zn than other plant parts (Katrin Voigt and Ute Krämer, unpublished data), and Zn is accumulated in specific subcellular structures of the plant embryo (Otegui et al. 2002). Although Zn accumulation in pollen is not generally high (Orzaez Villanueva et al. 2001), pollen production is impaired in the *A. thaliana hma2-2hma4-1* double mutant (Hussain et al. 2004). The development of embryos and seeds upon cross-pollination of *hma2hma4* plants with wild type pollen is also disrupted. The growing tip of pollen tubes was reported to contain 150 μg g^{-1} Zn, a concentration that is threefold higher than that in basal pollen tube regions (see also Marschner 1995).

The Zn ion has been selected for functions in biological chemistry because of several unique features of its chemistry (Berg and Shi 1996; Fraústo da Silva and Williams 2001). Firstly, the Zn ion is a strong Lewis acid, inferior only to Cu(I) and Cu(II) ions among the transition metal micronutrients, and it exhibits high binding affinity for soft bases, such as sulphide ligands, as well as for hard bases, such as amino, carboxylate, and hydroxyl ligands. Secondly, Zn(II) occurs in a single oxidation state in all biology. Consequently, the use of Zn(II) bears no risk of initiating free radical reactions. Thirdly, the coordination geometry of Zn(II) is more flexible than that of most other transition metal cations, and Zn is the only transition metal ion readily entering a tetrahedral coordination geometry. Thus, Zn ions are suitable for catalysis of reactions, during which ligands may move between different coordination geometries around the central Zn ion without ligand exchange. In addition, Zn selectivity can be enhanced by a tetrahedral coordination geometry, such as, for example, in most Zn fingers. Fourthly, ligand exchange for Zn(II) is fast when compared to ions of related chemical properties, i.e. Ni(II), Co(II), Cu(II), although it is several orders of magnitude slower than for the signaling ion Ca^{2+} (Fraústo da Silva and Williams 2001).

The chemical properties of Zn are reflected in its functions in biological chemistry. As Zn(II) is a strong Lewis acid with flexible geometry and fast ligand exchange, it is used for catalysis of hydrolytic and oxidoreductase reactions. As a strong binder devoid of redox activity Zn is also abundantly used for structural functions within proteins and in protein-protein interactions. The abundance of Zn in DNA handling functions is particularly striking. Finally, the lack of redox activity and the relatively fast ligand exchange of Zn could render Zn^{2+} suitable to function as a slow signaling ion, signaling modulator or "hormone" (Fraústo da Silva and Williams 2001). In the vertebrate central nervous system Zn^{2+} ions act as a modulator of neurotransmission (Baranano et al. 2001). Several surveys of proteins containing Zn ions with a catalytic or structural role and of their respective coordination geometries have been published elsewhere (Coleman 1998; Auld 2001). Here we focus mainly on novel and on plant-specific functions of Zn.

It has to be noted that the prominent role of Zn in DNA regulation, as exemplified by the abundance of Zn finger-containing proteins, appears to be largely restricted to eukaryotes, and is virtually absent in bacteria and archaea (Clarke and Berg 1998). For example, among the predicted proteins of *A. thaliana*, 4% contain at least one Zn finger (Kawagashira et al. 2001), a motif common in transcription factors as well as in protein-protein interaction domains. This eukaryote bias may reflect the increase in Zn bioavailability upon transition from a sulphide-rich to an oxygen-rich biosphere. At the subcellular level, the nucleus and the nucleoli can be predicted to contain high concentrations of Zn. In addition to numerous transcription factors, a number of enzymes in nucleic acid synthesis and maintenance contain Zn, for example, all type I, II, and III RNA polymerases present in plants, DNA polymerases, histone deacetylases and some splicing factors. Plant chloroplasts and mitochondria possess additional RNA polymerases homologous to the T7 phage RNA polymerase, which are independent of Zn. In plants, 456 editing events have been reported in mitochondrially encoded mRNAs (Giege and Brennicke 1999), and approximately 30 in chloroplast mRNAs (Sasaki et al. 2003). The editing of RNA involves the conversion predominantly of specific C into U nucleotides. The enzyme likely to perform this reaction, cytidine deaminase, is a Zn-dependent enzyme, although its involvement in plant RNA editing is still under debate (Mulligan et al. 1999; Takenaka and Brennicke 2003). There is no doubt, however, that overall Zn is important for plastid and mitochondrial nucleic acid metabolism and modification.

Zn also has a central role in the cytoplasm, primarily in the process of translation and as a cofactor of a number of tRNA synthetases. For example, the translation initiation factor elF-5 from maize exhibits RNA-binding activity dependent on the binding of Zn to its $CX_2CX_{18}CX_2C$ Zn finger motif (Lopez Ribera and Puigdomenech 1999). According to the functions of homologous eukaryotic proteins, the interaction of elF-5 with the 40S initiation complex, followed by GTP hydrolysis, is required for the formation of a functional 80S initiation complex. Translation in mitochondria and in chloroplasts is possibly less dependent on Zn. Interestingly, in bacteria Zn-dependent proteins of the translational machinery are replaced by Zn-independent isoforms under Zn deficiency (Panina et al. 2003).

Zn is required in processes leading to protein degradation. An example for the involvement of Zn in protein-protein interactions is the presence of a RING finger motif, $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}CX_2CX_{(4-48)}CX_2C$ (C3HC4-type) binding 2 Zn ions, in the constitutive photomorphogenic protein (COP1) of *A. thaliana* and other members of its class of ubiquitin E3 ligases (Torii et al. 1999; Saijo et al. 2003). The activity of the COP1 protein is required for the COP9-signalosome (CSN) mediated degradation of specific transcription factors in the dark (Schwechheimer et al. 2002; Sullivan et al. 2003). A query of the *Arabidopsis* proteome with the search term "ring finger" (TAIR) retrieves 451 proteins containing RING finger motifs of the C3HC4 or the C3H2C3 (RING-H2) type. The SCF (SKP1 – Cul1 – F-box protein) ubiquitin E3 ligase complex, which is thought to be regulated by the COP9 signalosome, also contains a RING finger-containing protein (RBX1).

Another example for the involvement of Zn in protein-protein interactions is the 60-amino-acid CHORD (cys- and his-rich domain) Zn-binding motif present in duplicate (CHORD-I and CHORD-II) in the barley Rar1 protein and orthologous proteins of other organisms (Shirasu et al. 1999). Plant Rar1-like proteins act downstream of pathogen perception and upstream of H_2O_2 production in pathogen resistance signaling (Liu et al. 2002). The CHORD-I domain of plant Rar1 is able to interact with the SGT1 protein, which itself interacts with the SCF (SKP1 – cullin – F-box protein) ubiquitin E3 ligase complex and with components of the COP9 signalosome multiprotein complex (Azevedo et al. 2002). The CHORD-II domain of Rar1 was reported to interact with the heat shock protein HSP90 (Takahashi et al. 2003a).

Not only do a number of protein-protein interactions associated with the ubiquitin 26S proteasome pathway require Zn, but Zn-dependent proteins are also of key importance in proteolytic processes in this pathway. Metalloproteases containing a Zn-binding Jab1/MPN-domain-associated metalloisopeptidase motif (JAMM), $EX_nHXHX_{10}D$, are necessary for the deneddylation and deubiquitination activities of the COP9 signalosome (CSN5 subunit) and mediate the deubiquitination activity of the lid complex of the 19S regulatory particle (RPN11 subunit) of the 26S proteasome (Sullivan et al. 2003; Wei and Deng 2003). These activities are required for the biological activities of the respective protein complexes and for the degradation of target proteins (Yao and Cohen 2002).

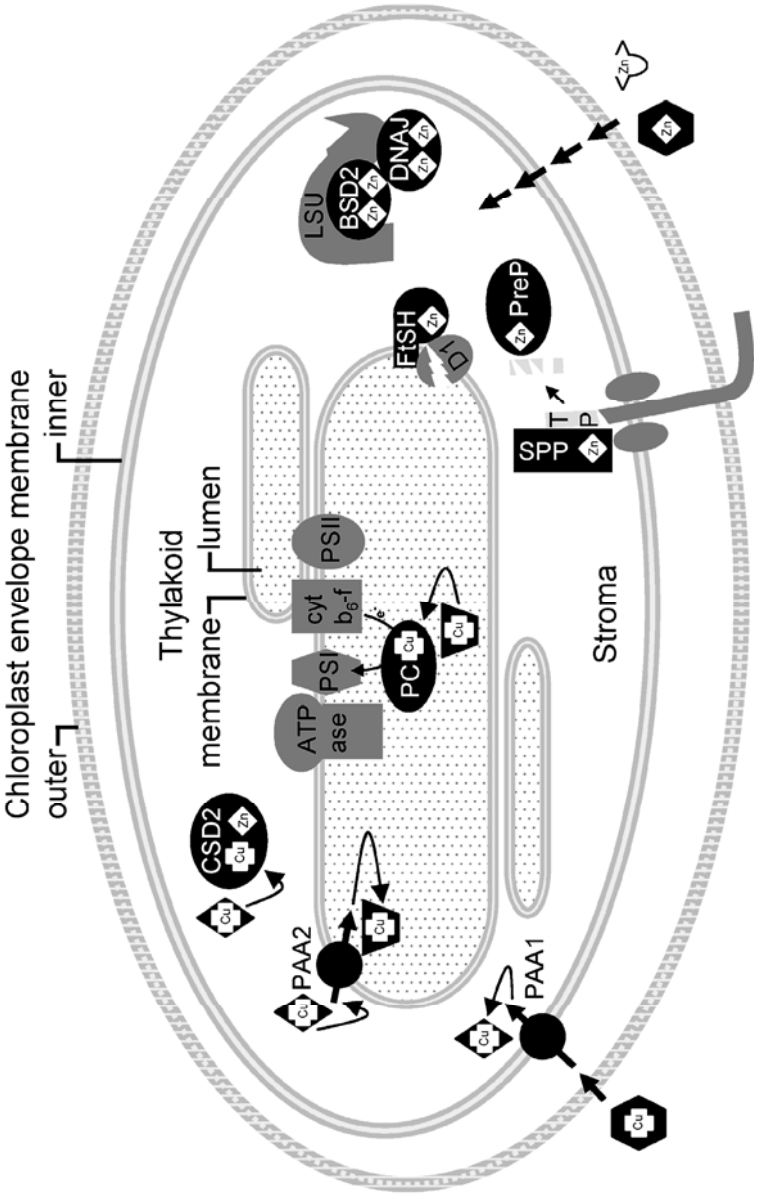
In mammals, the lysosome is a subcellular compartment exhibiting high Zn levels. This is probably due to the use of Zn in a number of hydrolytic enzymes, namely nucleases, carboxypeptidases, aminopeptidases (Bartling and Weiler 1992), and thermolysin (astacin) family proteases. In plants, vacuoles and the apoplast are also major compartments for hydrolytic enzymes. Some examples for known Zn-dependent hydrolytic enzymes of plants are the vacuolar Zn enzyme α-mannosidase (Snaith and Levvy 1968), plant Zn-dependent carboxypeptidases, purple acid phosphatases containing a binuclear Fe(III)-Zn(II) or Fe(III)-Mn(II) metallocenter (Li et al. 2002), and a protein family of at least five plant matrix metalloproteinases with a potential involvement in modulation of the plant extracellular matrix (Maidment et al. 1999). Matrix metalloproteinases belong to the metzincin superfamily of proteinases, and exhibit a characteristic Zn binding motif (HEXGHXXGXXH).

Plant chloroplasts contain a comparably small number of Zn-dependent proteins, which, however, perform crucial functions (Fig. 1; see also above). Approximately 3,500 plant proteins are encoded in the nuclear genome and have to be imported into the chloroplast after their translation. Recent work has implicated several Zn-dependent proteases in chloroplast protein import, as well as in the degradation of proteins following light-dependent damage (Adam and Clarke 2002). During plastid protein import, N-terminal chloroplast targeting sequences of proteins are cleaved off in an ATP-dependent manner by the Zn-dependent stromal processing peptidase SPP, which contains a characteristic HXXEH motif. The SPP protein belongs to the same metallopeptidase family as the mitochondrial matrix processing peptidase (MPP), which performs the analogous function in mitochondria (Luciano et al. 1998; Richter and Lamppa 1999, 2003). It should be

mentioned that carrier proteins of the mitochondrial inner membrane are imported by a distinct pathway, which involves a Zn-dependent function in the intermembrane space (Lister et al. 2002). After the targeting sequences have been cleaved off, they have to be proteolytically degraded, because an accumulation of these peptides disrupts organellar function (Stahl et al. 2002). This task is performed in the mitochondrial matrix by a Zn metalloprotease of the pitrilysin family (potato PreP), with a characteristic Zn-binding motif ($HILEHX_{74}E$). The PreP protein and its *A. thaliana* orthologue were shown to be dually targeted to both mitochondria and chloroplasts and to degrade both mitochondrial presequences and chloroplast targeting peptides (Stahl et al. 2002; Bhushan et al. 2003; Moberg et al. 2003).

Inside the stroma, the activities of both of two thylakoid membrane-anchored Zn metalloproteases of the AAA (ATPase associated with diverse cellular activities) ATPase family, FtsH2 (AtVAR2) and FtsH5 (AtVAR1) are crucial for the development of normal chloroplasts. *A. thaliana* mutants in either gene display a variegated[2] phenotype, which is more severe in a *var1var2* double mutant (Lindahl et al. 1996; Chen et al. 2000; Takechi et al. 2000; Sakamoto et al. 2002). Moreover, *var1* and *var2* mutants are hypersensitive to photoinhibition (Bailey et al. 2002; Sakamoto et al. 2002). The role in chloroplast development of VAR1 and VAR2 is not fully understood to date. The *Arabidopsis* chloroplast FtsH1 (Lindahl et al. 2000) and FtsH2 proteins (Bailey et al. 2002) are capable of turning over photo-damaged D1 protein (psbA) of photosystem II. The D1 protein is turning over rapidly in the light because it incurs irreversible damage from reactive oxygen species formed in photosystem II under conditions of photoinhibition. Damaged D1 protein is cleaved into a 10 kDa and a 23 kDa fragment by the serine protease DegP2 localized on the stromal side of the thylakoid membrane. Lindahl et al. (2000) showed that the 23 kDa fragment of D1 can be degraded by FtsH1 *in vitro*. Following photoinhibitory irradiance, cleavage of D1 protein occurs in wild type *A. thaliana*, but is disrupted in the *var2-2* mutant (Bailey et al. 2002). FtsH proteins have also been implicated in the degradation of other unassembled proteins in the stroma. There are 12 FtsH proteins in *A. thaliana*, all possessing two N-terminal transmembrane helices and a C-terminal protease domain containing an HEXXH motif. Eight of the *A. thaliana* FtsH proteins are thought to localize to the chloroplast. VAR1 and VAR2 occur together in a protein complex (Sakamoto et al. 2003), which appears to also contain subunits of the less abundant FtsH1 and FtsH8 proteins, presumably in a heterohexameric structure (Yu et al. 2004). The remaining four *A. thaliana* FtsH proteins are likely to localize to the mitochondria, as do all three known FtsH proteins from *S. cerevisiae*. A decrease in *FtsH* transcript levels has been implicated in the formation of necrotic lesions in the hypersensitive response of tobacco plants following tobacco mosaic virus infection (Seo et al. 2000). Since the single and essential *E. coli* FtsH protein has a number of diverse functions, additional functions may be identified for plant FtsH proteins.

[2] Variegated plants exhibit patches of different colors in their vegetative tissues. In this case normally green leaves contain yellow-whitish sectors. Rodermel S (2002) *Arabidopsis* Variegation Mutants. The *Arabidopsis* Book:1-28.



Zinc is also a cofactor in some of the chloroplast protein chaperones related to the *E. coli* protein chaperone DnaJ, which contains a Zn binding motif essential for the binding of protein substrates (Schlicher and Soll 1997). For example, the DnaJ-related protein BSD2 (bundle-sheath-defective 2) is required for the accumulation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in maize (Brutnell et al. 1999). The authors proposed that BSD2 could

Fig. 1 (overleaf). Zinc and Copper proteins and homeostasis of the higher plant chloroplast. Cu(I) or Cu(II) is most likely delivered to the chloroplast by an unknown metallochaperone and passes the outer envelope membrane by an unknown mechanism. It is transported across the inner envelope membrane by AtPAA1 (Shikanai et al. 2003). It is likely that copper is then bound to one or several distinct metallochaperone(s) for the delivery to the apo-Cu,Zn-SOD (AtCSD2) or to AtPAA2 for transport into the thylakoid lumen. In the thylakoid lumen Cu is incorporated into apo-plastocyanin. This may involve another Cu metallochaperone. Zn(II) is delivered to the chloroplast by a metallochaperone or in chelated form involving a low-molecular-weight chelator. Zn passes outer and inner envelope membranes by unknown mechanisms. Inside the stroma, Zn is passed by unknown mechanisms to its binding sites, for example, in HSP40/DnaJ-related chaperone proteins like ZmBSD2 and other proteins ("DNAJ") that may assist the folding of the large subunit of Rubisco (LSU) and of other chloroplast proteins (Brutnell et al. 1999). Alternatively, Zn is passed on to binding sites in the stromal Cu,Zn-SOD and in FtSH proteins associated with the thylakoid membrane (Lindahl et al. 1996), involving unknown mechanisms. Zn is also incorporated in the stromal processing peptidase (SPP), which removes targeting peptides (TP) from proteins during import into the chloroplast (Richter and Lamppa 1998), and in the presequence peptidase (PreP), which degrades targeting peptides (Moberg et al. 2003). Metallochaperones are represented by black hexagons, diamonds, or trapezoids. Low-molecular-weight chelator molecules are represented by curved lines. Cu binding sites are represented by white crosses. Zn binding sites are represented by white diamonds. Metalloproteins are represented by black shapes and labeled with specific or generic names. A small selection of additional proteins are represented by grey shapes.

be part of a protein complex that facilitates the assembly of the Rubisco enzyme and prevents the aggregation of nascent large subunit peptide chains in the stroma.

When plants are Zn-deficient, the first enzyme activities known to be reduced are those of carbonic anhydrase (see above) and Cu-Zn superoxide dismutase. *Arabidopsis* possesses 14 isoforms of the Zn enzyme carbonic anhydrase (Moroney et al. 2001). At least one isoform is localized in the chloroplast (Fett and Coleman 1994). Carbonic anhydrase activity is limiting for CO_2 assimilation in C_4 plants, which involves the use of bicarbonate anions by PEP carboxylase in the mesophyll chloroplasts. Carbonic anhydrase activity is thought not to limit the assimilation of CO_2 by Rubisco in C_3 plants (for details, see Marschner 1995). However, in cyanobacteria, carbonic anhydrase co-localizes with Rubisco in specialized compartments called carboxysomes where carbonic anhydrase converts bicarbonate into CO_2, thus concentrating the substrate in the vicinity of Rubisco (Smith and Ferry 2000; Badger and Price 2003). There is no doubt that Zn deficiency reduces net photosynthesis in plants, with a reduction in stomatal opening and chlorophyll content (Hu and Sparks 1991; Sharma et al. 1995), but the mechanisms are not yet fully understood. During Zn resupply to deficient cauliflower leaves, the recovery of carbonic anhydrase activity correlates with an increase in stomatal aperture.

The enzyme porphobilinogen synthase (E.C.4.2.1.24) catalyses the synthesis of porphobilinogen from 5-aminolevulinate, which is a common initial step in the biosynthesis of heme and chlorophyll. The enzyme generally uses Mg instead of

Zn in organisms carrying out oxygenic photosynthesis (Jaffe 2003). The authors speculate that performing chlorophyll biosynthesis in the presence of an excess of Mg, and under exclusion of Zn, may have been selected for because it may reduce the spontaneous insertion of a Zn ion into protoporphyrin XI. The structures of cytosolic and chloroplast isoforms of D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1), an enzyme of the Calvin cycle and the oxidative pentose phosphate pathway, respectively, have recently been solved (Jelakovic et al. 2003). The authors propose the presence of a Zn ion at the active site of both isoenzymes, although direct evidence was not obtained.

Superoxide dismutases, which catalyze the conversion of the superoxide anion radicals ($O_2^{-\cdot}$) to O_2 and H_2O_2, are important in the protection of plant cells from oxidative stress and have been implicated in the generation of the pathogen-induced oxidative burst (Delledonne et al. 2001). *Arabidopsis thaliana* possesses three Cu,Zn-SOD proteins: CSD1 is predicted to localize to the cytoplasm, CSD2 to the chloroplast and CSD3 to the peroxisome. The chloroplast possesses three additional, iron-dependent superoxide dismutase proteins (FSD1, 2 and 3). Finally, there is a single mitochondrial MnSOD enzyme. Expression levels of the cytosolic CSD1 protein, transcript levels for the peroxisomal *CSD3* and chloroplast FSD protein levels increase in rosette leaves in response to high light, UV and/or ozone treatments. In contrast, chloroplast CSD2 protein is constitutively expressed, or even downregulated, in response to UV light (Kliebenstein et al. 1998). The Zn(II) in Cu,Zn-SOD proteins has a structural function whereas the Cu metallocenter is directly involved in catalysis. The important role of superoxide dismutases in cellular redox control may constitute a molecular basis for the interdependence of Zn and Cu homeostasis (Wintz et al. 2003).

The number of Cu-dependent proteins in plants is generally smaller than the number of Zn-requiring proteins. Querying the *Arabidopsis* proteome with the search term “copper” yields 105 proteins. The search term “copper-binding” retrieves 21 proteins. These smaller numbers are probably largely attributable to the fact that Cu ions – unlike Zn ions - are not used as structural components because of their reactivity. As pointed out earlier, Cu and Fe are essential for numerous redox reactions in biological systems due to their ability to exist in two different oxidation states under physiological conditions. Furthermore, of all the metal ions available to biological systems, Cu(I) and Cu(II) are the monovalent and divalent cations with the highest affinity for O-, N- or S-containing functional groups (Fraústo da Silva and Williams 2001). It is assumed that the use of Cu redox chemistry was acquired rather late in evolution, namely only after the advent of photosynthesis when, as a consequence of O_2 generation, Cu became more available than in the reduced environment before. This is also reflected by the fact that many bacteria living in habitats where solubility of Cu is low – such as alkaline lakes - do not require Cu.

A particularly important and widely used feature of Cu(I) is its ability to bind small molecules such as O_2 as ligands. This explains why Cu is a co-factor of a large number of oxidases and why Cu-dependent oxidases are the principal catalysts of terminal oxidation reactions in cells. The best-known example is mitochondrial cytochrome c oxidase.

Cu centers in Cu oxidases can be grouped into three classes (McGuirl and Dooley 1999). Type I sites are the blue copper centers, in which one cysteine is the ligand of the sole Cu. Mononuclear sites with non-sulfur ligation are termed type II, dinuclear centers are termed type III. Cu oxidases often comprise various combinations of these centers, and members of the respective classes are present in plants. Oxidases with mononuclear copper sites are, for instance, amine oxidases that catalyze the oxidative deamination of primary amines. Plant amine oxidases are cell wall-associated enzymes catalyzing the oxidation of putrescine. The H_2O_2 formed in this reaction has been implicated in lignification, cross-linking of cell wall proteins and programmed cell death (Moller and McPherson 1998). At least 13 different amine oxidase genes are present in *Arabidopsis*. Important multicopper oxidases in plants are ascorbate oxidases and laccases (diphenol oxidases), both encoded by multi-gene families in *Arabidopsis* (5 and 20 members, respectively). Ascorbate oxidases are localized in the apoplast. They catalyze the oxidation of ascorbate to monodehydroascorbate and are proposed to play an important role in regulating the redox state of the apoplast (Pignocchi and Foyer 2003). Plant laccase activities are also apoplastic and functionally not nearly as well understood as their fungal counterparts. A role in lignification is assumed (Ranocha et al. 2002). Interestingly, there are apparently also multi-copper oxidase-like proteins such as SKU5, which are involved in cell wall formation yet lack any detectable oxidase activity (Sedbrook et al. 2002).

Some smaller proteins with one mononuclear blue copper (type I) center do not function as oxidases, but as electron carriers. The best-known and quantitatively most important example in plants is plastocyanin, which accounts for about 50% of the plastidic Cu (Marschner 1995). This protein mediates the electron transfer from the cytochrome b_6f complex to photosystem I (Fig. 1). Unlike in some algae and cyanobacteria that can use cytochrome c_6 for electron transfer in place of plastocyanin under Cu deficiency (Merchant and Bogorad 1986b; Cavet et al. 2003), plastocyanin is presumably indispensable in higher plants, as demonstrated for a plastocyanin-deficient *Arabidopsis* mutant (Weigel et al. 2003). In addition to plastocyanin, there is a large number (>32) of related proteins (blue-copper binding proteins) with unknown functions encoded in the *Arabidopsis* genome (Nersissian et al. 1998). Several of these have been reported to be transcriptionally stress-responsive (Richards et al. 1998). For a cell wall plantacyanin from lily stigma it was recently shown that it induces chemotropism of the pollen tube (Kim et al. 2003b).

Cu proteins are not only interacting with molecular dioxygen. Another important small molecule that is bound by a Cu protein is ethylene. The function of the ethylene receptor ETR1 is dependent on a Cu(I) ion bound by a Cys and a His ligand (Rodriguez et al. 1999). Molecular genetic dissection of the ethylene signaling pathway has yielded important insights into Cu metabolism in plant cells (Hirayama et al. 1999; Woeste and Kieber 2000).

A distinctive feature of the Cu use by living systems, congruent with the onset of Cu usage later in evolution, is the almost complete absence of Cu-dependent proteins from the cytoplasm. Most of the oxidases are extracellular, i.e., apoplastic in plants. Plastocyanin is found in the thylakoids, cytochrome c oxidase in mito-

chondria, ethylene receptors in the plasma membrane. The only known exception in plants and other eukaryotes is the cytosolic Cu,Zn-SOD.

The concentration of Cu required in vegetative plant parts is between 1 and 5 μg g^{-1} dry biomass (Marschner 1995). These numbers vary substantially depending on the plant species, the developmental stage and environmental factors such as N supply. Plants grown under high nitrogen supply require significantly more Cu. In agreement with the predominant use of Cu in electron transfer and in oxidases, the most pronounced effects of Cu deficiency in vegetative tissues are on photosystem I efficiency and on lignification. Understandably, the latter is accompanied by anatomical changes. Overall, reproductive tissues are most severely affected by Cu deficiency. Anthers and ovaries normally show the highest Cu content. Molecularly, little is known about the effects of and responses to Cu deficiency in plants. Leaf Cu levels above which toxicity symptoms can be observed are in the range of 20 to 30 μg g^{-1} dry biomass (see Marschner 1995).

Ni is required in vegetative tissues of plants only in trace amounts of between 2 and 4 ng g^{-1} dry biomass (Dalton et al. 1988) and up to 90 ng g^{-1} dry biomass in barley (Brown et al. 1987a). Ni toxicity can be observed above concentrations between 10 and 50 μg g^{-1} dry biomass. Ni deficiency has been reported in *A. thaliana* after cultivation of several subsequent generations in highly pure Ni-free growth media (Zonia et al. 1995). Ni deficiency symptoms are the accumulation of toxic concentrations of urea in several plant species (Eskew et al. 1983, 1984; Brown et al. 1987b), the inhibition of seed germination in cereals (Brown et al. 1987a) and in nitrogen-limited *Arabidopsis thaliana* (Zonia et al. 1995), and the disruption of the grain-filling process in barley (Brown et al. 1987a). Ni is found in a number of hydrogenases, dehydrogenases and methyl reductases in anaerobic bacteria and archaebacteria, but is hardly used as a cofactor in higher eukaryotes. This has been attributed to the fact that, in general, the favorable properties of Ni in redox catalysis are restricted to anoxic, sulfur-rich environments (Fraústo da Silva and Williams 2001).

Urease (E.C. 3.5.1.5), which catalyses the hydrolysis of urea to carbon dioxide and ammonia, is the only known Ni-requiring enzyme in higher plants. Curiously, jack bean (*Canavalia ensiformis*) urease was the first enzyme ever to be crystallized (Sumner 1926). Plant ureases form a homotrimer or a homohexamer, in which each subunit consists of three fused domains homologous to bacterial urease subunits α, β, and γ (encoded by *ureC*, *ureB* and *ureA*, respectively). Each plant urease subunit contains two Ni ions in its catalytic center. In soybean there are two isoforms of urease encoded by the *Eu1* and the *Eu4* gene, respectively (Meyer-Bothling and Polacco 1987; Torisky et al. 1994). In bacteria, urease operons also contain genes encoding accessory proteins necessary for urease activation, UreD, UreF and UreG, and a Ni metallochaperone protein required for the incorporation of Ni(II) into the urease apoenzyme named UreE. The structure of UreE is related to the yeast Atx1 Cu metallochaperone. Orthologues of UreD, UreF (Bacanamwo et al. 2002) and UreG (Freyermuth et al. 2000; Witte et al. 2001) have been identified in plants. There is one additional soybean gene, *Eu2*, which encodes an accessory protein essential for urease activity, the identity of which is still unknown (Meyer-Bothling and Polacco 1987). In soybean and

Arabidopsis, a protein with similarity to UreG contains a his-rich N-terminal extension and was reported to bind Ni ions (Freyermuth et al. 2000). A soybean *eu3* mutant devoid of the UreG-related protein lacks activity of both urease isoforms. These results suggest that in plants UreG may act as a Ni metallochaperone. It is possible that some, if only very few, additional Ni-dependent enzymes will be identified in plants.

No transporters specifically mediating Ni uptake or translocation have been identified. The *Thlaspi goesingense* MTP1 protein was proposed to mediate cellular Ni(II) efflux, but is also able to transport Zn(II) (Persans et al. 2001; Kim et al. 2004). The *in planta* role and substrate specificity of this transporter remain to be clarified.

2.2 Homeostasis of Zn, Cu, and Ni in higher plants

2.2.1 General aspects of metal homeostasis in plants

Higher plant metal homeostasis involves the mobilization, uptake, binding/chelation, trafficking, storage, long-distance, and short-distance transport of metals, as well as the regulation of these processes (Clemens 2001; Clemens et al. 2002).

The concentrations of free metal ions or metal chelates in the soil solution are generally rather low. An overwhelming proportion of the total soil metal content is associated with the mineral fraction. Metal ions in the soil solution have a high affinity for inorganic and organic binding sites of soil components, as well as for binding sites in the cell walls and on the outer membrane surface of plant root cells. All these binding sites act as a pool of exchange sites for metal and other cations, including protons, in the rhizosphere. In addition, metal ions are sparingly soluble at near-neutral pH, especially in the presence of phosphate, and readily form precipitates. In 15 soil samples from metal-contaminated and non-contaminated sites in the Netherlands and Belgium, which contained between 10 and 496 µmol kg^{-1} total Cu and between 98 and 7073 µmol kg^{-1} total Zn (Weng et al. 2001), between 0.046 and 2.06 µmol kg^{-1} Cu and between 0.68 and 84 µmol kg^{-1} Zn readily entered the soil solution (free aqueous metal ion concentration between 0.012 and 0.226 µmol kg^{-1} for Cu^{2+} and between 0.368 and 72.7 µmol kg^{-1} for Zn^{2+}). In the soil solution of non-contaminated soils, Zn concentrations are generally in the sub-micromolar to micromolar range, whereas Cu concentrations are in the nanomolar to micromolar range (del Castilho et al. 1993; Marschner 1995).

The first important process in plant metal homeostasis is the acquisition of micronutrient metals, beginning with the modification of metal bioavailability in the rhizosphere by the plant. Under Fe deficiency, plant roots modify the rhizosphere by acidification through increased plasma membrane proton pump activity and by electron transfer *via* ferric chelate reductases to reduce Fe(III) to Fe(II) and Cu(II) to Cu(I), as found in dicotyledenous and non-graminaceous monocotyledonous species (strategy I), such as *Arabidopsis* and tomato. Another Fe-deficiency re-

sponse is the secretion of metal chelator molecules, as primarily known in graminaceous species such as rice, maize, and wheat, which secrete phytosiderophores (strategy II; see Chapter 8). In addition, Fe deficiency induces changes in root morphology (Schmidt and Schikora 2001). Responses specific to other micronutrient deficiencies have not been extensively characterized in plants (see also below). Root Fe deficiency responses also affect the chemical speciation and availability of other metal ions for uptake by plant roots. For example, Fe chelator molecules form complexes also with other micronutrient cations (von Wiren et al. 1996), and plant roots are able to take up these complexes (see below).

Almost all plants establish root symbioses, for example, with vesicular-arbuscular mycorrhizae or with ectomycorrhizae, or associations with growth promoting rhizosphere bacteria (Strack et al. 2003). These microorganisms modify the rhizosphere and are believed to contribute to plant macronutrient (Rausch et al. 2001) and micronutrient acquisition, but the interaction is not well-understood to date (Marschner 1995). In addition, metal acquisition may involve root growth responses dependent on micronutrient demand and local availability. So far, such responses have only been observed under conditions of metal excess. The roots of Zn-sensitive plants were found to avoid Zn-rich patches in a heterogeneous soil, whereas the roots of a Zn-tolerant and hyperaccumulating plant, *Thlaspi caerulescens*, were found to proliferate preferentially in Zn-rich soil patches (Schwartz et al. 1999; Whiting et al. 2000). These results may reflect either or both differential metal tolerance of the two species and an acquisition response in *T. caerulescens*, because metal hyperaccumulation can be viewed as a strongly enhanced metal requirement.

The final step in plant micronutrient acquisition is their uptake. Micronutrient ions or chelates enter the symplasm of plant roots by passage through specific or non-specific membrane transport proteins (see below). In the uptake step, and possibly also prior to uptake within the plant cell wall, competition between cations appears to play an important role. For example, increasing the concentration of Ca^{2+} ions in the medium is well-known to decrease Cd^{2+} uptake (Clemens et al. 1998; Perfus-Barbeoch et al. 2002). The activity of metal uptake systems is generally induced under deficiency of the respective metal, and some of the transport proteins involved have been characterized at the molecular level in plants (see below). Soils experience extreme seasonal and local variations in nutrient and metal concentrations. Mechanical treatments, for example, freeze-thaw cycles or plowing, are known to increase metal bioavailability. Soil pH and redox state can rapidly change depending on microbial activity, water status and deposition of compounds on the soil, for example, by rainwater or fertilization. Finally, a fluctuating water status directly changes metal ion concentration, mitigated by the buffering capacity of soil binding sites and the precipitation of metal salts, mainly phosphates and oxides. It is clear that metal acquisition processes have to be regulated by the plant to maintain an adequate influx of metal ions.

Based on results from bacterial metal sensors and metallochaperones, the concentrations of free, hydrated metal ions in the symplasm of plant cells are believed to be in the femtomolar and attomolar range for Zn and Cu ions, respectively (Rae et al. 1999; O'Halloran and Culotta 2000; Outten and O'Halloran 2001). These low

values contrast sharply with total cellular Zn and Cu levels, which correspond to around 100 µM Zn and 2 µM Cu in rosette leaves of soil-grown *A. thaliana* plants. In plants, metal compartmentalization and binding by metallochaperone proteins (primarily for Cu), metal-binding ("buffer") proteins and high-affinity low-molecular-weight chelators (primarily for Zn) are thought to contribute to this, preventing the binding of metal ions to adventitious binding sites within the cell.

The non-proteinogenic amino acid nicotianamine (see below and Chapter 10), which is ubiquitous in higher plants, is an important chelator not only for Fe, but probably also for Zn, Ni, Mn, and Cu in plants (Stephan and Scholz 1993; Takahashi et al. 2003b; Vacchina et al. 2003; Becher et al. 2004; Weber et al. 2004). Thermodynamically, nicotianamine (as the L^{3-} anion containing three carboxylate groups) chelates Fe(II) (pK_s = 12.8), Co(II) (pK_s = 14.8), Zn(II) (pK_s = 15.4), Ni(II) (pK_s = 16.1), Cu(II) (pK_s = 18.6) and Fe(III) (pK_s = 20.6) with high affinities (Stephan and Scholz 1993; von Wiren et al. 1999; Reichman and Parker 2002). For predictions of chelation it is important to consider that the apparent stability constant of a complex defines the complex stability under the given conditions. The apparent stability constant is derived from the thermodynamic stability constant (pK_s), the protonation constant (pK_a) of a chelator molecule and the pH of the solution, which together determine the extent to which free electron pairs of the chelator molecule are protonated and thus unavailable for the complexation of metal ions (Dawson et al. 1986). In addition, other ligands and precipitation, above all the precipitation of Fe(III) as a hydroxide, may compete with chelation by nicotianamine at given pH values and solute concentrations (Reichman and Parker 2002). Computer programs, for example, Geochem-PC, are available for the modeling of metal chelation (Parker et al. 1979).

Nicotianamine concentrations are highest in regions of cell division, differentiation and expansion (Stephan et al. 1990). The nicotianamine-deficient tomato mutant *chloronerva* exhibits severe growth and developmental defects and constitutive Fe deficiency symptoms (Stephan and Scholz 1993). Mapping of the *chloronerva* mutation led to the identification of the gene encoding the enzyme nicotianamine synthase (EC 2.5.1.43), which catalyses the biosynthesis of nicotianamine from three molecules of S-adenosylmethionine, releasing three molecules of 5'-methylthioadenosine (Higuchi et al. 1994; Herbik et al. 1999; Ling et al. 1999). It should be noted that the biosynthesis of the important metal chelator nicotianamine originates in the sulfur assimilation and methionine biosynthesis pathways. The substrate S-adenosylmethionine is also a substrate for the synthesis of the precursor of ethylene, 1-aminocyclopropane-1-carboxylate, generating 5'-methylthioadenosine as a second product. The enzyme aminocyclopropane-1-carboxylate oxidase, which produces ethylene, is a non-heme iron enzyme. The molecule S-adenosylmethionine or its downstream products are also used in spermidine synthesis and in methyl transfer reactions. By modifying the flux at important branchpoints of these metabolic pathways, the plant may be able to regulate a subset of them in concert or alternate between them.

In most plant species, excess metal ions are primarily immobilized in the root – either in the apoplast or by sequestration, presumably inside the vacuoles. The proportion of metals that is kept mobile is translocated from cell to cell towards

and into the stele, followed by a final export from the cytoplasm of xylem parenchyma cells into the apoplastic xylem. Inside the xylem sap, chelation by ligands, for example, nicotianamine or organic acids, and the exchange with cell wall binding sites determine the rate of movement of a metal cation into the shoot with the transpiration stream (Clemens et al. 2002).

Inside the shoot, metals, or metal complexes are unloaded from the xylem by transport into cells in processes that are similar to the initial uptake into root cells. There are reports that different cell types differentially capture metal ions. For example, in the presence of excess metal supply epidermal cells (Brune et al. 1995), trichomes (Salt et al. 1995) and hydathodes (Tung and Temple 1996) have generally been reported to accumulate higher metal concentrations than, for example, mesophyll cells. In contrast, under exposure to low metal concentrations, metals were preferentially accumulated in mesophyll cells of barley leaves (Brune et al. 1995). Thus, a distribution system is likely to exist, which involves differential capture of metals in different cell types, and possibly efflux from cells and symplastic transfer from cell to cell. There is some circumstantial evidence to suggest that chelation, for example, by nicotianamine (Takahashi et al. 2003b), and putative cellular efflux systems such as *A. thaliana* FRD3 (ferric reductase defective 3, TC 2.A.66.1) could be of primary importance in the distribution of metals between cells (Delhaize 1996; Rogers and Guerinot 2002).

Inside each individual cell, metal ions are delivered to apo-metalloproteins in all compartments. The mechanisms for this are still poorly understood (see below). The central vacuole appears to be a primary storage site for excess or toxic metal ions (Brune et al. 1995; Krämer et al. 2000; Sarret et al. 2002; Dräger et al. 2004). In *Arabidopsis*, metal ion stores are known to be formed in internal membrane-delimited structures during embryo development (Otegui et al. 2002). These stores are remobilized from the vacuole during germination in a process dependent on the metal transporters NRAMP3 and NRAMP4 (Lanquar et al. 2004). In sink tissues cells are likely to require net metal import to assemble the machinery of metalloproteins needed for metabolism. When a metalloprotein is degraded in a mature plant cell, the bound metal is likely to be liberated and has to be captured and redistributed in the cell. The plant central (lytic) vacuole is a site for metal storage and detoxification as well as for the degradation of proteins, analogous to the mammalian lysosome (Vitale and Raikhel 1999). The vacuole may thus represent a transient storage site in the cycling of metal ions liberated during protein degradation, until these metal ions are exported from the vacuole and incorporated in newly synthesized apometalloproteins. Recently, a relatively small number of 46 proteins functionally involved in membrane transport were identified in purified vacuole preparations from rosette leaves of *Arabidopsis* in a proteomics study (Carter et al. 2004). It is interesting to note that only two metal transport proteins were among these, either fortuitously or because they are quite abundant: the natural-resistance-associated macrophage protein NRAMP4 (TC 2.A.55) and a member of the copper transporter-2 (CTR2; TC 9.A.12) family. The NRAMP4 protein has been shown to localize to the vacuolar membrane as a chimeric GFP fusion protein (Lanquar et al. 2004). According to heterologous expression in yeast and reverse genetics in *A. thaliana*, the homologous AtNRAMP3 was concluded to be

a rather non-specific metal transporter that can import metal cations - primarily Fe^{2+}, but also Mn^{2+} and Cd^{2+} - from the vacuole into the cytoplasm (Thomine et al. 2000; 2003). The yet uncharacterized putative tonoplast Cu transporter-2 from *A. thaliana* is most homologous to the yeast ScCtr2 protein, which has been shown to transport Cu ions – presumably as Cu^{+} – from the lumen of the vacuole into the cytoplasm (Rees et al. 2004). Taken together, this supports the idea that in vegetative photosynthetic tissues of *A. thaliana*, the export of metal ions from the vacuole could be an abundant and important function in the cycling of metal ions. Despite the function of the vacuolar lumen in metal storage it contains metallo- and other enzymes that maintain their functions. These enzymes are likely to be protected from the replacement of their metal ion cofactors or from deactivation by adventitious binding of metal ions. The molecular basis of this is unknown.

At a larger scale, metal remobilization *via* the phloem is widely important during the senescence of plant organs and possibly during major developmental transitions, for example, the formation of the inflorescence bolt in *Arabidopsis thaliana* or grain filling in rice. In source tissues, this requires the transport of metal ions or metal-nicotianamine complexes (see below), into companion cells of the vasculature, either symplastically or apoplastically (Oparka and Turgeon 1999). Subsequently, metals are symplastically released from the companion cells into the phloem. From the phloem sap of various plants, researchers have isolated nicotianamine complexes of various metals (Stephan and Scholz 1993) as well as a late embryogenesis abundant protein capable of metal binding (Krüger et al. 2002). Both may represent long-distance transport forms of metals. In sink tissues, metals have to be released from companion cells, a process that is believed to be symplastic in vegetative growing sink tissues, such as young roots and leaves, and apoplastic in other sink tissues, such as seeds or storage organs.

In the following sections, we will summarize our specific molecular knowledge of plant Cu and Zn homeostasis using two examples: Firstly, since Zn is a major factor determining human fertility, as well as pollen fertility in plants, we will describe how Zn reaches developing pollen grains of a flower. We will focus on the importance of nicotianamine and related low-molecular-weight metal chelator molecules in the maintenance of Zn mobility and homeostasis inside the plant. Secondly, we will outline the pathway of Cu into the major Cu protein plastocyanin in the thylakoid lumen. We will describe the important role of Cu in photosynthesis and consider the involvement of metallochaperones in the trafficking of Cu to apometalloprotein Cu binding sites.

2.2.2 The journey of a Zn^{2+} ion from the rhizosphere into a pollen grain

The precise root cell type(s) involved in primary Zn uptake, as well as its precise location are unknown. Based on the current state of knowledge it is likely that multiple transporters contribute to Zn uptake by plant roots (Fig. 2). These are primarily transporters of the ZRT-IRT-related (TC 2.A.5) transporter family (Mäser et al. 2001), which has 17 members in *A. thaliana* (Hanikenne et al. 2005). In *A. thaliana* ZIP1 and ZIP3 were the first proteins found to complement a yeast

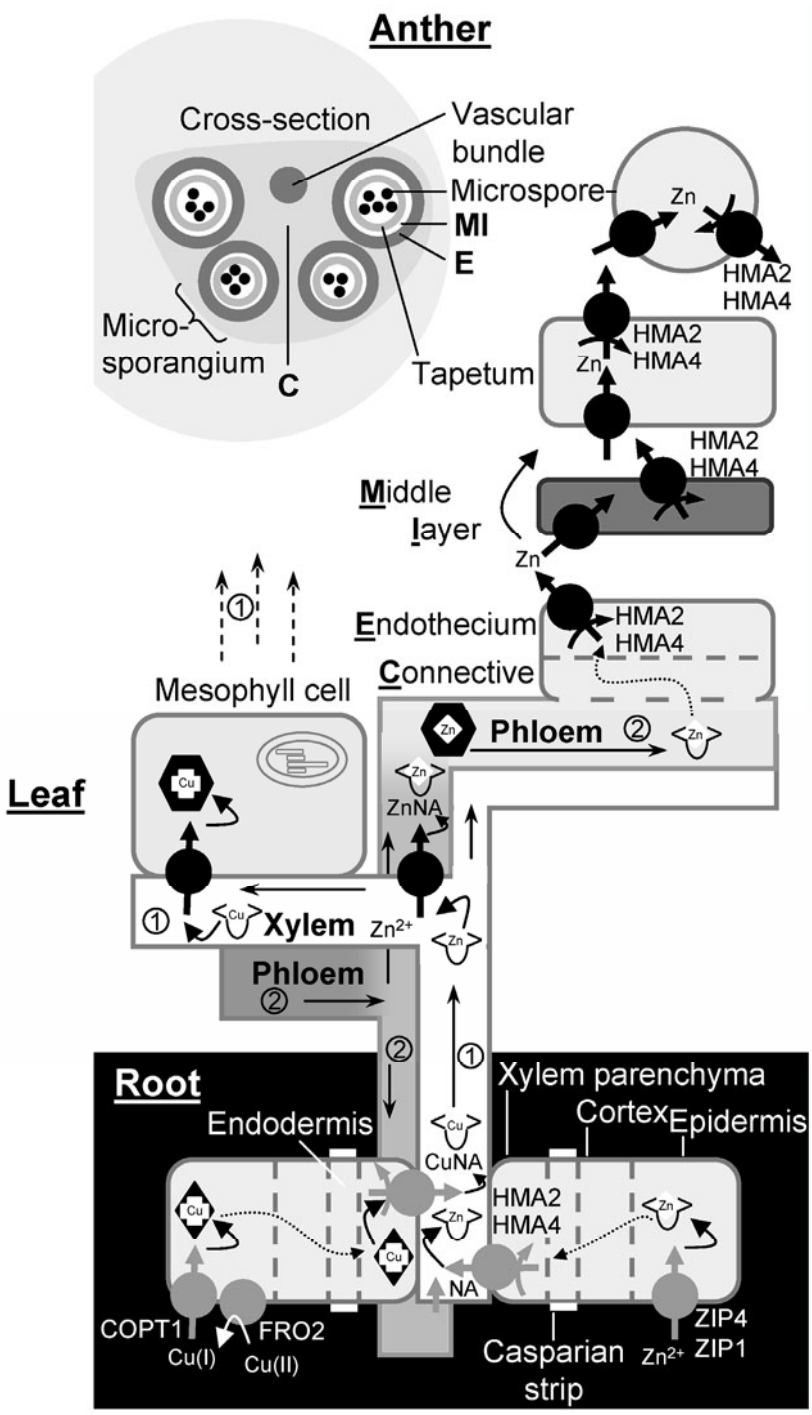

Anther
Cross-section
Vascular bundle
Microspore
MI
E
Micro-sporangium
C
Tapetum
Zn
HMA2
HMA4
Middle layer
Endothecium
Connective
Mesophyll cell
Leaf
Phloem
ZnNA
Cu
Xylem
Zn2+
Root
Endodermis
Xylem parenchyma
Cortex
Epidermis
CuNA
NA
COPT1
Cu(I)
FRO2
Cu(II)
Casparian strip
ZIP4
ZIP1

Fig. 2 (overleaf). A working model of zinc and copper homeostasis in *Arabidopsis thaliana*. Two examples are given: a possible pathway for a Zn^{2+} ion from the rhizosphere into developing pollen in an anther (top and right, see also Section 2.2.2) and a possible pathway for a Cu^{2+} ion from the rhizosphere into a leaf mesophyll cell (left, see also Section 2.2.3). Cu(I) may be transported into root cells via COPT1 following reduction of Cu(II) to Cu(I), presumably by a ferric chelate reductase similar to FRO2. Cu is likely to be transferred to a metallochaperone after uptake. It is not known whether or in which chemical form Cu moves symplastically from cell to cell *via* plasmodesmata. Cu efflux from the xylem parenchyma cells into the apoplastic xylem could be mediated by a P-type Cu-ATPase. The radial cell walls of the endodermis, which is the cell layer surrounding the vasculature, are lined with the hydrophobic substance suberin (Casparian strip) within differentiated regions of the root. It is generally accepted that the Casparian strip largely precludes an entirely apoplastic pathway for the entry of solutes from the rhizosphere into the xylem. Inside the xylem, Cu is present as a nicotianamine (NA) complex (CuNA). Free NA could be transported into the xylem in a distinct transport process. Alternatively, a Cu-NA complex could be transported into the xylem. Solutes move into the shoot in the xylem with the transpiration stream (1). Cu is transferred from the xylem into mesophyll cells in a cellular uptake step, which could involve a COPT1-related transporter. Alternatively, this step could involve a YS1-like transporter, which would transport a Cu-NA complex. On the cytoplasmic side of this uptake system, Cu is likely to be transferred to a metallochaperone. Zn^{2+} is probably taken up into root cells by transporters of the ZIP family. Inside the root symplasm, at least a proportion of Zn is likely to be present as a low-molecular-weight chelate, for example, as Zn-NA (ZnNA). This may allow symplastic mobility of Zn across the root cortex, endodermis and into the vascular cylinder *via* plasmodesmata. Release from the xylem parenchyma cells into the xylem involves the P-type metal ATPases HMA2 and HMA4. In the xylem, an equilibrium is likely to exist between various Zn species, for example, free aqueous Zn^{2+} ions, Zn-chelates such as Zn-NA and Zn bound to cell walls lining the xylem. The transfer of Zn from the xylem into the phloem system can be assumed to incorporate a key transport step resembling cellular uptake. Inside the phloem Zn undergoes mass flow-driven movement (2) from source (mainly photosynthetic leaves) towards sink tissues such as the anthers. In the phloem, Zn is likely to move as a Zn-NA complex, or possibly bound to proteins. Inside the developing anther plasmodesmata connect the phloem to the connective and to the endothecium (see Goldberg et al. 1993; Lee and Tegeder 2004). The subsequent passages from the endothecium through the middle layer and the tapetum into the microspore involve repeated transport across the plasma membrane of different cell types for Zn efflux and Zn uptake, respectively. Several of the cellular efflux steps are likely to involve HMA2 and/or HMA4. Low-molecular-weight chelator molecules are represented by curved lines. Metallochaperones are represented by black hexagons, diamonds, or trapezoids. Cu binding sites are generally represented by white crosses. Zn binding sites are represented by white diamonds. Movement down the electrochemical gradient for cations is symbolized by a transporter symbol containing a single straight arrow. Movement against the electrochemical gradient for cations is denoted by a transporter symbol containing an additional curved arrow. This could represent directly energized transport (ATP), such as HMA2-mediated transport, or transport energized by the proton gradient. Please note that the substrate for transport may in some cases be a non-cationic metal-chelate complex.

mutant defective in Zn uptake (Grotz et al. 1998). The steady-state transcript levels of *ZIP1, ZIP2*, *ZIP3*, *ZIP4*, *ZIP5*, and *ZIP9* (Grotz et al. 1998; Wintz et al. 2002) are all upregulated in roots in response to Zn deficiency. All of these and

possibly additional transporters may contribute to Zn uptake into the root symplasm of *Arabidopsis*, but we do not know the localization of the gene products in the root nor the subcellular localization in the root cell. The *A. thaliana irt1* mutant (Varotto et al. 2002; Vert et al. 2002), which is defective in high-affinity Fe uptake, also accumulates less Zn, suggesting that IRT1 makes a significant contribution to plant Zn uptake (Henrique et al. 2002). It is likely that Fe and Zn influx into *A. thaliana* roots are linked because of the lack of specificity of the IRT1 high-affinity iron uptake transporter of *A. thaliana*, which – in addition to Zn^{2+} and Fe^{2+} - can also mediate cellular influx of Mn^{2+}, Co^{2+} and Cd^{2+} ions (Korshunova et al. 1999). The *IRT1* transcript is present at very low levels, and induced under Fe deficiency specifically in epidermal cells of the root hair zone. The IRT1 protein transports Fe^{2+} ions following the reduction of poorly soluble extracellular Fe(III) by the NADPH-dependent ferric chelate reductase FRO2 (Robinson et al. 1999). Similar to *irt1* mutants, *fro2* mutants exhibit an Fe uptake defect. The contribution of FRO2 to Zn uptake is presumably minor because there is no need for Zn reduction prior to uptake, and FRO2 might influence Zn solubility only by the reduction of Fe(III) and subsequent breakdown of mixed oxides in the rhizosphere. However, FRO2 may link Fe uptake and Cu uptake (see below).

Under Fe deficiency, roots of graminaceous plants produce and secrete phytosiderophores, which chelate Fe(III) in the rhizosphere (Marschner 1995). Phytosiderophores are high-affinity low-molecular-weight chelator molecules, which are derived from the metal chelating amino acid nicotianamine by consecutive replacement of amino groups by hydroxyl groups at various positions and to different degrees. This is initiated by the formation of 2'-deoxymugineic acid involving the enzyme nicotianamine aminotransferase and the oxygenase deoxymugineic acid synthase (Takahashi et al. 1999; Kobayashi et al. 2001). Compared to nicotianamine, the replacement of amino by hydroxyl groups stabilizes the complexes with Fe(III) and renders phytosiderophore-metal complexes more stable at low pH values that may be encountered in the immediate surroundings of the roots (von Wiren et al. 2000). So far, phytosiderophores have only been detected in graminaceous plants.

The Fe(III)-phytosiderophore complexes are then taken up by specific transport systems, among which the yellow stripe 1 (YS1; TC 2.A.67.2.1) transporter is likely to be the primary uptake system in maize (von Wiren et al. 1994; Curie et al. 2001). Maize roots can also take up Zn(II)-phytosiderophore complexes (von Wiren et al. 1996). According to the complementation of yeast mutants and two-electrode voltage-clamp analyses of YS1-expressing oocytes of *Xenopus laevis*, YS1 operates as a metal-phytosiderophore proton cotransporter and can mediate cellular uptake of complexes of the phytosiderophore 2'-deoxymugineic acid with Fe(III), Ni(II), Zn(II), Cu(II) and, at lower rates, Mn(II) and Cd(II) (Schaaf et al. 2004). Evidence that YS1 is capable of mediating cellular import of nicotianamine (NA) complexes of Ni(II), Fe(II), and Fe(III) was also obtained. In another study, expression of YS1 did not complement a yeast mutant strain defective in Zn uptake in the presence of another phytosiderophore, mugineic acid (MA). Evidence for uptake into YS1-expressing yeast cells was obtained for Fe(III)-MA, Fe(II)-

NA, Cu(II)-MA and Co(II)-MA complexes (Roberts et al. 2004), only partially confirming the results of Schaaf et al. (2004).

The YS-like transporter subfamily, which belongs to the oligopeptide transporter family (OPT; TC 2.A.67), has 18 members in rice (OsYSL1 to OsYSL18), suggesting that cellular uptake of metal complexes is important and widespread in this plant (Koike et al. 2004). Transcript levels of *OsYSL2* are induced under Fe deficiency in leaves, and promoter activity is restricted primarily to phloem companion cells and the embryo and outer endosperm layer in developing seeds. An OsYSL2-GFP fusion protein was localized to the plasma membrane (Koike et al. 2004). Two-electrode voltage clamp analyses in *Xenopus* oocytes suggested that the protein transports NA complexes, but not DMA complexes, of Fe(II) and Mn(II), and not of Zn(II) and Cu(II). A similar role, the transport of metal-NA complexes within the plant, is being envisaged for the eight YS-like transporters of *A. thaliana* AtYSL1 to AtYSL8 (DiDonato et al. 2004). Heterologously expressed AtYSL2 is capable of conferring Fe(II)-NA and Cu(II)-NA, as well as Fe(II)-MA uptake to yeast cells (DiDonato et al. 2004). AtYSL2 was proposed to mediate the lateral movement of Fe and Cu in the vascular system (DiDonato et al. 2004). In conclusion, although YS-like transporters are clear candidates for transport of Zn(II)-NA or Zn(II)-phytosiderophore complexes, direct evidence is scarce.

In fact, ZIP-like transport proteins are not only important for Zn uptake in non-graminaceous species, but appear to contribute to Zn uptake by the roots of graminaceous plant species, as well. The *OsZIP1* and *OsZIP3* cDNAs complement a yeast mutant defective in Zn uptake. Transcript levels are upregulated under Zn deficiency and localize to the root epidermis and vasculature, as well as to the vascular bundles of leaves and stems of rice (Ramesh et al. 2003).

Inside the cytosol, incoming Zn^{2+} ions are likely to undergo controlled binding, either to metallochaperone or metal buffering proteins or to low-molecular-weight chelator molecules. The latter may involve glutathione (GSH), which has a high affinity for Zn at cytoplasmic pH values, and GSH-derived molecules. The role of nicotianamine in Zn chelation has been characterized most extensively to date (see also Chapter 10). Expression of various nicotianamine synthase isoforms partially complemented Zn hypersensitivity of mutant yeast strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, or conferred Ni tolerance to yeast cells (Vacchina et al. 2003; Becher et al. 2004; Weber et al. 2004). Moreover, roots of the highly Zn-tolerant Zn hyperaccumulator *A. halleri* contain higher steady-state nicotianamine concentrations than the roots of its Zn-sensitive relative *A. thaliana* (Weber et al. 2004). Cytoplasmic chelation of Zn(II) may contribute to maintaining its symplastic mobility for transfer into the vascular bundle.

The P_{1B}-ATPases HMA2 and HMA4 have an important role in the translocation of Zn(II) from the root to the shoot of *A. thaliana* (Eren and Arguello 2004; Hussain et al. 2004; Verret et al. 2004). Both proteins are metal ion pumps of the P-type ATPase superfamily, which use ATP to drive metal ion transport and form a phosphorylated intermediate during the reaction cycle (Axelsen and Palmgren 2001). Compared to the wild type, the *A. thaliana hma2-2hma4-1* double mutant accumulates only about half the Zn concentrations in the shoot and approximately

twofold higher Zn concentrations in the root. Shoots of double mutant plants display severe symptoms of Zn deficiency (see above). Chimeric GFP fusions of both proteins have been localized to the plasma membrane (Hussain et al. 2004; Verret et al. 2004), suggesting that they function in the export of Zn(II) from the cell. Consistent with a role in root-to-shoot transport of Zn, promoter activities of *AtHMA2* and *AtHMA4* are localized to the vascular system of roots, stems and cauline leaves, and were detected in the vicinity of both sieve elements and xylem vessels. In agreement with these results, leaves of *A. thaliana* overexpressing AtHMA4 accumulate higher concentrations of Zn and Cd, when compared to the wild type. Microsomal vesicles prepared from yeast cells expressing AtHMA2 displayed ATP-dependent Zn accumulation (Eren and Arguello 2004). ATPase activation was highest in the presence of Zn(II) and Cd(II), and approximately 50% in the presence of Cu(I), Cu(II), Ni(II), or Co(II). Taken together, these data provide solid support for an involvement of HMA2 and HMA4 in cellular export of Zn(II), enhancing root-to-shoot translocation and possibly decreasing Zn(II) retention by vascular cells along the xylem transport pathway.

The speciation of Zn(II) during transport in the xylem is unknown. During the vegetative phase of growth of *A. thaliana*, Zn is transported into rosette leaves with the transpiration stream. The proteins involved in the retrieval of Zn(II) from the xylem have not been identified. The phenotypes of tobacco and tomato plants lacking nicotianamine suggest a model, in which a significant proportion of Zn, as well as major proportions of Cu and Fe, enters young developing leaves and flowers in a nicotianamine-dependent manner *via* the phloem. The remainder may be delivered *via* the phloem in other binding forms or *via* the xylem. Nicotianamine is reduced to virtually undetectable levels in both the tomato mutant *chloronerva*, which has a defect in a nicotianamine synthase gene (Ling et al. 1999), and tobacco ectopically expressing a barley nicotianamine aminotransferase (*naat-A*, Takahashi et al. 2003). In both these systems, only young leaves exhibit interveinal chlorosis, which is a typical symptom of Fe deficiency. This chlorosis disappears gradually when leaves mature (Stephan and Scholz 1993; Takahashi et al. 2003b). In *chloronerva*, leaves have been reported to gradually accumulate an excess of several metals. In leaves of *naat-A*-expressing plants, there is an enhanced gradient of metal ion concentrations between the leaf veins and interveinal regions, suggesting that NA is needed for the distribution of metals within the leaf blade. In addition, young leaves contain decreased concentrations of Cu, Fe, Zn, and Mn.

Both *chloronerva* and *naat-A* plants have severe flowering defects, with *chloronerva* being unable to develop floral organs and *naat-A* developing sterile flowers of abnormal morphology. Pollen development is disrupted in *naat-A* plants, anthers and stigma are deformed and pistils shortened. The concentrations of Cu, Fe and Zn, but not Mn is strongly reduced in flowers and floral organs of *naat-A* plants. The defects were partly alleviated by supply of exogenous NA, or by grafting of *naat-A* shoots onto rootstocks of NAS-overexpressing tobacco plants. These data suggest that a deficiency in NA mainly affects the supply of nutrient metals to sink organs (compare Section 2.2.3). Since NA is mainly accumulated in sink organs a proportion of nutrient metal ions including Zn(II) are likely

to move in the phloem as nicotianamine complexes. Prior to the long-distance movement inside the phloem these metals may be transported into the phloem companion cells as NA complexes by YSL transporters. Tobacco plants engineered to overexpress NAS accumulate higher Fe, Zn, and Cu concentrations in young leaves, flowers, and all floral organs (sepals, petals, filaments, anthers, pollen, pistils) and higher Fe and Zn concentrations in seeds, when compared to wild type tobacco plants.

If considerable proportions of micronutrient metals are delivered into sink tissues *via* the sieve tubes, as implicated by the work mentioned above, transfer is required from the xylem into the phloem. The molecular basis for this is unknown. It is likely that some, but not all floral abnormalities in nicotianamine-deficient plants are caused by suboptimal Zn supply to floral organs, because nicotianamine deficiency also causes Cu and Fe deficiency in flowers. There is convergent evidence for an important role for Zn from another study. In addition to exhibiting Zn deficiency symptoms in the shoot, the *A. thaliana hma2hma4* double mutant (see also above) is unable to form mature pollen and may also exhibit defects in ovule or embryo development (Hussain et al. 2004). The phenotype may be caused by a general lack of Zn in shoot tissues, and by a specific function for HMA2 and HMA4 inside developing anthers. The *HMA2* and the *HMA4* promoters are active in the endothecium, middle layer and tapetum regions of developing anthers and in developing immature pollen grains (Goldberg et al. 1993; Hussain et al. 2004; Lee and Tegeder 2004). Tapetum cells secrete nutrients into the locule fluid that surrounds the pollen grains, and Zn secreted by HMA2 and HMA4 may be among these nutrients. The activities of *HMA2* and *HMA4* promoters inside developing pollen grains, however, suggest that the microspores also exhibit Zn(II) efflux pump activity. More information concerning the precise localizations of the HMA2 and HMA4 proteins is needed to fully understand their role in pollen development.

2.2.3 The journey of a Cu^{2+} ion from the rhizosphere into the plastocyanin protein

Cu(II) ions are tightly bound to soil particles because of their tendency to form stable complexes with, for instance, carboxyl or phenol groups. Still, it is not clear if and to what extent plant roots actively mobilize Cu ions. Phytosiderophore secretion by monocots is known to enhance Cu mobilization (Römheld 1991). However, there is no evidence for the uptake of Cu-phytosiderophore complexes by plant roots.

Uptake of Cu ions is apparently mediated by members of the CTR family (Fig. 2), which is ubiquitous in eukaryotes (Puig and Thiele 2002). The first CTR-like transporter in *Arabidopsis thaliana*, COPT1, was cloned by complementation of the *S. cerevisiae* high-affinity Cu uptake-defective mutant *ctr1ctr3* (Kampfenkel et al. 1995). The *Arabidopsis* genome contains 6 genes encoding CTR-like transporters, i.e. COPT1 to 5 and an unnamed CTR-like protein (Sancenon et al. 2003; Carter et al. 2004). COPT1, COPT2, COPT3, and COPT5 have been shown to complement the *ctr1ctr3* mutant (Sancenon et al. 2003). These proteins have not been

functionally characterized in sufficient detail. COPT1 is expressed in various tissues including most embryonic cells during heart stage. In mature plants, expression is detected in stomata, trichomes, pollen, and root tips (Sancenon et al. 2004). COPT1 is hypothesized to represent an important component of Cu uptake in *Arabidopsis* roots. Antisense lines show reduced ^{64}Cu uptake rates, lower leaf Cu content and reduced growth in the presence of the Cu chelator BCS. However, the subcellular localization of COPTs has not been determined so that the pathways for entry of Cu ions into plant cells remain to be identified. Recently, the exclusive role of CTR-like transporters in plant Cu uptake has been questioned. Transporters of the ZIP family have been suggested to contribute to Cu uptake into plant cells. ZIP2 and 4 were both found to rescue a yeast Cu uptake-defective mutant. In addition, ZIP2 is transcriptionally upregulated under Cu deficiency (Wintz et al. 2003). Thus, although the analogy to the better-characterized eukaryotic model systems yeast and mice strongly suggests a key role for CTR-like transporters in plant Cu uptake, we cannot rule out the contribution of other transporter families.

The actual substrates of CTR transporters – Cu(I), Cu(II) or a Cu-ligand complex - have not been unequivocally determined yet. It is presumed that Cu(I) is the substrate of yeast and mammalian Ctr transporters (Lee et al. 2002). The same is suggested for the plant COPTs (Sancenon et al. 2003). This would obviously require reduction of Cu(II) prior to uptake. Physiological studies showed that Fe-deficient dicot roots express not only ferric chelate reductase activity but also Cu(II) reductase activity (Welch et al. 1993; Holden et al. 1995). It appears likely that ferric chelate reductases such as FRO2 account for this activity. In *S. cerevisiae*, the ferric reductases Fre1 and Fre2 possess cupric reductase activity, as well, and have been shown to be involved in Cu uptake (Georgatsou et al. 1997). This would also help to explain why Cu excess causes Fe deficiency (see below).

Because of the high affinity of Cu(I) and Cu(II) for various functional groups in biological molecules and based on the available evidence from *S. cerevisiae*, it is generally accepted that there are no free hydrated Cu ions inside a cell (Rae et al. 1999). Instead, specific distribution systems ensure delivery of Cu ions to Cu-requiring organelles and proteins (O'Halloran and Culotta 2000). In *Arabidopsis*, orthologues of the known *S. cerevisiae* metallochaperones ATX1, CCS and COX17 – which are required for Cu delivery to the P-type ATPase CCC2, the cytosolic Cu,Zn-SOD and cytochrome c oxidase, respectively – have been identified (Himelblau et al. 1998; Balandin and Castresana 2002; Wintz and Vulpe 2002; Abdel-Ghany et al. 2005a).

As in the other model systems, namely *S. cerevisiae* and mammalian cells, the transfer of Cu ions taken up *via* CTR transporters to metallochaperones is not understood. It is unknown whether there is a direct interaction of the chaperones with the transporters or rather the involvement of as yet unidentified proteins. In root cells – just like in all other cells – some of the Cu will be channeled to sites such as the mitochondria and the cell walls where Cu is needed as a cofactor.

A fraction of the acquired Cu needs to be maintained in a mobile form in order to be available for transport into the shoot. Prime candidate as ligand for Cu ions in the cytosol as well as during transfer through xylem and phloem is nicotianamine (NA). NA forms stable complexes with Cu(II) *in vitro* (Benes et al.

1983). The NA deficient tomato mutant *chloronerva* displays symptoms of Cu deficiency in the leaves – Cu concentration in leaves is reduced by 80% - while root Cu accumulation is increased (Pich et al. 1994). Similarly, tobacco plants artificially rendered NA-deficient by overexpression of a barley nicotianamine aminotransferase display lower Cu contents in young leaves (Takahashi et al. 2003b). Analysis of the xylem sap of *chloronerva* plants showed much lower Cu content as compared to wild type (Pich et al. 1994). Furthermore, NA is detectable in the xylem sap of wild type tomato in sufficient amounts and based on the stability constants, the pH and the concentrations of NA and metals in the xylem sap, NA is predicted to chelate all of the Cu (von Wiren et al. 1999). Thus, a strong case can be made for NA as the major Cu chelator in the xylem (Pich and Scholz 1996) and against previous assumptions that asparagine, histidine, and citrate would serve this function (Rauser 1999).

The path of Cu ions from the cytosol of root cells into the xylem is far less understood. It is difficult to test whether NA is the main binding partner for Cu also in the cytosol because the number of potential binding sites is far higher than in the xylem sap, and because pure cytoplasm cannot be sampled in sufficient amounts for metal-ligand analysis. Assuming that Cu enters the xylem following symplastic passage across the endodermis – just like other nutrients – transfer of Cu into the xylem requires a membrane passage. It is conceivable that Cu is transported in a complex with NA. Alternatively, NA and Cu ions might be transported separately. Candidate mediators for Cu efflux into the xylem are P-type ATPases (compare HMA2/HMA4 and Zn loading into the xylem). In *S. cerevisiae* and mammalian cells it was shown that metallochaperones deliver Cu ions to Cu pumps which remove Cu ions from the cytosol (see above and Chapters 2 and 5). Most likely, as yet unidentified proteins are required in plant cells as well.

It is quite possible that metallochaperones also play a role in long distance transport of Cu. There are >30 genes in *Arabidopsis* encoding proteins that carry a heavy-metal binding domain similar to the one present in Atx1 (Wintz and Vulpe 2002). The functions of these proteins – which according to sequence-based predictions are targeted to different cellular compartments including the nucleus, the secretory pathway and plastids – are largely unknown. The CCH protein was detected extracellularly and is hypothesized to be involved in Cu mobilization from senescing tissues (Mira et al. 2001). The Cd^{2+}-induced protein CdI19 (Suzuki et al. 2002) is able to bind Cu as shown by CD spectra, and *CdI19* transcript levels are increased under conditions of excess Cu.

Cu arriving *via* the xylem might be taken up into leaf cells as Cu ion or as a Cu-ligand complex. For the latter alternative, Cu-NA complexes are the most likely candidates according to the current state of knowledge. The transporter AtYSL2 is the first characterized *Arabidopsis* transporter of the YSL family showing similarity to the Fe-phytosiderophore uptake system yellow stripe 1 (YS1) from maize (Curie et al. 2001). Expression of AtYSL2 has been shown to enable cells to take up a Cu-NA complex (DiDonato et al. 2004). The *Arabidopsis* genome encodes 8 predicted YS-like transporters that need to be studied with respect to tissue distribution, subcellular localization, regulation and substrate specificity.

Once taken up into a leaf mesophyll cell there is still a long way to go for a Cu ion to reach plastocyanin. Assembly of Cu-plastocyanin occurs only after import of apoplastocyanin into the thylakoid lumen (Merchant and Dreyfuss 1998) so that Cu ions have to be transported through the cytosol, across the outer and inner chloroplast envelope membrane, through the stroma and across the thylakoid membrane. A metallochaperone pathway to the plastids of plant cells has not been identified to date. In light of the knowledge about Cu homeostasis in other eukaryotic cells, however, there is no doubt that such a delivery system exists (see Chapters 2 and 5). The best-characterized example for Cu distribution in plant cells to date was uncovered through studies on the ethylene signaling pathway. The *Arabidopsis ran1* mutant displays typical ethylene responses when treated with an ethylene antagonist. When the corresponding gene was cloned it was found to encode a P-type ATPase similar to CCC2 from *S. cerevisiae* (Hirayama et al. 1999). This ATPase is homologous to the human Menke's disease protein, which pumps Cu ions into post-Golgi vesicles. By analogy, RAN1 is hypothesized to mediate the respective activity in plant cells, thereby delivering Cu to the ethylene receptor. The interacting metallochaperone, which is expected to be orthologous to *S. cerevisiae* Atx1 or human HAH1 (Hamza et al. 1999), is unknown.

Basic mechanisms of plastid Cu homeostasis can probably be inferred from knowledge gained on Cu homeostasis in cyanobacteria (Cavet et al. 2003). A P-type ATPase, CtaA, is required for Cu import into *Synechocystis* PCC 6803 cells. An Atx1 homolog is hypothesized to shuttle Cu between CtaA and a second P-type-ATPase, PacS, which is located in the thylakoid membrane and is essential for the synthesis of holo-plastocyanin. *Synechocystis* Atx1 has been shown to interact with both CtaA and PacS.

The two analogous plastidic Cu pumps have recently been identified in *Arabidopsis*. The P-type ATPase PAA1 is proposed to transport Cu across the inner envelope membrane (Fig. 1). *Arabidopsis paa1* mutants are impaired in photosynthetic electron transport and show reduced plastid Cu levels (Shikanai et al. 2003). A second P-type ATPase, PAA2, is responsible for the final membrane passage into the thylakoid lumen (Abdel-Ghany et al. 2005b). The metallochaperone shuttle between the two pumps remains to be identified. Also, it is unknown whether specific chaperones are required for the synthesis of mature plastocyanin. *Chlamydomonas reinhardtii pcy2* mutants are known to carry the wild type plastocyanin allele, yet, they accumulate apoplastocyanin (Li et al. 1996). The role in plastid Cu homeostasis of the *Arabidopsis* Cu-binding protein CUTA, which is assumed to be localized in the intermembrane space, remains to be elucidated (Burkhead et al. 2003).

3 Metal Regulation

3.1 Known and likely levels of metal regulation in plants

Plants respond to a change in metal supply by marked alterations in their steady-state transcriptome. The first known example was *IRT1* (Eide et al. 1996), which encodes the major high-affinity Fe uptake system of *A. thaliana* and is induced under Fe-deficient conditions in *A. thaliana* (Vert et al. 2002). Several genes encoding Zn transport proteins, namely *ZIP1*, *ZIP3* and *ZIP4* (Grotz et al. 1998), were shown to be induced under Zn-deficient conditions. Since then, microarray studies have identified numerous additional genes that respond to micronutrient deficiencies at the transcript level (Wintz et al. 2003; Colangelo and Guerinot 2004). In contrast, only a small number of genes appear to respond specifically to an excess of Zn, Cd, or Cu (Becher et al. 2004; Weber and Clemens, unpublished). Instead, transcriptional responses to metal excess largely resemble general stress responses.

It has been shown that Fe-deficiency-induced transcripts, for example, *IRT1* and *FRO2*, are also induced under mild excess of Zn (Connolly et al. 2002, 2003; Becher et al. 2004). When present in excess, Zn^{2+} ions are likely to replace Fe ions in metalloproteins, and are thus capable of inducing Fe deficiency as a result of a metal imbalance. Alternatively, there may be an underlying crosstalk between Zn and Fe directly in the Fe sensing mechanism of an Fe sensor protein. The *cis* elements and *trans* factors involved in transcriptional responses of plants to their Zn, Cu or Ni status are unknown.

The only higher plant metal regulatory transcription factors identified so far are involved in Fe regulation (Ling et al. 2002), for example, *Arabidopsis* BHLH29 (basic helix-loop-helix 29), also designated FIT1 (Fe-deficiency-induced transcription factor 1, Colangelo and Guerinot 2004). In a *fit1* mutant, a number of Fe-deficiency-induced transcripts, for example, *FRO2* encoding a root ferric chelate reductase, are no longer upregulated, suggesting that FIT1 is required for their transcriptional induction (Colangelo and Guerinot 2004; Jakoby et al. 2004). Steady-state *FIT1* transcript levels are upregulated under Fe deficiency. The *FIT1* gene is expressed in the epidermis of the root hair zone of iron-deficient seedlings, as observed previously for *IRT1* and *FRO2* expression. This highlights the tissue-specific expression of many metal homeostasis genes. Another example is the expression pattern of the putative cellular Cu uptake system *COPT1*, which is restricted to specific cell types, for example, the external cell layers of a small apical zone in the root (Sancenon et al. 2004). Promoter sequence elements have been identified, which confer iron-regulated expression of reporter genes (Wei and Theil 2000; Petit et al. 2001; Kobayashi et al. 2003).

There is also evidence for post-transcriptional regulation in iron-deficiency-induced gene expression. Plants transformed with *p35S::IRT1* or *p35S::FRO2* display constitutive ectopic overexpression of *IRT1* or *FRO2* transcripts, respectively, but protein levels remain iron-regulated as in wild type plants (Connolly et al. 2002; 2003). So far, nothing is known about post-transcriptional regulation in response to changes in Zn, Ni, and Cu supply.

In higher plants, there is no published evidence for the post-translational regulation of metal homeostasis proteins. However, there is solid evidence for the regulation of sub-cellular protein localization and stability in the yeast *Saccharomyces cerevisiae* and in humans (see below). It is, thus, likely that similar mechanisms operate in higher plants.

Plants can be postulated to contain specific metal sensors that detect changes in metal status, i.e., deficiency or excess, and trigger signaling cascades that activate the appropriate responses. In higher plants, the involved signal transduction pathways have not been identified. The activation of distinct mitogen-activated protein kinase pathways has been reported upon exposure of *Medicago sativa* seedlings to very high, toxic concentrations of Cu or Cd, respectively (Jonak et al. 2004). It remains to be established to which extent the activation of the respective MAP kinase cascades is metal-dependent or an effect of oxidative stress and thiol group reactivity of high concentrations of Cu and Cd.

3.2 Long distance signaling in metal homeostasis

In plants, photosynthetic organs or the shoot apex are likely to be important metal sinks, but the site of metal uptake is at a distance in the root. There is a need for long distance signaling for the maintenance of metal homeostasis. Grafting experiments employing the pea *dgl* and *brz* mutants suggested that a shoot-derived signal is sufficient to induce Fe deficiency responses in pea roots (Grusak and Pezeshgi 1996; Vert et al. 2003). The *chloronerva* mutant of tomato, which is nicotianamine-deficient, contains lower shoot Cu than wild type plants, but accumulates higher concentrations of Mn, Fe, and Zn in all leaves except the apex (Pich et al. 1994). Yet the *chloronerva* mutant constitutively exhibits root Fe deficiency responses and chlorosis of young leaves.

Constitutive root Fe deficiency responses have also been described in the *A. thaliana frd3* (*man1*) mutant lacking a putative transporter of the MATE (multidrug and toxin efflux; multi-antimicrobial extrusion) family, which is mainly expressed in the root vasculature (Delhaize 1996; Rogers and Guerinot 2002). Green and Rogers (2004) observed ferric Fe accumulation in the root vasculature of *frd3*. Although *frd3* mutants accumulate Mn, Cu, Fe, and Zn in their leaves, intracellular Fe concentrations are lower in *frd3* leaves than in wild type leaves. Grafting experiments suggested that a *frd3* root is sufficient to generate a *frd3* phenotype. This led the authors to suggest that FRD3 is involved in the transport into the xylem of an Fe chelator or another compound that maintains Fe in - or is necessary for the transfer of Fe into - a chemical form that can be taken up by cells inside the shoot (Green and Rogers 2004).

Both the *chloronerva* and the *frd3* mutants have in common that they generally accumulate higher concentrations of several metals, including Fe, around the vasculature and in the apoplast of their leaves, respectively, when compared to wild type plants. Despite metal accumulation in the leaves, Fe deficiency responses are induced in the roots, probably because the metal ions do not reach their destination in the leaves. Thus, plant cells may sense their Fe status in close proximity to Fe-

requiring sites, for example, in the leaves, and generate signals that act in a long-distance manner, for example, in the root. Similar regulatory mechanisms can be expected to operate for other metals.

3.3 Principles of Cu and Zn regulation in model organisms

3.3.1 Cu regulation in Chlamydomonas reinhardtii

Cu-dependent transcriptional regulation has been analyzed in the unicellular green alga *Chlamydomonas reinhardtii*. When no Cu is available for the formation of holoplastocyanin in *C. reinhardtii*, apoplastocyanin is rapidly degraded, presumably through a non-specific pathway (Merchant and Bogorad 1986a). Instead, several Cu-deficiency-induced proteins are induced at the transcriptional level, including a heme-containing cytochrome c_6 (Cyc6), which acts as a functional substitute for the Cu-dependent plastocyanin in the photosynthetic electron transport chain when Cu availability is low (Merchant and Bogorad 1986b; Hill and Merchant 1995; Quinn and Merchant 1995; Moseley et al. 2000; Quinn et al. 2000, 2002). The upregulation of genes in response to Cu deficiency is dependent on a Cu-responsive element (CuRE) in the 5' upstream region of Cu-deficiency induced genes, containing a core GATC sequence. The transcriptional activator responsible for Cu-deficiency-induced gene expression is Crr1 (copper-responsive regulator 1). The Crr1 protein shares some similarity with the plant-specific squamosa-promoter-binding protein family (SBP) and contains Zn fingers in its DNA-binding domain. Binding of a Cu ion to a C-terminal cysteine-rich domain of Crr1 is thought to result in its dissociation from the CuRE (Merchant et al. 2004).

The Crr1 protein is also required for growth under hypoxia, and some Cu-deficiency-induced genes, including *Cyc6*, are also induced under hypoxia. In addition to Crr1, hypoxia-dependent gene expression requires a second *cis* element named hypoxia-responsive element. Other hypoxia-induced genes are induced *via* a Crr1-independent pathway (Quinn et al. 2002). Hypoxia alone does not induce a reduction in Cu absorption or internal Cu availability in *C. reinhardtii*. This suggests that the coupling of hypoxia and Cu-deficiency signaling may be an indirect effect, for example, related to Fe acquisition, or may have an evolutionary or an environmental significance. Another interesting feature of Crr1-dependent gene regulation is that the Crr1 protein can function as a transcriptional activator or as a repressor, depending on the position of the CuRE (Moseley et al. 2002).

3.3.2 Known principles of metal regulation

The example of Cu regulation in *C. reinhardtii* outlined above highlights a number of principles of metal regulation that have been found in several model organisms. Metal-sensing transcription factors controlling the transcription of target genes are a common feature in metal regulation. These are examples for very direct metal-dependent regulation, not requiring upstream signal transduction cas-

cades. For example, under conditions of Cu deficiency the Cu-binding Mac1p (metal-binding activator 1, for details see Chapter 2) transcription factor of *S. cerevisiae* binds as a homodimer to copper-responsive sequence elements (CuRE) in the promoters of the genes *ScCTR1* and *ScCTR3*, which encode Cu(I) uptake systems (Jungmann et al. 1993; Labbe et al. 1997; Yamaguchi-Iwai et al. 1997; Zhu et al. 1998; Rutherford and Bird 2004). Mac1p is able to bind 4 Cu(I) ions in a poly-copper cluster within its transactivation domain. The binding of Cu(I) to Mac1p triggers an interaction between the transactivation and DNA binding domains of Mac1p, thereby inhibiting the functions of both domains. Under conditions of Cu excess, the Cu-dependent transcriptional activator ScAce1p is activated by the binding of four Cu(I) ions and activates the transcription of genes involved in the protection of yeast cells from Cu toxicity, for example, the gene encoding the Cu-buffering cysteine-rich Cup1 protein (Rutherford and Bird 2004).

Two other well-known examples are the Zn-sensing transcription factors *Saccharomyces cerevisiae* Zap1 (Zn-dependent transcriptional activator protein 1, for details see Chapter 3), which regulates Zn deficiency responses (Zhao and Eide 1997), and the human MTF1 (metal-regulatory transcription factor 1, for details see Chapter 6), which is involved in the responses to an excess of Zn and other metals, and other stresses (Westin and Schaffner 1988; Smirnova et al. 2000; Andrews 2001; LaRochelle et al. 2001). Both proteins possess high-affinity Zn fingers in their DNA-binding domain and regulatory Zn fingers with Zn affinities in the nanomolar to sub-nanomolar range. Similar to Crr1, Zap1 can also act as an activator or repressor of transcription of a target gene depending on the position of the Zn-responsive *cis* element (Bird et al. 2004). In many cases, regulation by metal-sensing transcription factors involves additional levels of regulation, for example, nucleo-cytoplasmic shuttling (for example, MTF-1; Smirnova et al. 2000), phosphorylation (MTF-1; LaRochelle et al. 2001) and protein degradation (for example, Mac1p; Zhu et al. 1998).

In plants no homologues of Zap1 or MTF1 can be clearly identified. Moreover, the 5' sequences upstream of Zn deficiency induced genes like *AtZIP1* or *AtZIP4* do not contain sequences related to the *S. cerevisiae* Zn responsive elements found in the ZAP1 regulon. Further research is needed to identify plant Zn- and Cu-regulatory *cis* elements and *trans* factors and to investigate whether plants possess transcription factors that directly sense metal ions.

A second important level of metal regulation is the metal-dependent regulation of transcript stability. The expression of the *S. cerevisiae CTH2* gene is induced under Fe deficiency. The Cth2 protein binds to AU-rich elements in specific mRNAs encoding Fe-metalloproteins and targets them for degradation (Puig et al. 2005).

Availability and insertion of the metal cofactor into apoplastocyanin controls the stability of the translation product in the chloroplast of *C. reinhardtii* (see above). Although not all apometalloproteins are unstable, the insertion of metal ion cofactors is likely to be an important factor controlling the activity and/or stability of proteins, and possibly of biological processes.

A well-known post-translational mechanism of metal regulation is the metal-dependent re-localization or degradation of metal transport proteins. The human

Cu-pumping ATPase ATP7A or MNK (for Menkes), mutations of which are responsible for Menkes disease (see Chapter 5), exhibits Cu-dependent sub-cellular localization (Petris et al. 1996). The protein was proposed to cycle continuously between the Golgi and the plasma membrane. Under most conditions the major proportion of the MNK protein localizes predominantly to the *trans*-Golgi, supplying Cu ions to the lumen of this compartment. Under exposure to high Cu concentrations, the steady state is shifted towards localization in the plasma membrane, where the bulk of MNK protein exports Cu to the exterior of the cell (Petris and Mercer 1999). In Cu-deficient cells, the human copper uptake transporter hCtr1 localizes to the plasma membrane, but undergoes Cu-stimulated endocytosis under Cu re-supply. Two putative Cu-binding methionine-rich sequence elements of hCtr1 are involved in the regulation of endocytosis, suggesting that direct Cu sensing by hCtr1 may be controlling hCtr1 localization (Guo et al. 2004).

Under Zn-deficient conditions the *S. cerevisiae* high affinity Zn uptake system Zrt1 is expressed at high levels in a Zap1-dependent manner. When Zn-deficient yeast cells are transferred to a Zn-supplemented medium, the Zrt1 protein is ubiquinated and internalized by endocytosis, followed by its degradation in the vacuole (Gitan et al. 1998, 2003; Gitan and Eide 2000). Zinc-stimulated endocytosis also controls plasma membrane localization and, thus, Zn uptake activity through the mouse ZIP1, ZIP3, and ZIP4 transporters, which are homologues of ScZrt1p (Kim et al. 2003a; Wang et al. 2004a, 2004b). Zn-dependent localization has also been reported for Zn transporters of the so-called cation diffusion facilitator (SLC30 or TC 2.A.4) protein family (Kelleher and Lonnerdal 2003). It is very likely that some plant metal transport proteins also undergo metal dependent relocalization.

Some of the known metal regulatory events exemplify very direct regulation: binding of a metal ion modifies the biological activity of a protein involved in the homeostasis of this metal ion. As pointed out earlier, the delivery or withholding of a metal ion through the metal homeostatic machinery could also modify the activity of biological processes. For example, Zn^{2+} ions modulate signal transduction in animal neurotransmission and development (Baranano et al. 2001; Hajnal 2002). The presence of metal ions in hormone receptors and related proteins, for example, of Cu(I) in the ethylene receptor (Rodriguez et al. 1999) or Zn(II) in the auxin-binding protein ABP1 (Napier 2004), may provide micronutrient checkpoints in plant signaling cascades. In this context, it is interesting to note that the EIN2 (ethylene-insensitive 2) protein possesses an N-terminal domain sharing strong homology with NRAMP (natural-resistance associated macrophage protein) family metal transport proteins, although no metal transport activity could be demonstrated for EIN2 (Alonso et al. 1999). Along a similar line, IAR1 (IAA-alanine resistance protein 1) is a member of the ZIP family of divalent transition metal cation transport proteins (Lasswell et al. 2000).

Protein modifications, for example, ubiquitination or phosphorylation, appear to be common in metal homeostasis and are likely to involve metal-dependent signaling cascades. These are largely unknown, although Zn sensing and signaling pathways in mammalian cells are being discussed (Hershfinkel et al. 2001; Maret 2001; Azriel-Tamir et al. 2004). In addition, future work will have to clarify to

which extent Zn^{2+} acts as a signaling ion beyond the regulation by Zn of its own homeostasis.

4 Toxicity and tolerance in plants of Cu, Ni, Zn

4.1 Toxicity of excess concentrations of Cu, Ni, and Zn

When present in excess in the growth medium, metal ions can cause toxicity in plants. Easily detectable symptoms are a reduction in photosynthetic electron transport and photosynthetic efficiency, the inhibition of root elongation, reduced shoot growth, leaf chlorosis, and necrosis of tissues. The precise mechanistic sequence of events in metal toxicity is poorly understood. Critical toxicity levels for Zn range between 100 and 300 μg g^{-1} dry weight (Marschner 1995). For Ni, critical leaf toxicity thresholds vary between 10 and approximately 50 μg g^{-1} dry biomass. Plants are very sensitive to Cu, with critical toxicity concentrations around 20 to 30 μg g^{-1} dry biomass in the leaves of crop plants (Marschner 1995). The latter figures have been confirmed for young maize seedlings (Mocquot et al. 1996) by using the Cu-elicited increase in peroxidase activity as a marker for toxicity. Few other recent studies have attempted to determine the toxicity threshold based on sensitive indicators. Most reports instead describe effects of exposure to Cu concentrations that result in root or foliar Cu contents, which are substantially above the minimal toxic concentration.

Fertilization, primarily with nutrient metals like Fe or Ca, has repeatedly been reported to alleviate metal toxicity (Marschner 1995). This highlights one major mechanism of toxic action of all transition metal ions: the efficient competition of metal ions, which are found at the left end of the Irving-Williams series, for binding sites of metal ions that are found downstream in the Irving-Williams series. Primary effects of this are displacements of essential metal ions from their binding sites, for example, of Mg in chlorophyll, which generally result in deactivation of the respective protein. It is difficult to distinguish primary displacement from secondary displacement effects. The latter are nutrient deficiencies caused by an excess of another metal ion. For example, an excess of Zn^{2+} or Cu^{2+} can cause Fe or Mn deficiency (Marschner 1995). Photosynthesis, especially the functioning of photosystem II, is particularly sensitive to the toxicity of a number of metals including Zn, Ni, and Cu (Jegerschold et al. 1999; Pätsikkä et al. 2002; Cho et al. 2003). For example, it was shown for intact plants and *in vitro* for isolated thylakoids that Cu^{2+} enhances the sensitivity of photosystem II (PS II) to photoinhibition (Yruela et al. 1996a, 1996b). It is, however, still a matter of debate whether this is due to a direct effect of Cu^{2+} on PS II components or rather due to an indirect effect. A recent study suggests that the primary cause is a reduction in chlorophyll content, possibly attributable to a competition between Fe and Cu ions in the root (Pätsikkä et al. 2002).

A further effect of the presence of excess metal ions is the inactivation of metabolites or proteins by adventitious binding, for example, to thiol groups of en-

zymes (Van Assche and Clijsters 1990). Metal-induced disruption of electron transport chains and of enzymatic reactions can lead to secondary oxidative stress. In addition, oxidative stress can be caused indirectly by a depletion of reduced glutathione, the major redox buffer in plant cells, through formation of metal-glutathione complexes. Excess Cu ions elicit the synthesis of phytochelatins (PC) from GSH, and it is well-documented that the glutathione pool decreases transiently upon the onset of PC synthesis (De Vos et al. 1992).

Two possible modes of Cu toxicity have been discussed predominantly: inhibitory effects on photosystem II (see above) and membrane damage due to lipid peroxidation. Due to the ability to change its redox state under biological conditions, Cu – but not Ni or Zn – can cause oxidative stress directly. Cu(I) ions can react in a Fenton-type reaction with H_2O_2 to generate hydroxyl radicals ($^{\cdot}OH$), one of the most reactive molecules occurring in biological systems. In a Haber-Weiss reaction oxidized Cu(II) can be reduced by superoxide radicals ($O_2^{\cdot-}$) and then react with H_2O_2 (Halliwell and Gutteridge 1984). Indeed, root cells exposed to external growth-inhibiting Cu^{2+} concentrations show K^+ leakage (De Vos et al. 1991; Murphy et al. 1999; Quartacci et al. 2001). Copper-induced lipid peroxidation was observed to begin after a lag period of 4 h in *Arabidopsis* roots. Copper-induced long-term net K^+ leakage between 0 h and 36 h correlated with Cu sensitivity in different accessions of *A. thaliana* (Murphy and Taiz 1997). However, short-term K^+ and citrate release from roots within 4 h of Cu^{2+} exposure was inversely correlated with Cu sensitivity in these *Arabidopsis* accessions, and was attributed to the activation of ion channels.

4.2 Basal metal tolerance in plants

In order to avoid metal toxicity all plants possess basal tolerance mechanisms. At the physiological level, this involves the immobilization of excess metals in the root, thus excluding the metals from the shoot. In the cytoplasm, this could involve the binding of free metal ions to metal-buffering proteins, e.g., metallothioneins or metallochaperones, or to low-molecular-weight metal chelator molecules (for details see Chapter 10). Definitive molecular proof for a direct involvement of metallothioneins in Zn or Cu detoxification is still scarce (Murphy and Taiz 1995; Cobbett and Goldsbrough 2002). Phytochelatins, low molecular-weight cysteine-rich metal-binding peptides of the general formula $(\gamma\text{-EC})_nG$ (n = 2 to 11), are known to be of major importance in the basal tolerance of plants to Cd, Cu and arsenic (Grill et al. 1985; Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999; Schmöger et al. 2000; Hartley-Whitaker et al. 2001). Phytochelatins are synthesized from glutathione by the enzyme phytochelatin synthase. The sequestration of excess metal ions in the vacuole is also important for detoxification. Metal-phytochelatin or metal-glutathione complexes are likely to be transported into the vacuole *via* transporters of the ATP-binding cassette transporter family (Ortiz et al. 1995; Rea et al. 1998). Zinc transporters of the so-called cation diffusion facilitator family, such as ZAT (Zn transporter of *Arabidopsis thaliana*) or MTP1 (metal tolerance protein 1), participate in the cellular detoxifi-

cation of Zn in the vacuole (Van der Zaal et al. 1999; Kobae et al. 2004; Desbrosses-Fonrouge et al. 2005; Krämer 2005). When ectopically overexpressed in *A. thaliana* the poplar PtdMTP1 as well as the *A. thaliana* ZAT/MTP1 confer enhanced Zn tolerance (Van der Zaal et al. 1999; Blaudez et al. 2003). However, *ZAT* or *PtdMTP1* transcript levels are not upregulated in response to Zn treatment. This suggests that these transporters may have a housekeeping function instead of a specific detoxification function, or that they may be regulated post-transcriptionally or post-translationally in response to excess Zn. Another detoxification mechanism is the direction of excess metals to specific cell types, for example, trichomes or the epidermis of leaves (Brune et al. 1995). The molecular basis for this is unknown.

4.3 Naturally selected metal hypertolerance and hyperaccumulation

Some plants have evolved naturally selected metal tolerance. For example, specific accessions of *Silene vulgaris*, which originate from various metal-contaminated sites (Ernst 1974), are known to be tolerant to either Zn, Cd, or Cu (Schat et al. 1996). A segregation analysis of crosses between accessions of *S. vulgaris* exhibiting tolerance to different metals suggested the involvement of a small number of genes in each Zn, Cd, and Cu tolerance, respectively (Schat et al. 1993; Schat and Vooijs 1997). A similar conclusion has been reached in a genetic analysis of Cu tolerance in a naturally selected Cu-tolerant population of *Mimulus guttatus* (Macnair 1993). Tonoplast-enriched membrane fractions isolated from roots of a Zn-tolerant accession of *S. vulgaris* exhibited distinct Zn^{2+} transport characteristics, including an increased rate of Zn^{2+} transport, when compared to a Zn-sensitive accession (Chardonnens et al. 1999). An enhanced sequestration of Zn in the vacuoles of roots, which removes Zn from the cytoplasm and is likely to reduce the mobility of Zn for movement into the shoot, may thus contribute to Zn tolerance in *S. vulgaris*.

It was demonstrated for *Silene cucubalus (vulgaris)* that K^+ efflux is measurable at the minimal inhibitory Cu^{2+} concentration and is correlated with membrane damage (De Vos et al. 1991). No differences were found in plasma membrane lipid composition between Cu-sensitive and Cu-tolerant *Silene* populations. It was proposed that naturally selected Cu hypertolerance involves enhanced efflux of excess Cu ions, which was suggested to reduce damage to the membranes (De Vos et al. 1991). Indeed, comparing Cu-tolerant and Cu-sensitive accessions of *S. vulgaris*, an enhanced ATP-dependent cellular Cu efflux activity was reported in plasma membrane preparations of the Cu tolerant accession (Van Hoof et al. 2001b). This supports the idea that Cu tolerance in *S. vulgaris* is based on Cu exclusion from the plant. Using the progeny of crosses between Cu-sensitive and Cu-tolerant accessions of *S. vulgaris*, a co-segregation analysis was carried out to analyze the role of enhanced transcript levels of a metallothionein gene related to *A. thaliana MT2b* in Cu tolerance in *S. vulgaris* (Van Hoof et al. 2001a). The authors concluded that high *MT2b* transcript levels conferred enhanced Cu tolerance

only to Cu-tolerant plants, which excludes *MT2b* as a major gene in naturally selected Cu tolerance of *S. vulgaris*.

A small group of metal-tolerant plant taxa are designated metal hyperaccumulators (for more see Chapter 11), because they specifically accumulate very high concentrations of metals in their shoot biomass without developing any toxicity symptoms (Baker and Brooks 1989). Since geologically Ni-rich soils, so-called ultramafic soils, are quite widespread worldwide, Ni hyperaccumulation is most common, with approximately 350 taxa (R. Reeves, personal communication) known to date, which accumulate between 1000 μg g^{-1} Ni and just below 40,000 μg g^{-1} Ni. There are also 11 known Zn hyperaccumulator taxa (R. Reeves, personal communication). Copper hyperaccumulation to leaf concentrations above 1000 μg g^{-1} dry biomass was reported in plant specimens of 34 plant taxa (R. Reeves, personal communication). Although these concentrations may be the result of contamination, the Cu concentrations accumulated in leaves of these plants may still be substantially higher than in other plants. In metal hyperaccumulators the concentration of the hyperaccumulated metal is generally higher in the aboveground biomass than in the roots (Baker et al. 1994; Krämer et al. 1996). Metals are detoxified primarily by sequestration in shoot vacuoles (Krämer et al. 2000; Cosio et al. 2004; Ma et al. 2005).

To date, we know little about the mechanisms underlying metal hyperaccumulation. A microarray-based comparison of the transcriptome of a Zn and Cd-tolerant Zn hyperaccumulator, *Arabidopsis halleri*, and the sensitive non-accumulator *A. thaliana* has recently resulted in the identification of a number of candidate genes, which together may be responsible for hyperaccumulation and associated tolerance (Becher et al. 2004; Weber et al. 2004). Surprisingly, there were only minor gene expression changes upon exposure of the hyperaccumulator or the non-accumulator to high, but non-toxic, Zn or Cd concentrations. Instead, a number of metal homeostasis genes were constitutively very highly expressed in *A. halleri*. In roots and shoots, the encoded proteins were distinct isoforms of membrane transport proteins of the ZIP family (see above) with a predicted function in cytoplasmic import of metal ions. In addition, a P_{1B}-type metal ATPase and an MTP1-like protein with a putative function in effluxing metal ions from the cytoplasm were implicated primarily in the shoots. Finally, distinct isoforms of nicotianamine synthase were identified in roots and shoots and shown to mediate cellular Zn detoxification when expressed in yeast (see also Section 2.2.1). These results suggest that the low-molecular-weight chelator molecule nicotianamine is involved in naturally selected Zn tolerance of *A. halleri* and possibly in the hyperaccumulation of Zn (Weber et al. 2004; see also Chapter 10).

Increased rates of Zn^{2+} influx into roots of the Zn hyperaccumulator *T. caerulescens* were reported to correlate with high transcript levels for the ZIP family genes *ZnT1* and *ZnT2*, when compared to the non-accumulator *T. arvense* (Lasat et al. 2000; Pence et al. 2000; Assunção et al. 2001). In *T. arvense* the *ZnT1* orthologue was upregulated after two weeks of growth in a hydroponic solution lacking Zn, and no expression was detected at 1 μM Zn. This is analogous to the known regulation of several *ZIP* genes in *A. thaliana* (see above). A downregulation of *ZnT1* transcript levels in roots of *T. caerulescens*, however, was observed

only at a high Zn concentration of 50 μM. Similarly, the nicotianamine synthase gene *NAS2* was found to be highly expressed under control conditions in *A. halleri* and upregulated in a Zn-deficient medium in *A. thaliana* (Weber et al. 2004). This supports the idea that Zn hyperaccumulation may involve an alteration of the regulation of Zn homeostasis. A decreased Zn-sensitivity of a Zn sensor or downstream signal transduction pathway may contribute to metal hyperaccumulation. Based on the direct Zn regulatory mechanisms known to operate in the yeast *Saccharomyces cerevisiae*, this could involve, for example, a decreased affinity for Zn of a Zn-sensing transcription factor that negatively regulates the expression of *ZIP* and *NAS* genes upon Zn binding (see also above). Additionally, the enhanced sequestration and chelation of Zn(II) in Zn hyperaccumulators may maintain Zn concentrations at the site of sensing, for example, in the nucleus, at lower levels over a wider range of external Zn concentrations when compared to non-accumulators.

Ni hyperaccumulator species of the genus *Alyssum* contain constitutively high concentrations of the free amino acid histidine, which was shown to act as a ligand for Ni ions in the plants (Krämer et al. 1996; Persans et al. 1999; Kerkeb and Krämer 2003). Supplying the closely related non-tolerant non-accumulator plant *Alyssum montanum* with exogenous free histidine increased its tolerance to Ni (Krämer et al. 1996). *Arabidopsis thaliana* plants were generated which express a microbial gene encoding an ATP phosphoribosyl transferase, the enzyme catalyzing the first committed, rate-limiting step in the histidine biosynthesis pathway of bacteria, yeast, and plants (Wycisk et al. 2004). These transgenic plants contained increased concentrations of free histidine and were more Ni-tolerant than wild type plants. This supports a role for the Ni chelator free histidine in naturally selected Ni tolerance. Furthermore, when excised root systems of *A. lesbiacum* were immersed in a Ni-containing root medium, both Ni and histidine were translocated into the xylem at increased rates, when compared to plants immersed in a medium without added Ni (Krämer et al. 1996). By contrast, in the closely related non-accumulators *Alyssum montanum* and *Brassica juncea* much lower amounts of Ni were translocated into the xylem. However, when *A. montanum* or *B. juncea* root systems were pre-treated or concomitantly supplied with exogenous free histidine, Ni translocation into the xylem was strongly enhanced and accompanied by histidine release into the xylem (Krämer et al. 1996; Kerkeb and Krämer 2003). Histidine may thus also have an important role in the high rate of root-to-shoot transport of Ni in *Alyssum* hyperaccumulators. However, the histidine-overproducing transgenic *A. thaliana* plants did not accumulate higher leaf Ni concentrations than wild type plants (Wycisk et al. 2004; Ingle et al. 2005). In Zn hyperaccumulators, transcript levels of orthologues of *AtHMA4* are very high (compare Section 2.2.1). Thus, HMA4 proteins may account for the accumulation of Zn predominantly in the above-ground tissues of these species (Bernard et al. 2004; Hussain et al. 2004; Papoyan and Kochian 2004).

Transcript analyses revealed a high and generally constitutive expression of genes encoding proteins similar to the *A. thaliana* ZAT (also MTP1), primarily in the leaves of the Ni hyperaccumulator *T. goesingense* (Persans et al. 2001; Kim et al. 2004) and the Zn hyperaccumulators *T. caerulescens* (Assunção et al. 2001)

and *Arabidopsis halleri* (Dräger et al. 2004). Expression of the *T. goesingense* and *A. halleri* proteins, respectively, rescued a Zn hypersensitive yeast mutant defective in the sequestration of Zn in the vacuole. A chimeric GFP fusion of the *A. halleri* MTP1 protein was localized to the vacuolar membrane, suggesting a function for MTP1 in vacuolar sequestration and thus detoxification of Zn (Dräger et al. 2004). Two of a total of three genetically unlinked and highly similar *A. halleri MTP1* gene copies co-segregated with metal tolerance in the back-cross 1 (BC1) population of a cross between *A. halleri* and the Zn-sensitive non-accumulator species *A. lyrata*. These two gene copies largely accounted for the *MTP1* transcripts detected in leaves of BC1 individuals, whereas the third *AhMTP1* gene was expressed at a low level comparable to the single *AlMTP1* locus originating from *A. lyrata* (Dräger et al. 2004). This is strong evidence supporting the involvement of enhanced vacuolar metal sequestration in naturally selected metal tolerance of a hyperaccumulator. An increase in gene copy number may provide the ability to synthesize *MTP1* transcripts in higher amounts and may have allowed the different *MTP1* gene copies to acquire distinct regulation. A gene copy number amplification has also been proposed for the *S. vulgaris* metallothionein gene *MT2b* (Van Hoof et al. 2001a). Future work will show whether additional examples of copy number amplification can be identified for other genes involved in naturally selected metal tolerance or hyperaccumulation.

5 Prospects and developments

During the past 15 years, the small fields of plant metal nutrition and plant metal tolerance have merged into the much more comprehensive and complex research field of plant metal homeostasis. From the understanding of functions of single genes and proteins involved in metal homeostasis, we are gradually progressing towards the understanding of their regulation and beginning to develop a more integrated understanding of the functioning of the plant metal homeostasis network as a whole. The availability of genome sequence information for more and more plant species will enable us to address the evolution and conservation of metal homeostasis proteins and systems.

At present, however, huge gaps remain in our understanding of plant metal homeostasis. So far, we have not identified all molecular players and functions. For example, we do not know precisely which protein(s) mediate(s) the uptake of Zn(II) from the rhizosphere into the root symplast, and how metals move symplastically from cell to cell. We do not have complete knowledge of all plant metal-dependent proteins and their metal requirements. We do not know whether specific metallochaperone proteins control the trafficking of other metals in addition to Cu and Ni. We do not know the subcellular localization of most plant Cu metallochaperones. We also have not identified the target proteins for Cu delivery by each of these metallochaperones. Predictions indicate that there are virtually no free aqueous transition metal ions in the cytoplasm. What are then the real substrates for metal transporters like MTP proteins, which pick up their metal sub-

strates, believed to be the free aqueous metal ions, in the cytoplasm? Even if the affinities of these metal transporters for free metal ions were extremely high, a diffusion-controlled acquisition of cytoplasmic free metal ions by these transporters would be unlikely because transition metal ion dissociation kinetics are slow. How do the substrates reach the metal transporters within biologically reasonable time spans? What is the speciation of metal ions in the cytoplasm and in other organelles of plant cells, and are there any fluctuations? Moreover, we have only a very limited understanding of whether and how metal specificity is achieved by plants. A number of metal transporters appear to transport multiple metals and the Irving-Williams series largely determines the stability of metal complexes formed by many different types of ligands.

At the whole-plant level, we have limited knowledge of differential accumulation of metals in different cell types, and of metal remobilization and relocalization, throughout the plant life cycle. In humans, defense against invading pathogens involves the active withdrawal of micronutrients, primarily Mn (Supek et al. 1997) and Fe (Laham and Ehrlich 2004). So far, we have no clear evidence that similar processes contribute to plant defenses against pathogens.

In order to achieve a better understanding of how metals reach their binding sites in apometalloproteins, we will need to elucidate the structures of plant metalloproteins, metal transporters and metallochaperones. It will be particularly rewarding to obtain information on structural transitions in protein-protein and protein-metal-chelate interactions during metal transfer. Future research will also address the organ and cell-type specific needs and functions of metals, functioning, regulation, and coordination of the metal homeostasis network, as well as its interaction and integration with metabolism, stress responses, and plant development.

Acknowledgements

The collaboration between the laboratories of the two authors is financially supported by the EU Research and Training Network “Molecular mechanisms of metal homeostasis in higher plants” (METALHOME, contract no. HPRN-CT-2002-00243). This work was supported by a “Biofuture” grant from the German Federal Ministry of Education and Research (no. 0311877 to U.K.). Research in the laboratories of U.K. and S.C. is also funded by the Deutsche Forschungsgemeinschaft.

References

Abdel-Ghany SE, Burkhead JL, Gogolin KA, Andres-Colas N, Bodecker JR, Puig S, Penarrubia L, Pilon M (2005a) AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Lett 579:2307-2312

Abdel-Ghany SE, Muller-Moule P, Niyogi KK, Pilon M, Shikanai T (2005b) Two P-Type ATPases are required for Copper delivery in *Arabidopsis thaliana* chloroplasts. Plant Cell 17:1233-1251

Adam Z, Clarke AK (2002) Cutting edge of chloroplast proteolysis. Trends Plant Sci 7:451-456

Adamson P (2004) VITAMIN & MINERAL DEFICIENCY - A GLOBAL PROGRESS REPORT. The Micronutrient Initiative and UNICEF, Ottawa, Canada

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. Science 284:2148-2152

Andrews GK (2001) Cellular zinc sensors: MTF-1 regulation of gene expression. Biometals 14:223-237

Assunção AGL, Da Costa Martins P, De Folter S, Vooijs R, Schat H, Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 24:217-226

Auld DS (2001) Zinc coordination sphere in biochemical zinc sites. Biometals 14:271-313

Axelsen KB, Palmgren MG (2001) Inventory of the superfamily of P-type ion pumps in *Arabidopsis*. Plant Physiol 126:696-706

Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K, Schulze-Lefert P (2002) The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. Science 295:2073-2076

Azriel-Tamir H, Sharir H, Schwartz B, Hershfinkel M (2004) Extracellular zinc triggers ERK-dependent activation of Na^+/H^+ exchange in colonocytes mediated by the zinc-sensing receptor. J Biol Chem 279:51804-51816

Bacanamwo M, Witte CP, Lubbers MW, Polacco JC (2002) Activation of the urease of *Schizosaccharomyces pombe* by the UreF accessory protein from soybean. Mol Genet Genomics 268:525-534

Badger MR, Price GD (2003) CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. J Exp Bot 54:609-622

Bailey S, Thompson E, Nixon PJ, Horton P, Mullineaux CW, Robinson C, Mann NH (2002) A critical role for the Var2 FtsH homologue of *Arabidopsis thaliana* in the photosystem II repair cycle *in vivo*. J Biol Chem 277:2006-2011

Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metallic elements - a review of their distribution, ecology and phytochemistry. Biorecovery 1:81-126

Baker AJM, Reeves RD, Hajar ASM (1994) Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C. Presl (*Brassicaceae*). New Phytol 127:61-68

Balandin T, Castresana C (2002) AtCOX17, an *Arabidopsis* homolog of the yeast copper chaperone COX17. Plant Physiol 129:1852-1857

Baranano DE, Ferris CD, Snyder SH (2001) Atypical neural messengers. Trends Neurosci 24:99-106

Bartling D, Weiler EW (1992) Leucine aminopeptidase from *Arabidopsis thaliana*. Molecular evidence for a phylogenetically conserved enzyme of protein turnover in higher plants. Eur J Biochem 205:425-431

Becher M, Talke IN, Krall L, Krämer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J 37:251-268

Benes I, Schreiber K, Ripperger H, Kircheiss A (1983) Metal complex formation by nicotianamine, a possible phytosiderophore. Experientia 39:261-262

Berg JM, Shi Y (1996) The galvanization of biology: a growing appreciation for the roles of zinc. Science 271:1081-1085

Bernard C, Roosens N, Czernic P, Lebrun M, Verbruggen N (2004) A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. FEBS Letters 569:140-148

Bhushan S, Lefebvre B, Stahl A, Wright SJ, Bruce BD, Boutry M, Glaser E (2003) Dual targeting and function of a protease in mitochondria and chloroplasts. EMBO Rep 4:1073-1078

Bird AJ, Blankman E, Stillman DJ, Eide DJ, Winge DR (2004) The Zap1 transcriptional activator also acts as a repressor by binding downstream of the TATA box in *ZRT2*. EMBO J 23:1123-1132

Blaudez D, Kohler A, Martin F, Sanders D, Chalot M (2003) Poplar metal tolerance protein 1 (MTP1) confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. Plant Cell 15:2911-2928

Brown PH, Welch RM, Cary EE (1987a) Nickel: a micronutrient essential for all higher plants. Plant Physiol 85:801-803

Brown PH, Welch RM, Cary EE, Checkai RT (1987b) Beneficial effects of nickel on plant growth. J Plant Nutr 10:2125-2135

Brune A, Urbach W, Dietz KJ (1995) Differential toxicity of heavy metals is partly related to a loss of preferential extraplasmic compartmentation: a comparison of Cd-, Mo-, Ni- and Zn-stress. New Phytol 129:403-409

Brutnell TP, Sawers RJ, Mant A, Langdale JA (1999) BUNDLE SHEATH DEFECTIVE2, a novel protein required for post-translational regulation of the *rbcL* gene of maize. Plant Cell 11:849-864

Buchanan BB, Gruissem W, Jones RL (2000) Biochemistry & Molecular Biology of Plants, 1st edn. John Wiley & Sons Ltd., Bognor Regis, West Sussex, England

Burkhead JL, Abdel-Ghany SE, Morrill JM, Pilon-Smits EA, Pilon M (2003) The *Arabidopsis thaliana CUTA* gene encodes an evolutionarily conserved copper binding chloroplast protein. Plant J 34:856-867

Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16:3285-3303

Castilho Pd, Chardon WJ, Salomons W (1993) Influence of cattle-manure slurry application on the solubility of cadmium, copper, and zinc in a manured acidic, loamy-sand soil. J Environ Qual 22:689-697

Cavet JS, Borrelly GP, Robinson NJ (2003) Zn, Cu and Co in cyanobacteria: selective control of metal availability. FEMS Microbiol Rev 27:165-181

Chardonnens AN, Koevoets PL, van Zanten A, Schat H, Verkleij JA (1999) Properties of enhanced tonoplast zinc transport in naturally selected zinc-tolerant *Silene vulgaris*. Plant Physiol 120:779-786

Chen M, Choi Y, Voytas DF, Rodermel S (2000) Mutations in the *Arabidopsis* VAR2 locus cause leaf variegation due to the loss of a chloroplast FtsH protease. Plant J 22:303-313

Cho M, Chardonnens AN, Dietz K-J (2003) Differential heavy metal tolerance of *Arabidopsis halleri* and *Arabidopsis thaliana*: A leaf slice test. New Phytol 158:287-293

Clarke ND, Berg JM (1998) Zinc fingers in *Caenorhabditis elegans*: finding families and probing pathways. Science 282:2018-2022

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212:475-486

Clemens S, Antosiewicz DM, Ward JM, Schachtman DP, Schroeder JI (1998) The plant cDNA *LCT1* mediates the uptake of calcium and cadmium in yeast. Proc Natl Acad Sci USA 95:12043-12048

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 18:3325-3333

Clemens S, Palmgren MG, Kraemer U (2002) A long way ahead: Understanding and engineering plant metal accumulation. Trends Plant Sci 7:309-315

Clemens S, Simm C (2003) *Schizosaccharomyces pombe* as a model for metal homeostasis in plant cells: the phytochelatin-dependent pathway is the main cadmium detoxification mechanism. New Phytol 159:323-330

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:159-182

Colangelo EP, Guerinot ML (2004) The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell 16:3400-3412

Coleman JE (1998) Zinc enzymes. Curr Opin Chem Biol 2:222-234

Connolly EL, Campbell NH, Grotz N, Prichard CL, Guerinot ML (2003) Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. Plant Physiol 133:1102-1110

Connolly EL, Fett JP, Guerinot ML (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell 14:1347-1357

Cosio C, Martinoia E, Keller C (2004) Hyperaccumulation of cadmium and zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. Plant Physiol 134:716-725

Curie C, Panaviene Z, Loulergue C, Dellaporta SL, Briat JF, Walker EL (2001) Maize *yellow stripe1* encodes a membrane protein directly involved in Fe(III) uptake. Nature 409:346-349

Dalton AD, Russell SA, Evans HJ (1988) Nickel as a micronutrient element for plants. Biofactors 1:11-16

Dawson RM, Elliott DC, Elliott HE, Jones KM (1986) Data for Biochemical Research, 3rd edn. Oxford University Press, Oxford, UK

De Vos CHR, Schat H, De Waal MAM, Vooijs R, Ernst WHO (1991) Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. Physiol Plant 82:523-528

De Vos CHR, Ten Bookum WM, Vooijs R, Schat H, De Kok LJ (1992) Effect of copper on fatty acid composition and peroxidation of lipids in the roots of copper tolerant and sensitive *Silene cucubalus*. Plant Physiol Biochem 31:151-158

del Castilho P, Chardon WJ, Salomons W (1993) Influence of cattle-manure slurry application on the solubility of cadmium, copper, and zinc in a manured acidic, loamy-sand soil. Journal Environ Quality 22:689-697

Delhaize E (1996) A metal-accumulator mutant of *Arabidopsis thaliana*. Plant Physiology 111:849-855

Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci U S A 98:13454-13459

Desbrosses-Fonrouge AG, Voigt K, Schröder A, Arrivault S, Thomine S, Krämer U (2005) *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. FEBS Letters in press

DiDonato RJ Jr, Roberts LA, Sanderson T, Eisley RB, Walker EL (2004) *Arabidopsis Yellow Stripe-Like2* (*YSL2*): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine-metal complexes. Plant J 39:403-414

Dräger DB, Desbrosses-Fonrouge AG, Krach C, Chardonnens AN, Meyer RC, Saumitou-Laprade P, Krämer U (2004) Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. Plant J 39:425-439

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci USA 93:5624-5628

Eren E, Arguello JM (2004) *Arabidopsis* HMA2, a divalent heavy metal-transporting P_{IB}-type ATPase, is involved in cytoplasmic Zn^{2+} homeostasis. Plant Physiol 136:3712-3723

Ernst WHO (1974) Schwermetallvegetationen der Erde. Gustav Fischer Verlag, Stuttgart, Germany

Eskew DL, Welch RM, Cary EE (1983) Nickel: an essential micronutrient for legumes and possibly all higher plants. Science 222:621-623

Eskew DL, Welch RM, Norvell WA (1984) Nickel in higher plants. Plant Physiol 76:691-693

Fett JP, Coleman JR (1994) Characterization and expression of two cDNAs encoding carbonic anhydrase in *Arabidopsis thaliana*. Plant Physiol 105:707-713

Finney LA, O'Halloran TV (2003) Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300:931-936

Fraústo da Silva JJR, Williams RJP (2001) The Biological Chemistry of the Elements, 2nd edn. Clarendon Press, Oxford, UK

Freyermuth SK, Bacanamwo M, Polacco JC (2000) The soybean *Eu3* gene encodes an Ni-binding protein necessary for urease activity. Plant J 21:53-60

Georgatsou E, Mavrogiannis LA, Fragiadakis GS, Alexandraki D (1997) The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. J Biol Chem 272:13786-13792

Giege P, Brennicke A (1999) RNA editing in *Arabidopsis* mitochondria effects 441 C to U changes in ORFs. Proc Natl Acad Sci USA 96:15324-15329

Gitan RS, Eide DJ (2000) Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter. Biochem J 346:329-336

Gitan RS, Luo H, Rodgers J, Broderius M, Eide DJ (1998) Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. J Biol Chem 273:28617-28624

Gitan RS, Shababi M, Kramer M, Eide DJ (2003) A cytosolic domain of the yeast Zrt1 zinc transporter is required for its post-translational inactivation in response to zinc and cadmium. J Biol Chem 278:39558-39564

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell 5:1217-1229

Graham RD, Rengel Z (1993) Genotypic variation in zinc uptake and utilization by plants. In: Robson AD (ed) Developments in Plant and Soil Sciences: Zinc in soils and plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 107-118

Green LS, Rogers EE (2004) FRD3 controls iron localization in *Arabidopsis*. Plant Physiol 136:2523-2531

Grill E, Winnacker EL, Zenk MH (1985) Phytochelatins, the principal heavy-metal complexing peptides of higher plants. Science 230:674-676

Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. Proc Natl Acad Sci USA 95:7220-7224

Grusak MA, Pezeshgi S (1996) Shoot-to-root signal transmission regulates root Fe(III) reductase activity in the *dgl* mutant of pea. Plant Physiology 110:329-334

Guo Y, Smith K, Lee J, Thiele DJ, Petris MJ (2004) Identification of methionine-rich clusters that regulate copper-stimulated endocytosis of the human Ctr1 copper transporter. J Biol Chem 279:17428-17433

Ha S-B, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. Plant Cell 11:1153-1163

Hacisalihoglu G, Hart JJ, Kochian LV (2001) High- and low-affinity zinc transport systems and their possible role in zinc efficiency in bread wheat. Plant Physiol 125:456-463

Hacisalihoglu G, Hart JJ, Vallejos CE, Kochian LV (2004) The role of shoot-localized processes in the mechanism of Zn efficiency in common bean. Planta 218:704-711

Hacisalihoglu G, Hart JJ, Wang YH, Cakmak I, Kochian LV (2003) Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. Plant Physiol 131:595-602

Hajnal A (2002) Fine-tuning the RAS signaling pathway: Zn^{2+} makes the difference. Mol Cell 9:927-928

Halliwell B, Gutteridge JM (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1-14

Hamza I, Schaefer M, Klomp LW, Gitlin JD (1999) Interaction of the copper chaperone HAH1 with the Wilson disease protein is essential for copper homeostasis. Proc Natl Acad Sci USA 96:13363-13368

Hanikenne M, Krämer U, Demoulin V, Baurain D (2005) A comparative inventory of metal transporters in the green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschyzon merolae*. Plant Physiol 137:428-446

Hartley-Whitaker J, Ainsworth G, Vooijs R, Bookum WT, Schat H, Meharg AA (2001) Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. Plant Physiol 126:299-306

Haslett BS, Reid RJ, Rengel Z (2001) Zinc mobility in wheat: Uptake and distribution of zinc applied to leaves or roots. Ann Bot 87:379-386

Henrique R, Jasik J, Klein M, Martinoia E, Feller U, Schell J, Pais MS, Koncz C (2002) Knock-out of *Arabidopsis* metal transporter gene *IRT1* results in iron deficiency accompanied by cell differentiation defects. Plant Mol Biol 50:587-597

Herbik A, Koch G, Mock HP, Dushkov D, Czihal A, Thielmann J, Stephan UW, Bäumlein H (1999) Isolation, characterization and cDNA cloning of nicotianamine synthase from barley. A key enzyme for iron homeostasis in plants. Eur J Biochem 265:231-239

Hershfinkel M, Moran A, Grossman N, Sekler I (2001) A zinc-sensing receptor triggers the release of intracellular Ca2+ and regulates ion transport. Proc Natl Acad Sci USA 98:11749-11754

Higuchi K, Kanazawa K, Nishizawa N-K, Chino M, Mori S (1994) Purification and characterization of nicotianamine synthase from Fe-deficient barley roots. Plant Soil 165:173-179

Hill KL, Merchant S (1995) Coordinate expression of coproporphyrinogen oxidase and cytochrome c6 in the green alga *Chlamydomonas reinhardtii* in response to changes in copper availability. EMBO J 14:857-865

Himelblau E, Mira H, Lin SJ, Culotta VC, Penarrubia L, Amasino RM (1998) Identification of a functional homolog of the yeast copper homeostasis gene *ATX1* from *Arabidopsis*. Plant Physiol 117:1227-1234

Hirayama T, Kieber JJ, Hirayama N, Kogan M, Guzman P, Nourizadeh S, Alonso JM, Dailey WP, Dancis A, Ecker JR (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97:383-393

Holden MJ, Crimmins TJ Jr, Chaney RL (1995) Cu^{2+} reduction by tomato root plasma membrane vesicles. Plant Physiol 108:1093-1098

Hu H, Sparks D (1991) Zinc deficiency inhibits chlorophyll synthesis and gas exchange in "Stuart" pecan. HortScience 26:267-268

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. Plant Cell 16:1327-1339

Ingle RA, Mugford ST, Rees JD, Campbell MM, Smith JAC (2005) Constitutively high expression of the histidine biosynthetic pathway contributes to nickel tolerance in hyperaccumulator plants. Plant Cell 17:2089-2106

Jaffe EK (2003) An unusual phylogenetic variation in the metal ion binding sites of porphobilinogen synthase. Chem Biol 10:25-34

Jakoby M, Wang HY, Reidt W, Weisshaar B, Bauer P (2004) *FRU* (*BHLH029*) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. FEBS Lett 577:528-534

Jegerschold C, MacMillan F, Lubitz W, Rutherford AW (1999) Effects of copper and zinc ions on photosystem II studied by EPR spectroscopy. Biochemistry 38:12439-12445

Jelakovic S, Kopriva S, Suss KH, Schulz GE (2003) Structure and catalytic mechanism of the cytosolic D-ribulose-5-phosphate 3-epimerase from rice. J Mol Biol 326:127-135

Jonak C, Nakagami H, Hirt H (2004) Heavy metal stress. Activation of distinct mitogen-activated protein kinase pathways by copper and cadmium. Plant Physiol 136:3276-3283

Jungmann J, Reins HA, Lee J, Romeo A, Hassett R, Kosman D, Jentsch S (1993) MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. EMBO J 12:5051-5056

Kampfenkel K, Kushnir S, Babiychuk E, Inze D, Van Montagu M (1995) Molecular characterization of a putative *Arabidopsis thaliana* copper transporter and its yeast homologue. J Biol Chem 270:28479-28486

Kawagashira N, Ohtomo Y, Murakami K, Matsubara K, Kawai J, Carninci P, Hayashizaki P, Kikuchi S, Higo K (2001) Multiple zinc finger motifs with comparison of plant and insect. In: Matsuda H, Miyano S, Takagi T, Wong L (eds) 12th International Conference on Genome Informatics, The Garden Hall, Yebisu Garden Place, Tokyo, Japan, pp 368-369

Kelleher SL, Lonnerdal B (2003) Zn transporter levels and localization change throughout lactation in rat mammary gland and are regulated by Zn in mammary cells. J Nutr 133:3378-3385

Kerkeb L, Krämer U (2003) The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. Plant Physiol 131:716-724

Kim BE, Wang F, Dufner-Beattie J, Andrews GK, Eide DJ, Petris MJ (2003a) Zn^{2+}-stimulated endocytosis of the mZIP4 zinc transporter regulates its location at the plasma membrane. J Biol Chem 279:4523-4530

Kim D, Gustin JL, Lahner B, Persans MW, Baek D, Yun DJ, Salt DE (2004) The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. Plant J 39:237-251

Kim S, Mollet JC, Dong J, Zhang KL, Park SY, Lord EM (2003b) Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. Proc Natl Acad Sci USA 100:16125-16130

Kliebenstein DJ, Monde RA, Last RL (1998) Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118:637-650

Kobae Y, Uemura T, Sato MH, Ohnishi M, Mimura T, Nakagawa T, Maeshima M (2004) Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. Plant Cell Physiol 45:1749-1758

Kobayashi T, Nakanishi H, Takahashi M, Kawasaki S, Nishizawa NK, Mori S (2001) *In vivo* evidence that *Ids3* from *Hordeum vulgare* encodes a dioxygenase that converts 2'-deoxymugineic acid to mugineic acid in transgenic rice. Planta 212:864-871

Kobayashi T, Nakayama Y, Itai RN, Nakanishi H, Yoshihara T, Mori S, Nishizawa NK (2003) Identification of novel *cis*-acting elements, *IDE1* and *IDE2*, of the barley *IDS2* gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants. Plant J 36:780-793

Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant J 39:415-424

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB (1999) The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Mol Biol 40:37-44

Krämer U (2005) MTP1 mops up excess zinc in *Arabidopsis* cells. Trends Plant Sci 10:313-315

Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM, Smith JAC (1996) Free histidine as a metal chelator in plants that accumulate nickel. Nature 379:635-638

Krämer U, Pickering IJ, Prince RC, Raskin I, Salt DE (2000) Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. Plant Physiol 122:1343-1353

Krüger C, Berkowitz O, Stephan UW, Hell R (2002) A metal-binding member of the late embryogenesis abundant protein family transports iron in the phloem of *Ricinus communis* L. J Biol Chem 277:25062-25069

Labbe S, Zhu Z, Thiele DJ (1997) Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. J Biol Chem 272:15951-15958

Laham N, Ehrlich R (2004) Manipulation of iron to determine survival: competition between host and pathogen. Immunol Res 30:15-28

Lahner B, Gong J, Mahmoudian M, Smith EL, Abid KB, Rogers EE, Guerinot ML, Harper JF, Ward JM, McIntyre L, Schroeder JI, Salt DE (2003) Genomic scale profiling of nutrient and trace elements in *Arabidopsis thaliana*. Nat Biotechnol 21:1215-1221

Lane TW, Morel FM (2000) A biological function for cadmium in marine diatoms. Proc Natl Acad Sci USA 97:4627-4631

Lanquar V, Lelièvre1 F, Vansuyt G, Curie C, Schröder A, Krämer U, Barbier-Brygoo H, Thomine S (2004) *AtNRAMP3* and *AtNRAMP4* encode reduntant metal transported involved in the mobilization of vacuolar iron pools. In: The XII International Symposium on Iron Nutrition and Interactions in Plants, Tokyo, Japan

LaRochelle O, Gagne V, Charron J, Soh JW, Seguin C (2001) Phosphorylation is involved in the activation of metal-regulatory transcription factor 1 in response to metal ions. J Biol Chem 276:41879-41888

Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV (2000) Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. J Exp Bot 51:71-79

Lasswell J, Rogg LE, Nelson DC, Rongey C, Bartel B (2000) Cloning and characterization of IAR1, a gene required for auxin conjugate sensitivity in Arabidopsis. Plant Cell 12:2395-2408

Lee J, Pena MM, Nose Y, Thiele DJ (2002) Biochemical characterization of the human copper transporter Ctr1. J Biol Chem 277:4380-4387

Lee YH, Tegeder M (2004) Selective expression of a novel high-affinity transport system for acidic and neutral amino acids in the tapetum cells of *Arabidopsis* flowers. Plant J 40:60-74

Li D, Zhu H, Liu K, Liu X, Leggewie G, Udvardi M, Wang D (2002) Purple acid phosphatases of *Arabidopsis thaliana*. Comparative analysis and differential regulation by phosphate deprivation. J Biol Chem 277:27772-27781

Li HH, Quinn J, Culler D, Girard-Bascou J, Merchant S (1996) Molecular genetic analysis of plastocyanin biosynthesis in *Chlamydomonas reinhardtii*. J Biol Chem 271:31283-31289

Lindahl M, Spetea C, Hundal T, Oppenheim AB, Adam Z, Andersson B (2000) The thylakoid FtsH protease plays a role in the light-induced turnover of the photosystem II D1 protein. Plant Cell 12:419-431

Lindahl M, Tabak S, Cseke L, Pichersky E, Andersson B, Adam Z (1996) Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants. J Biol Chem 271:29329-29334

Ling H-Q, Bauer P, Bereczky Z, Keller B, Ganal M (2002) The tomato *fer* gene encoding a bHLH protein controls iron-uptake responses in roots. Proc Natl Acad Sci USA 99:13938-13943

Ling H-Q, Koch G, Bäumlein H, Ganal MW (1999) Map-based cloning of *chloronerva*, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. Proc Natl Acad Sci USA 96:7098-7103

Lister R, Mowday B, Whelan J, Millar AH (2002) Zinc-dependent intermembrane space proteins stimulate import of carrier proteins into plant mitochondria. Plant J 30:555-566

Liu Y, Schiff M, Serino G, Deng XW, Dinesh-Kumar SP (2002) Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to tobacco mosaic virus. Plant Cell 14:1483-1496

Lopez Ribera I, Puigdomenech P (1999) Structure, organization and expression of the *eukaryotic translation initiation factor 5*, *eIF-5*, gene in *Zea mays*. Gene 240:355-359

Luciano P, Tokatlidis K, Chambre I, Germanique JC, Geli V (1998) The mitochondrial processing peptidase behaves as a zinc-metallopeptidase. J Mol Biol 280:193-199

Ma JF, Ueno D, Zhao FJ, McGrath SP (2004) Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. Planta 220:731-736

Macnair MR (1993) Tansley Review No. 49: The genetics of metal tolerance in vascular plants. New Phytol 124:541-559

Maidment JM, Moore D, Murphy GP, Murphy G, Clark IM (1999) Matrix metalloproteinase homologues from *Arabidopsis thaliana*. Expression and activity. J Biol Chem 274:34706-34710

Maret W (2001) Crosstalk of the group IIa and IIb metals calcium and zinc in cellular signaling. Proc Natl Acad Sci USA 98:12325-12327

Marschner H (1995) Mineral Nutrition of Higher Plants, 2nd edn. Academic Press Ltd., London

Mäser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJ, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. Plant Physiol 126:1646-1667

McGuirl MA, Dooley DM (1999) Copper-containing oxidases. Curr Opin Chem Biol 3:138-144

Merchant S, Allen M, Eriksson M, Kropat J, Moseley J, Quinn J, Tottey S (2004) Metabolic adaptation in a photosynthetic eukaryote to nutritional copper deficiency occurs through a novel DNA binding protein, Crr1. In: Trace Element Metabolsim: Integrating Basic and Applied Research, FASEB Summer Research Conferences, Snowmass, Colorado, USA

Merchant S, Bogorad L (1986a) Rapid degradation of apoplastocyanin in Cu(II)-deficient cells of *Chlamydomonas reinhardtii*. J Biol Chem 261:15850-15853

Merchant S, Bogorad L (1986b) Regulation by copper of the expression of plastocyanin and cytochrome c552 in *Chlamydomonas reinhardtii*. Mol Cell Biol 6:462-469

Merchant S, Dreyfuss BW (1998) Posttranslational assembly of photosynthetic metalloproteins. Annu Rev Plant Physiol Plant Mol Biol 49:25-51

Meyer-Bothling LE, Polacco JC (1987) Mutational analysis of the embryo-specific urease locus of soybean. Mol Gen Genet 209:439-444

Mira H, Martinez-Garcia F, Penarrubia L (2001) Evidence for the plant-specific intercellular transport of the Arabidopsis copper chaperone CCH. Plant J 25:521-528

Moberg P, Stahl A, Bhushan S, Wright SJ, Eriksson A, Bruce BD, Glaser E (2003) Characterization of a novel zinc metalloprotease involved in degrading targeting peptides in mitochondria and chloroplasts. Plant J 36:616-628

Mocquot B, Vangronsveld J, Clijsters H, Mench M (1996) Copper toxicity in young maize (*Zea mays* L.) plants: Effects on growth, mineral and chlorophyll contents, and enzyme activities. Plant Soil 182:287-300

Moller SG, McPherson MJ (1998) Developmental expression and biochemical analysis of the *Arabidopsis atao1* gene encoding an H_2O_2-generating diamine oxidase. Plant J 13:781-791

Moroney JV, Bartlett SG, Samuelsson G (2001) Carbonic anhydrases in plants and algae. Plant Cell Environ 24:141-153

Moseley J, Quinn J, Eriksson M, Merchant S (2000) The *Crd1* gene encodes a putative di-iron enzyme required for photosystem I accumulation in copper deficiency and hypoxia in *Chlamydomonas reinhardtii*. EMBO J 19:2139-2151

Moseley JL, Page MD, Alder NP, Eriksson M, Quinn J, Soto F, Theg SM, Hippler M, Merchant S (2002) Reciprocal expression of two candidate di-iron enzymes affecting photosystem I and light-harvesting complex accumulation. Plant Cell 14:673-688

Mulligan RM, Williams MA, Shanahan MT (1999) RNA editing site recognition in higher plant mitochondria. J Hered 90:338-344

Murphy A, Taiz L (1995) Comparison of metallothionein gene expression and nonprotein thiols in ten *Arabidopsis* ecotypes. Correlation with copper tolerance. Plant Physiol 109:945-954

Murphy A, Taiz L (1997) Correlation between potassium efflux and copper sensitivity in 10 *Arabidopsis* ecotypes. New Phytol 136:211-222

Murphy AS, Eisinger WR, Shaff JE, Kochian LV, Taiz L (1999) Early copper-induced leakage of K^+ from *Arabidopsis* seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiol 121:1375-1382

Napier R (2004) Plant hormone binding sites. Ann Bot 93:227-233

Nersissian AM, Immoos C, Hill MG, Hart PJ, Williams G, Herrmann RG, Valentine JS (1998) Uclacyanins, stellacyanins, and plantacyanins are distinct subfamilies of phytocyanins: Plant-specific mononuclear blue copper proteins. Protein Sci 7:1915-1929

Nieboer E, Richardson DES (1980) The replacement of the nondescript term "heavy metals" by a biologically and chemically significant classification of metal ions. Environ Pollut Ser B 1:3-26

Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air water and soils by trace metals. Nature 333:134-139

O'Halloran TV, Culotta VC (2000) Metallochaperones, an intracellular shuttle service for metal ions. J Biol Chem 275:25057-25060

Oparka KJ, Turgeon R (1999) Sieve elements and companion cells-traffic control centers of the phloem. Plant Cell 11:739-750

Ortiz DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270:4721-4728

Orzaez VMT, Diaz MA, Bravo R, Blazquez AG (2001) Mineral content of commercial pollen. Int J Food Sci Nutr 52:243-249

Otegui MS, Capp R, Staehelin LA (2002) Developing seeds of *Arabidopsis* store different minerals in two types of vacuoles and in the endoplasmic reticulum. Plant Cell 14:1311-1327

Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488-2492

Panina EM, Mironov AA, Gelfand MS (2003) Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc Natl Acad Sci USA 100:9912-9917

Papoyan A, Kochian LV (2004) Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. Plant Physiol 136:3814-3823

Parker DR, Norvell WA, Chaney RL (1979) GEOCHEM-PC--a chemical speciation program for IBM and compatible personal computers. Soil Sci Soc Am J 42:253-269

Pätsikkä E, Kairavuo M, Sersen F, Aro EM, Tyystjarvi E (2002) Excess copper predisposes photosystem II to photoinhibition *in vivo* by outcompeting iron and causing decrease in leaf chlorophyll. Plant Physiol 129:1359-1367

Pence NS, Larsen PB, Ebbs SD, Letham DL, Lasat MM, Garvin DF, Eide D, Kochian LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proc Natl Acad Sci USA 97:4956-4960

Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. Plant J 32:539-548

Persans MW, Nieman K, Salt DE (2001) Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. Proc Natl Acad Sci USA 98:9995-10000

Persans MW, Yan X, Patnoe J-MML, Krämer U, Salt DE (1999) Molecular dissection of the role of histidine in nickel hyperaccumulation in *Thlaspi goesingense* (Halacsy). Plant Physiol 121:1117-1126

Petit JM, van Wuytswinkel O, Briat JF, Lobreaux S (2001) Characterization of an iron-dependent regulatory sequence involved in the transcriptional control of *AtFer1* and *ZmFer1* plant ferritin genes by iron. J Biol Chem 276:5584-5590

Petris MJ, Mercer JF (1999) The Menkes protein (ATP7A; MNK) cycles via the plasma membrane both in basal and elevated extracellular copper using a C-terminal di-leucine endocytic signal. Hum Mol Genet 8:2107-2115

Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J (1996) Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. EMBO J 15:6084-6095

Pich A, Scholz G (1996) Translocation of copper and other micronutrient in tomato plants (*Lycopersicon esculentum* Mill.): nicotianamine-stimulated copper transport in the xylem. Journal of Experimental Botany 47:41-47

Pich A, Scholz G, Stephan UW (1994) Iron-dependent changes of heavy metals, nicotianamine, and citrate in different plant organs and in the xylem exudate of two tomato genotypes. Nicotianamine as possible copper translocator. Plant Soil 165:189-196

Pignocchi C, Foyer CH (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. Curr Opin Plant Biol 6:379-389

Puig S, Askeland E, Thiele DJ (2005) Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. Cell 120:99-110

Puig S, Thiele DJ (2002) Molecular mechanisms of copper uptake and distribution. Curr Opin Chem Biol 6:171-180

Quartacci MF, Cosi E, Navari-Izzo F (2001) Lipids and NADPH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. J Exp Bot 52:77-84

Quinn JM, Barraco P, Eriksson M, Merchant S (2000) Coordinate copper- and oxygen-responsive *Cyc6* and *Cpx1* expression in *Chlamydomonas* is mediated by the same element. J Biol Chem 275:6080-6089

Quinn JM, Eriksson M, Moseley JL, Merchant S (2002) Oxygen deficiency responsive gene expression in *Chlamydomonas reinhardtii* through a copper-sensing signal transduction pathway. Plant Physiol 128:463-471

Quinn JM, Merchant S (1995) Two copper-responsive elements associated with the *Chlamydomonas Cyc6* gene function as targets for transcriptional activators. Plant Cell 7:623-628

Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 284:805-808

Ramesh SA, Shin R, Eide DJ, Schachtman DP (2003) Differential metal selectivity and gene expression of two zinc transporters from rice. Plant Physiol 133:126-134

Ranocha P, Chabannes M, Chamayou S, Danoun S, Jauneau A, Boudet AM, Goffner D (2002) Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiol 129:145-155

Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N, Bucher M (2001) A phosphate transporter expressed in arbuscule-containing cells in potato. Nature 414:462-470

Rauser WE (1999) Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin, and metallothioneins. Cell Biochem Biophys 31:19-48

Rea PA, Li ZS, Lu YP, Drozdowicz YM, Martinoia E (1998) From Vacuolar Gs-X Pumps to Multispecific Abc Transporters. Annu Rev Plant Physiol Plant Mol Biol 49:727-760

Rees EM, Lee J, Thiele DJ (2004) Mobilization of intracellular copper stores by the Ctr2 vacuolar copper transporter. J Biol Chem 279:54221-54229

Reichman SM, Parker DR (2002) Revisiting the metal-binding chemistry of nicotianamine and 2'-deoxymugineic acid. Implications for iron nutrition in strategy II plants. Plant Physiol 129:1435-1438

Richards KD, Schott EJ, Sharma YK, Davis KR, Gardner RC (1998) Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. Plant Physiol 116:409-418

Richter S, Lamppa GK (1999) Stromal processing peptidase binds transit peptides and initiates their ATP-dependent turnover in chloroplasts. J Cell Biol 147:33-44

Richter S, Lamppa GK (2003) Structural properties of the chloroplast stromal processing peptidase required for its function in transit peptide removal. J Biol Chem 278:39497-39502

Roberts LA, Pierson AJ, Panaviene Z, Walker EL (2004) Yellow stripe1. Expanded roles for the maize iron-phytosiderophore transporter. Plant Physiol 135:112-120

Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric-chelate reductase for iron uptake from soils. Nature 397:694-697

Robson AD (1993) Zinc in Soils and Plants. Kluwer Academic Publishers, Dordrecht, Boston and London

Rodermel S (2002) *Arabidopsis* Variegation Mutants. The *Arabidopsis* Book:1-28

Rodriguez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleecker AB (1999) A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*. Science 283:996-998

Rogers EE, Guerinot ML (2002) FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in *Arabidopsis*. Plant Cell 14:1787-1799

Römheld V (1991) The role of phytosiderophores in acquisition of iron and other micronutrients in gramineous species - an ecological approach. Plant Soil 130:127-134

Rutherford JC, Bird AJ (2004) Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. Eukaryot Cell 3:1-13

Saijo Y, Sullivan JA, Wang H, Yang J, Shen Y, Rubio V, Ma L, Hoecker U, Deng XW (2003) The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. Genes Dev 17:2642-2647

Sakamoto W, Tamura T, Hanba-Tomita Y, Murata M (2002) The *VAR1* locus of *Arabidopsis* encodes a chloroplastic FtsH and is responsible for leaf variegation in the mutant alleles. Genes Cells 7:769-780

Sakamoto W, Zaltsman A, Adam Z, Takahashi Y (2003) Coordinated regulation and complex formation of yellow variegated1 and yellow variegated2, chloroplastic FtsH metalloproteases involved in the repair cycle of photosystem II in Arabidopsis thylakoid membranes. Plant Cell 15:2843-2855

Salt DE, Krämer U (2000) Mechanisms of metal hyperaccumulation in plants. In: Raskin I, Ensley, B.D. (ed) Phytoremediation of toxic metals: Using plants to clean up the environment. John Wiley & Sons, Inc., Hoboken, NJ, pp 231-246

Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. Plant Physiol 109:1427-1433

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu Rev Plant Physiol Plant Mol Biol 49:643-668

Sancenon V, Puig S, Mateu-Andres I, Dorcey E, Thiele DJ, Penarrubia L (2004) The *Arabidopsis* copper transporter COPT1 functions in root elongation and pollen development. J Biol Chem 279:15348-15355

Sancenon V, Puig S, Mira H, Thiele DJ, Penarrubia L (2003) Identification of a copper transporter family in *Arabidopsis thaliana*. Plant Mol Biol 51:577-587

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse A, Marcus MA, Manceau A (2002) Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiol 130:1815-1826

Sasaki T, Yukawa Y, Miyamoto T, Obokata J, Sugiura M (2003) Identification of RNA editing sites in chloroplast transcripts from the maternal and paternal progenitors of tobacco (*Nicotiana tabacum*): comparative analysis shows the involvement of distinct trans-factors for *ndhB* editing. Mol Biol Evol 20:1028-1035

Schaaf G, Ludewig U, Erenoglu BE, Mori S, Kitahara T, von Wiren N (2004) ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. J Biol Chem 279:9091-9096

Schat H, Kuiper E, Ten Bookum WM, Vooijs R (1993) A general model for the genetic control of copper tolerance in *Silene vulgaris*: Evidence from crosses between plants from different tolerant populations. Heredity 70:142-147

Schat H, Vooijs R (1997) Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: A co-segregation analysis. New Phytol 136:489-496

Schat H, Vooijs R, Kuiper E (1996) Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. Evolution 50:1888-1895

Schlicher T, Soll J (1997) Chloroplastic isoforms of DnaJ and GrpE in pea. Plant Mol Biol 33:181-185

Schmidt W, Schikora A (2001) Different pathways are involved in phosphate and iron stress-induced alterations of root epidermal cell development. Plant Physiol 125:2078-2084

Schmöger ME, Oven M, Grill E (2000) Detoxification of arsenic by phytochelatins in plants. Plant Physiol 122:793-801

Schwartz C, Morel JL, Saumier S, Whiting SN, Baker AJM (1999) Root development of the Zinc-hyperaccumulator plant *Thlaspi caerulescens* as affected by metal origin, content and localization in soil. Plant & Soil 208:103-115

Schwechheimer C, Serino G, Deng XW (2002) Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. Plant Cell 14:2553-2563

Sedbrook JC, Carroll KL, Hung KF, Masson PH, Somerville CR (2002) The *Arabidopsis* SKU5 gene encodes an extracellular glycosyl phosphatidylinositol-anchored glycoprotein involved in directional root growth. Plant Cell 14:1635-1648

Seo S, Okamoto M, Iwai T, Iwano M, Fukui K, Isogai A, Nakajima N, Ohashi Y (2000) Reduced levels of chloroplast FtsH protein in tobacco mosaic virus-infected tobacco leaves accelerate the hypersensitive reaction. Plant Cell 12:917-932

Sharma PN, Tripathi A, Bisht SS (1995) Zinc requirement for stomatal opening in cauliflower. Plant Physiol 107:751-756

Shikanai T, Muller-Moule P, Munekage Y, Niyogi KK, Pilon M (2003) PAA1, a P-type ATPase of *Arabidopsis*, functions in copper transport in chloroplasts. Plant Cell 15:1333-1346

Shirasu K, Lahaye T, Tan MW, Zhou F, Azevedo C, Schulze-Lefert P (1999) A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell 99:355-366

Smirnova IV, Bittel DC, Ravindra R, Jiang H, Andrews GK (2000) Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. J Biol Chem 275:9377-9384

Smith KS, Ferry JG (2000) Prokaryotic carbonic anhydrases. FEMS Microbiol Rev 24:335-366

Snaith SM, Levvy GA (1968) Alpha-mannosidase as a zinc-dependent enzyme. Nature 218:91-92

Stahl A, Moberg P, Ytterberg J, Panfilov O, Brockenhuus Von Lowenhielm H, Nilsson F, Glaser E (2002) Isolation and identification of a novel mitochondrial metalloprotease (PreP) that degrades targeting presequences in plants. J Biol Chem 277:41931-41939

Stephan UW, Scholz G (1993) Nicotianamine: mediator of transport of iron and heavy metals in the phloem? Physiol Plant 88:522-529

Stephan UW, Scholz G, Rudolph A (1990) Distribution of nicotianamine, a presumed symplast iron transporter, in different organs of sunflower and of tomato wild type and its mutant *chloronerva*. Biochem Physiol Pflanzen 186:81-88

Strack D, Fester T, Hause B, Schliemann W, Walter MH (2003) Arbuscular mycorrhiza: biological, chemical, and molecular aspects. J Chem Ecol 29:1955-1979

Sullivan JA, Shirasu K, Deng XW (2003) The diverse roles of ubiquitin and the 26S proteasome in the life of plants. Nat Rev Genet 4:948-958

Sumner JB (1926) The isolation and crystallization of the enzyme urease: preliminary paper. J Biol Chem 69:435-441

Supek F, Supekova L, Nelson H, Nelson N (1997) Function of metal-ion homeostasis in the cell division cycle, mitochondrial protein processing, sensitivity to mycobacterial infection and brain function. J Exp Biol 200 (Pt 2):321-330

Suzuki N, Yamaguchi Y, Koizumi N, Sano H (2002) Functional characterization of a heavy metal binding protein CdI19 from *Arabidopsis*. Plant J 32:165-173

Takahashi A, Casais C, Ichimura K, Shirasu K (2003a) HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in *Arabidopsis*. Proc Natl Acad Sci USA 100:11777-11782

Takahashi M, Terada Y, Nakai I, Nakanishi H, Yoshimura E, Mori S, Nishizawa NK (2003b) Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. Plant Cell 15:1263-1280

Takahashi M, Yamaguchi H, Nakanishi H, Shioiri T, Nishizawa NK, Mori S (1999) Cloning two genes for nicotianamine aminotransferase, a critical enzyme in iron acquisition (Strategy II) in graminaceous plants. Plant Physiol 121:947-956

Takechi K, Sodmergen, Murata M, Motoyoshi F, Sakamoto W (2000) The *YELLOW VARIEGATED* (*VAR2*) locus encodes a homologue of FtsH, an ATP-dependent protease in *Arabidopsis*. Plant Cell Physiol 41:1334-1346

Takenaka M, Brennicke A (2003) In vitro RNA editing in pea mitochondria requires NTP or dNTP, suggesting involvement of an RNA helicase. J Biol Chem 278:47526-47533

Thomine S, Wang R, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. Proc Natl Acad Sci USA 97:4991-4996

Torii KU, Stoop-Myer CD, Okamoto H, Coleman JE, Matsui M, Deng XW (1999) The RING finger motif of photomorphogenic repressor COP1 specifically interacts with the RING-H2 motif of a novel *Arabidopsis* protein. J Biol Chem 274:27674-27681

Torisky RS, Griffin JD, Yenofsky RL, Polacco JC (1994) A single gene (*Eu4*) encodes the tissue-ubiquitous urease of soybean. Mol Gen Genet 242:404-414

Tung G, Temple PJ (1996) Histochemical detection of lead in plant tissues. Environ Toxicol Chem 15:906-914

Vacchina V, Mari S, Czernic P, Marques L, Pianelli K, Schaumloffel D, Lebrun M, Lobinski R (2003) Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray MS/MS assisted by cloning using yeast complementation. Anal Chem 75:2740-2745

Van Assche F, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ 13:195-206

Van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JAC, Hooykaas PJJ (1999) Overexpression of a novel *Arabidopsis* gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiol 119:1047-1055

Van Hoof NA, Koevoets PLM, Hakvoort HWJ, Ten Bookum WM, Schat H, Verkleij JAC, Ernst WHO (2001) Enhanced ATP-dependent copper efflux across the root cell plasma membrane in copper-tolerant *Silene vulgaris*. Physiologia Plantarum 113:225-232

Varotto C, Maiwald D, Pesaresi P, Jahns P, Salamini F, Leister D (2002) The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. Plant J 31:589-599

Vatamaniuk OK, Mari S, Lu Y-P, Rea PA (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: Isolation and in vitro reconstitution. Proc Natl Acad Sci USA 96:7110-7115

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Lett 576:306-312

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briat JF, Curie C (2002) IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. Plant Cell 14:1223-1233

Vert GA, Briat JF, Curie C (2003) Dual regulation of the *Arabidopsis* high-affinity root iron uptake system by local and long-distance signals. Plant Physiol 132:796-804

Vitale A, Raikhel NV (1999) What do proteins need to reach different vacuoles? Trends Plant Sci 4:149-155

von Wiren N, Khodr H, Hider RC (2000) Hydroxylated phytosiderophore species possess an enhanced chelate stability and affinity for iron(III). Plant Physiol 124:1149-1158

von Wiren N, Klair S, Bansal S, Briat JF, Khodr H, Shioiri T, Leigh RA, Hider RC (1999) Nicotianamine chelates both Fe(III) and Fe(II). Implications for metal transport in plants. Plant Physiol 119:1107-1114

von Wiren N, Marschner H, Römheld V (1996) Roots of iron-efficient maize also absorb phytosiderophore-chelated zinc. Plant Physiol 111:1119-1125

von Wiren N, Mori S, Marschner H, Römheld V (1994) Iron inefficiency in maize mutant *ys1* (*Zea mays* L. cv Yellow-Stripe) is caused by a defect in uptake of iron phytosiderophores. Plant Physiol 106:71-77

Vreugdenhil D, Aarts MGM, Koornneef M, Nelissen H, Ernst WHO (2004) Natural variation and QTL analysis for cationic mineral content in seeds of *Arabidopsis thaliana*. Plant Cell Environ 27:828-839

Wang F, Dufner-Beattie J, Kim BE, Petris MJ, Andrews G, Eide DJ (2004a) Zinc-stimulated endocytosis controls activity of the mouse ZIP1 and ZIP3 zinc uptake transporters. J Biol Chem 279:24631-24639

Wang F, Kim BE, Dufner-Beattie J, Petris MJ, Andrews G, Eide DJ (2004b) Acrodermatitis enteropathica mutations affect transport activity, localization and zinc-responsive trafficking of the mouse ZIP4 zinc transporter. Hum Mol Genet 13:563-571

Weber M, Harada E, Vess C, von Roepenack-Lahaye E, Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. Plant J 37:269-281

Wei J, Theil EC (2000) Identification and characterization of the iron regulatory element in the ferritin gene of a plant (soybean). J Biol Chem 275:17488-17493

Wei N, Deng XW (2003) The COP9 signalosome. Annu Rev Cell Dev Biol 19:261-286

Weigel M, Varotto C, Pesaresi P, Finazzi G, Rappaport F, Salamini F, Leister D (2003) Plastocyanin is indispensable for photosynthetic electron flow in *Arabidopsis thaliana*. J Biol Chem 278:31286-31289

Welch RM, Norvell WA, Schaefer SC, Shaff JE, Kochian LV (1993) Induction of Iron(III) and Copper(II) Reduction in Pea (*Pisum-Sativum* L) roots by Fe and Cu status - Does the root-cell plasmalemma Fe(III)-chelate reductase perform a general role in regulating cation uptake? Planta 190:555-561

Weng L, Temminghoff EJM, Van Riemsdijk WH (2001) Determination of the free ion concentration of trace metals in soil solution using a soil column Donnan membrane technique. Eur J Soil Sci 52:629-637

Westin G, Schaffner W (1988) A zinc-responsive factor interacts with a metal-regulated enhancer element (MRE) of the mouse metallothionein-I gene. EMBO J 7:3763-3770

Whiting SN, Leake JR, McGrath SP, Baker AJM (2000) Positive responses to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. New Phytol 145:199-210

Wintz H, Fox T, Vulpe C (2002) Responses of plants to iron, zinc and copper deficiencies. Biochem Soc Trans 30:766-768

Wintz H, Fox T, Wu YY, Feng V, Chen W, Chang HS, Zhu T, Vulpe C (2003) Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. J Biol Chem 278:47644-47653

Wintz H, Vulpe C (2002) Plant copper chaperones. Biochem Soc Trans 30:732-735

Witte CP, Isidore E, Tiller SA, Davies HV, Taylor MA (2001) Functional characterisation of urease accessory protein G (ureG) from potato. Plant Mol Biol 45:169-179

Woeste KE, Kieber JJ (2000) A strong loss-of-function mutation in RAN1 results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. Plant Cell 12:443-455

Wycisk K, Kim EJ, Schroeder JI, Krämer U (2004) Enhancing the first enzymatic step in the histidine biosynthesis pathway increases the free histidine pool and nickel tolerance in *Arabidopsis thaliana*. FEBS Lett 578:128-134

Yamaguchi-Iwai Y, Serpe M, Haile D, Yang W, Kosman DJ, Klausner RD, Dancis A (1997) Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. J Biol Chem 272:17711-17718

Yao T, Cohen RE (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. Nature 419:403-407

Yruela I, Gatzen G, Picorel R, Holzwarth AR (1996a) Cu(II)-inhibitory effect on photosystem II from higher plants. A picosecond time-resolved fluorescence study. Biochemistry 35:9469-9474

Yruela I, Pueyo JJ, Alonso PJ, Picorel R (1996b) Photoinhibition of photosystem II from higher plants. Effect of copper inhibition. J Biol Chem 271:27408-27415

Yu F, Park S, Rodermel SR (2004) The *Arabidopsis* FtsH metalloprotease gene family: interchangeability of subunits in chloroplast oligomeric complexes. Plant J 37:864-876

Zhao H, Eide DJ (1997) Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*. Mol Cell Biol 17:5044-5052

Zhu Z, Labbe S, Pena MM, Thiele DJ (1998) Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. J Biol Chem 273:1277-1280

Zonia LE, Stebbins NE, Polacco JC (1995) Essential role of urease in germination of nitrogen-limited *Arabidopsis thaliana* seeds. Plant Physiol 107:1097-1103

Clemens, Stephan
Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Saale, Germany

Krämer, Ute
Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Golm, Germany
kraemer@mpimp-golm.mpg.de

Metal immobilization: where and how?

Stéphane Mari and Michel Lebrun

Abstract

Metal immobilization away from metabolically active sites within the cell represents the last step in both the homeostasis of metals and the detoxification of metal in excess. Assessment of the importance of this step requires having access to the *in vivo* speciation of metals. Evolving techniques have made it possible to acquire more reliable *in situ* profiling of: *(i)* spatio-temporal accumulation of metal, *(ii)* characterization of the metal-ligands complexes and determination of the structure of the different bio-ligands involved. The chapter "metal immobilization: where and how?" presents the role of different metal-chelators in plants, based on examples from works using non-invasive techniques and genetic approaches at both the whole plant, cellular and subcellular levels. The aim of the chapter is to give a survey of the key molecules and processes involved in metal immobilization in plants, on the basis of direct and robust evidences of the *in vivo* speciation of metals.

1 Introduction

The last step of heavy metal detoxification is the immobilization, in chemical forms as inactive as possible, with the aim of protecting metabolically active cells. By far, the mechanisms controlling the metal immobilization have been the least studied, when compared to metal ion uptake and transport in the plants. The difficulties to address this question lay on a major technical challenge: the need to develop and adapt non-invasive and non-destructive techniques aimed at identifying precisely the localization of metal ions within a tissue and the ligands involved in their chelation. As a consequence, few mechanisms have been identified so far, limiting the targets for molecular and genetic approaches.

The permanent concern, when using for example mass spectrometry approaches on extracts from metal-enriched plant tissues, is the artefactual formation of metal complexes by mixing high affinity chelators with metal ions that were in different cell compartments or tissues, leading to conclusions far from the *in vivo* situations.

This chapter deals with metal chelators, the different categories, their capacities, and their relative role in metal chelation. However, it has to be kept in mind that in this field the main limitation lays on the development and improvement of non-invasive techniques allowing the direct visualisation of the metal speciation *in*

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_103 / Published online: 15 September 2005

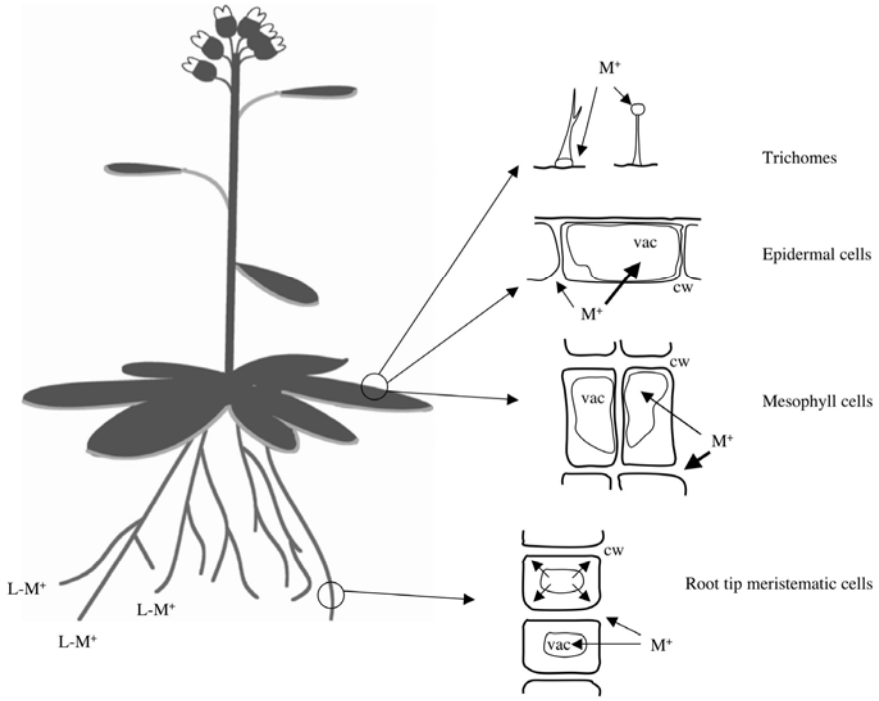


Fig. 1. Synthetic view of the mechanisms involved in metal immobilization. L-M^+ represents the complexation of metal ions in the rhizosphere (Section 1); following uptake by the root system, metal ions are bound to the cell wall compartment and induce an increased vacuolisation, in meristematic root cells (Section 2); in the leaves two different strategies have been identified from works on hyperaccumulator species: (*i*) a massive accumulation of metals in the vacuole of epidermal cells and a lower immobilization in the cell wall, (*ii*) the concentration of metals in the basal part of trichomes and a distribution of the remaining metal ions in the mesophyll cells with a relatively higher binding to the cell wall, compared to the vacuole. In the non-accumulator species studied, metals in the leaves are concentrated in the trichomes (Section 3). Abbreviations: L-M^+, metals complexed in the rhizosphere; M^+, metal ions; cw, cell wall, vac, vacuole . The thickness of the arrows are indicative of the relative amount of metals accumulated in the corresponding compartment.

situ and *in vivo*, to avoid artefacts created by decompartmentation. The examples chosen to illustrate our purpose (see Table 1 for a compilation of the examples cited in this section) will, therefore, come from works based on (*i*) non-invasive approaches coupled to electron/atomic spectroscopy, (*ii*) histo and cytological studies allowing the identification of organelles/cell types/tissues/specific organs involved in metal chelation, (*iii*) genetic approaches with mutants affected in the synthesis of potential chelators.

2 Immobilization in the rhizosphere

The metabolic activity of plants might be involved in mobilization or immobilization of metal outside the cell, *i.e.*, interfering upstream of the transport across the plasma membrane of metals (Fig. 1). Metal mobilization is well documented in the case of iron uptake, which is developed elsewhere in this book (Chapter 8). In brief, in conditions of iron starvation non-graminaceous plants species increases the H^+-ATPase activity at the plasma membrane in iron deficiency conditions. The resulting decrease in the apoplastic pH induces higher solubility of ferric iron and improves the capacity of plant to take up the metal after reduction by the root ferric reductase activity; this strategy was termed "strategy I". In contrast, strategy II plants excrete derivatives of mugineic acids that act as external phytosiderophores that chelate iron (and other metals, to different extent) allowing its absorption by the graminaceous plant where this mechanisms occurs specifically (so called pH independent mechanism). The quantitative contribution of these mechanisms, seemingly devoted to iron, to the uptake of other metals and in hyperaccumulating plants have not been analysed to any extent and remains unknown. In *Zea mays*, a potential loop between cadmium and iron uptake has been proposed. Cadmium stress induces iron deficiency symptoms, due to the speciation of cadmium with the phytosiderophore 2'-deoxymugineic acid (DMA) and thus a decrease in iron uptake, which in turn induces an increase secretion of DMA in both iron-sufficient and iron-limiting conditions, that would chelate more and more cadmium and thus reduce cadmium accumulation in plant (Hill et al. 2002).

Metal immobilization by outside chelation with organic compounds secreted by the plants has been analyzed and demonstrated for Pb and more thoroughly in one particular example, the tolerance to Al (Al^{3+}). The analysis of the tolerance of rice cultivars towards Pb has shown that tolerant cultivars secreted oxalate in the medium to chelate Pb and reduce its bioavailability (Yang et al. 2000). On acid soils, which comprise about 40% of arable lands in the world, Al is solubilized as the trivalent Al^{3+} cation, which rapidly inhibits root growth at micromolar concentrations. Organic acids have been shown to play an important role in detoxifying Al both externally or internally, notwithstanding, the various strategy the plants set up to cope with Al toxicity. Organic acids have been shown to form stable complexes with Al (Fig. 2); thereby, preventing the binding of Al to the (unknown) cellular targets. External detoxification of Al by external immobilization by secreted organic anion, thus, preventing entry and toxicity of Al have received major attention during the last decade. Many Al resistant species and crops have been shown to respond to Al stress by a secretion of organic anions. A robust correlation has been established between this anion secretion and the resistance achieved (reviewed in Ma and Furukawa 2003). Depending on the plant species, citrate, malate and oxalate are the organic ions that are secreted in response to Al. Although correlative genetic evidences have been brought over years, direct link between organic anion excretion and Al tolerance have been first obtained using transgenic tobacco lines that overexpressed a citrate synthase gene from the bacteria *Pseudomonas aeruginosa*. One line shows a tenfold increase in internal citrate

Table 1. Compilation of the information available concerning different metals, their localisation in the plants, the identification of the type of ligand and the corresponding techniques.

Species	Metal	Organ/Tissue localisation	Cell/Sub cellular localisation	Ligands (%)	Detection technique	Reference
Allium sativum	Copper	Root tips	vacuole		TEM + EELS	Liu & Kottke 2004
Alyssum bertolonii	Nickel	Leaves	Epidermis			Küpper et al. 2001
		Stems	Epidermis+boundary cell of vascular cylinder			
		trichomes	Basal cells			
Alyssum euboeum	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000
Alyssum heldreichii	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000
Alyssum lesbiacum	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000
Alyssum smolikanum	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000
Arabidopsis halleri	Zinc	Leaves		Malate	EXAFS, µEXAFS,X-ray microfluorescence EDX	Sarret et al. 2002
			Trichome base			
	Zinc & cadmium	leaves	Trichome base mesophyll			Küpper et al. 2000
	Zinc	Roots		Malate (29) citrate (39) phosphate (32)		Sarret et al. 2002
	Zinc & cadmium	Root epidermis		Phophate precipitates		Küpper et al. 2000
Arabidopsis lyrata	Zinc	Leaves		Phosphate		Sarret et al. 2002
		roots		phosphate		
Bornmuellera baldacii	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000
Bornmuellera tymphaea	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000
Brassica juncea	Cadmium	trichomes			autoradiography	Salt et al. 1995
Leptoplax emarginata	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000
Nicotiana tabacum	Cadmium	trichomes			SEM EDX	Choi et al. 2001
Nympheae	Cadmium	Lamina, petiole, rhizome	Epidermal glands		SEM EDX	Lavid et al. 2000
Thlaspi arvense	Nickel	leaves	Cell wall> cytoplasm>vacuole			Krämer et al. 2000
Thlaspi arvense	Zinc	Leaf veins only			X-ray fluorescence	Toaspern et al. 2000

Species	Metal	Organ/Tissue localisation	Cell/Sub cellular localisation	Ligands (%)	Detection technique	Reference
Thlaspi caerulescens (Ganges)	Cadmium	Young leaves Mature leaves Senescente leaves		O(46) S(35) His(20) O(38) S(34) His(26) O(59) His(25) S(16)	EXAFS	Küpper et al. 20004
	Zinc	Roots		His(70%) Cell wall(30%)		Salt et al. 1999
		Xylem		H_2O (79%) Citrate (21%)		
		Leaf epidermis (90%)	Vac (83%) cell wall (13%)		EDX	Küpper et al. 1999 ; and Frey et al. 2000
		Mesophyll (10%)	Vac (3,4%) cell wall (96%)		EDX	
		Whole leaves			X-ray fluorescence	Toaspern et al. 2000
		Shoots		H_2O (26%) Citrate (38%) Oxalate (9%) His (16%) Cell wall (12)	EXAFS	Salt et al. 1999
		Mature petioles		O (75) S (25)		Küpper et al. 20004
		Senescent petioles		O(70) S(27) His(20)		
		Mature stems		O(36) S(37) His(27)		
		Senescent stems		O(68) S(29) His(3)		
	Zinc (+Cd)	Young leaves Mature leaves Senescent leaves		O (80) His (20) O (99) His (1)		
Thlaspi goesingense	Nickel	Leaves	Epidermis			Küpper et al. 2001
Thlaspi goesingense	Nickel	Leaves	Cell wall> vacuole = cytoplasm	Citrate (vacuole) His (cytoplasm)	XAS	Krämer et al. 2000
Thlaspi pindicum	Nickel	leaves	epidermis		SEM X ray microanalysis	Psaras et al. 2000

Abreviations : EDX, energy dispersive X-ray analysis ; EELS, electron energy loss spectroscopy ; TEM, transmission electron microscopy ; SEM, scanning electron microscopy ; EXAFS, extended X-ray absorption fine structure ; XAS, X-ray absorption spectroscopy ; vac, vacuole, His, histidine, O, oxygen, S, sulfur

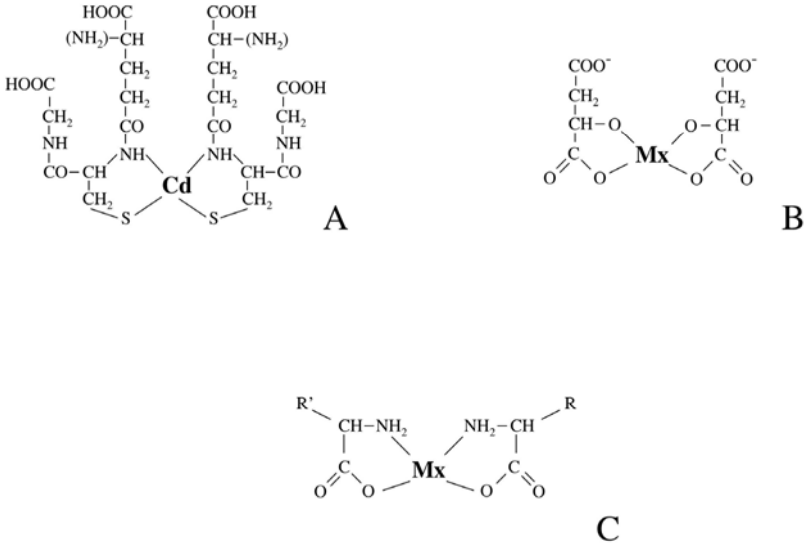


Fig. 2. Structure of three types of metal chelates. A. The structure of bis(glutathionato) cadmium (Li et al. 1997) illustrates the chelation by a thiol-containing molecule, like phytochelatins or metallothioneins; B. chelation of a metal ion (Mx) by organic acids, in this case two malate molecules coordinate one Mx with two hydroxy and two carboxy groups; C. chelation of a metal ion (Mx) by two amino acids involving their amino and carboxy groups, this type of chelation is found for nicotianamine for which the three carboxy and the three amino groups form an hexadentate coordination with one metal ion. In the case of histidine, the amino groups of the heterocycle are involved in chelation rather than the terminal amino group of the amino acid.

concentration and a 4-fold increase in citrate secretion and was reported to improve both Al tolerance and decrease internal Al accumulation (de la Fuente et al. 1997). However, repeating this approach did not lead to the same observations (Delhaize et al. 2001). Overexpression of citrate synthase from carrot mitochondria in *Arabidopsis thaliana* and of malate dehydrogenase in *Medicago sativa* resulted in enhanced organic acid synthesis, excretion, and Al resistance (Koyama et al. 2000; Tesfaye et al. 2001). Taken together, these results showed a modest Al tolerance that did not reach the level achieved in Al-resistant bred cultivars. It was, thus, hypothesized that, whereas an increased ability to synthesize organic acid might be important, the rate-limiting step would be the transport of the molecules in the external medium (Ryan et al. 2001). A pair of near isogenic lines of wheat differing in tolerance to Al at a single genetic locus and the tolerant genotype showed a greater Al-induced efflux of malate (Delhaize et al. 1993a, 1993b). The gene encoding ALMT1 was isolated from a wheat resistant line (Sasaki et al. 2004) and displayed properties of malate transport associated with malate efflux and Al tolerance on the basis of: membrane bound localization, co-segregation with Al tolerance, increased Al tolerance upon ectopic expression in plant and animal cells. Furthermore, expression in transgenic barley plants of the wheat

genes under the control of the ubiquitin promoter conferred an Al-activated efflux of malate and a higher tolerance to toxic concentration of Al in both hydroponic culture and acidic soils, thus, proving that a single gene is able to confer a high level of tolerance (Delhaize et al. 2004). Altogether these results reinforce the "malate hypothesis" which proposes that secreted malate binds and immobilize Al into non-toxic form, thus, protecting the root apex from damage. This is the best-documented example of an external immobilization of metal by a secreted plant product.

3 Immobilization in the roots

3.1 The case of phytochelatins

In 1985, an article published by Grill and co-workers in Science was entitled "Phytochelatins: the principal heavy-metal complexing peptides of higher plants". This concept has slightly changed over the years. Phytochelatins (PCs) are enzymatically synthesized from the tripeptide glutathione (GSH, γ-Glu-Cys-Gly). Phytochelatins consist in repeated units of $(\gamma\text{-Glu-Cys})_n$-Xaa, with n generally comprised between 2 and 5 although in some cases it can reach 11 and Xaa being almost exclusively Gly, although in some species it can be β-Ala, Ser or Glu (Grill et al. 1985; Rauser 1995, 1999; Zenk 1996). Increasing the amount of Cys residues per oligomer results in an increase in the affinity for metal ions, particularly cadmium (Fig. 2), through the formation of thermodynamically stable thiolate bonds (Merha et al. 1994). The overall mechanism of detoxification is completed by the storage of PC-metal complexes in the vacuolar compartment where the complexes can incorporate acid labile sulphide resulting in an increase in metal ion incorporation in the complexes (Mehra et al. 1994). The transport of PC-metal ions, particularly PC-Cd across the tonoplast, is catalyzed by an ABC (for ATP Binding Cassette) transporter encoded by HMT1 in the yeast *Schizosaccharomyces pombe* (Ortiz et al. 1992, 1995). In plants, the transport of PC-Cd across the tonoplast has the biochemical characteristics of an ABC transporter (Vögeli-Lange and Wagner 1989; Salt and Rauser 1995); nevertheless, the genes encoding such transporters remain unidentified.

The reaction catalysed by the phytochelatin synthase (EC 2.3.2.15) is a γ-Glu-Cys dipeptidyl transpeptidation (Grill et al. 1989; Vatamaniuk et al. 2000). The initial step of PC synthesis, namely the synthesis of PC_2, is achieved by the transpeptidation of a γ-Glu-Cys moiety from a donor GSH to an acceptor GSH, leading to the formation of γ-Glu-Cys-γ-Glu-Cys-Gly (PC_2) and the releasing of a Gly residue. Later on, the acceptor molecule can be an already synthesized PCn, to produce a n+1 polymer. For more than a decade, the molecular identification of the genes encoding PC synthase has been a challenge for plant biologists until 1999, when three groups, independently and simultaneously, isolated PC synthase genes from *Arabidopsis* and wheat (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999). Two groups used yeast complementation screens for cad-

mium (Cd) tolerance (Clemens et al. 1999; Vatamaniuk et al. 1999) and the other group (Ha et al. 1999) realized the positional cloning of the Cd-sensitive and PC deficient CAD1 locus of *Arabidopsis thaliana* (described by Howden et al. 1995). These findings have opened new perspectives in the field of heavy metal tolerance.

First, from a mechanistic point of view, the heterologous expression and reconstitution of a functional epitope-tagged enzyme has led to the purification of the protein to homogeneity (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999). Enzymatical studies realized with FLAG-tagged AtPCS1 have shown that the metal requirement and activation of the enzyme reported earlier (Grill et al. 1989; Loeffler et al. 1989) is the result of the formation of metal-GS complexes in the incubation medium and the requirement of both free GSH and metal-GS as co-substrate for AtPCS1-catalyzed PC synthesis (Vatamaniuk et al. 2000). Moreover, the capacity of different metal ions to activate the enzyme (Cd, Cu, Hg, Zn, Pb) was in good correlation with their respective reactivity towards GSH. Furthermore, the kinetics of PC_2 synthesis from GSH and Cd-GS_2 are consistent with a "ping-pong" mechanism, where the enzyme forms an acyl intermediate with a γ-Glu-Cys moiety at one site of the enzyme, further identified as being the residue Cys56 (Vatamaniuk et al. 2000, 2004).

Second, at a more physiological level, the regulation of PC synthase genes could be analyzed, in relation to metal exposure and organ/tissue specificity. Earlier reports indicated that the extractable PC synthase activity of cell suspensions was not stimulated by exposure to heavy metal (Grill et al. 1989; Loeffler et al. 1989). In *Arabidopsis*, expression analyses realized with RNA from seedlings exposed to Cd and Cu have shown a constitutive expression of *AtPCS1* (Ha et al. 1999; Vatamaniuk et al. 1999). In wheat, however, TaPCS1 expression can be induced by Cd exposure (Clemens et al. 1999). These discrepancies have somehow been reconciliated in another study using a construction with the AtPCS1 promoter fused to the GUS reporter gene, expressed in *A. thaliana* (Lee and Korban 2002). With this material, it was shown that the AtPCS1 promoter is active in the roots and its activity is increased twice with a Cd treatment. However, the stimulation of AtPCS1 expression is only visible in young (five day old) seedlings and gradually decreases until 21 days where no difference is visible between control and treated plants, consistent with the results of Vatamaniuk and co-workers who also used 21-day old plants. In terms of localization of the expression, PC synthase genes are mainly expressed in the roots and to a lesser extend in the stems (Chen et al. 1997; Lee and Korban 2002). Likewise, PCs are almost exclusively detected in the roots (reviewed in Cobbett and Goldsbrough 2002).

Third, the cloning of PC synthase genes was the requisite to confirm and continue genetic approaches. The only loss-of-function mutant is the *Arabidopsis cad1* mutant (Howden et al. 1995). The phenotypical analyses of this mutant revealed a high sensitivity to Cd and arsenate whereas Cu, Hg and Ag have only a very limited effect on the mutant compared to wild type plants (Ha et al. 1999). These results are extremely important because they demonstrate that although PCs are able to chelate several metal ions *in vitro* (Cd, Ag, Hg, As, Pb; Mehra et al. 1994, 1995, 1996; Rauser et al. 1999); the tolerance mechanisms *in planta* are

likely to be completely different. On the other hand, several studies have been conducted aiming at increasing the synthesis of PC by the generation of transgenic lines. This was achieved either by overexpressing genes encoding enzymes involved in GSH synthesis (γ-Glu-Cys synthase and GSH synthase; Zhu et al. 1999a, 1999b respectively) or PC synthase itself (Lee et al. 2003; Gisbert et al. 2003; Li et al. 2004). These strategies have led to increased tolerance and accumulation of Cd (Zhu et al. 1999a, 1999b; Gisbert et al. 2003), Pb (Gisbert et al. 2003), and As (Li et al. 2004), in correlation with an increase in PC accumulation. Although promising as biotechnological tools for phytoremediation, these results do not demonstrate that in the transgenic plants generated PCs are involved in Cd and Pb immobilization.

The unambiguous demonstration of the role of PCs in heavy metal immobilization has come from more analytical and chemical approaches. This was first realized by Maitani and co-workers (1996) by coupling high performance liquid chromatography (HPLC) separation to an inductively coupled plasma-atomic emission spectrometer (ICP-AES), allowing the simultaneous analysis of the chemical state of various metals from extracts obtained from roots of *Rubia tinctorum* exposed to a wide variety of metal ions. Although all the metal ions used induced the accumulation of PCs, only Ag, Cu, and Cd were bound to PCs they induced. Furthermore, Cu was also found bound to PCs from roots exposed to Ag, As, and Cd. More recently, the coupling of HPLC with ICP-MS (metal(loid) detection) and electrospray ionisation-mass spectrometry (organic ligand) in parallel was used to isolate and characterize As ligands in the As-tolerant grass *Holcus lanatus* and the As hyperaccumulator *Pteris cretica* (Raab et al. 2004). In both species, As-PC complexes have been identified, $As^{(III)}$-PC_3 and GS-$As^{(III)}$-PC_2 for *Holcus lanatus* and *Pteris cretica*, respectively. Arsenic-PC complexes represented 13% in *H. lanatus* and only 1% of the total As in *P. cretica*, illustrating the minor role of PCs in As immobilization. However, these data have to be interpreted with caution since artefactual chelation of As by PCs and GSH could along the process of extraction and chromatography.

More recently, the development of X-ray absorption spectroscopy (XAS) has opened new possibilities in the analysis, *in situ* and *in vivo*, of the speciation of metal ions. XAS is a very powerful technique, based on synchrotron radiation that provides information on the coordination (extended X-ray absorption fine structure, EXAFS, one section of the spectrum) and the oxidation state of elements (X-ray absorption near edge structure, XANES, the other section of a spectrum). The main limitation of this technique is the detection threshold that requires high concentration of metal ions. Therefore, these approaches are almost exclusively restricted to plants able to accumulate high concentrations of metal ions in their organs without showing toxicity symptoms. Such plants are called hyperaccumulators and they represent new model systems used to unravel metal tolerance and accumulation mechanisms. In tumbleweed (*Salsola kali*), a potential Cd hyperaccumulator, XAS studies have shown that in the leaves Cd is coordinated to oxygen and sulphur (de la Rosa et al. 2004). Most likely, Cd is transported in the leaves chelated to organic acids and is then stored bound to the cell wall (Cd-O coordination) and to PCs (Cd-S coordination). In a more detailed

work, Cd-S but not Zn-S coordinations have been found in young leaves and stems of the Cd/Zn hyperaccumulator *Thlaspi caerulescens* and the proportion of Cd-S coordination was lower in mature and senescent leaves (Küpper et al. 2004). This dynamic view of metal coordination is of great interest since, in the global context of PC synthase expression, induction by metals and localization, it becomes clearer that phytochelatins are involved in heavy metal immobilization, mainly in young organs and tissues. Indeed, the inductibility of PC synthase genes has only been visualized in roots of young seedlings (Lee and Korban 2002). As cells become mature, more vacuolized, less metabolically active, Cd-S coordination is proportionally lower. It can be hypothesized that PCs are involved in heavy metal chelation principally in young, metabolically active, less vacuolized, less tolerant cells where high-affinity (but high energy demanding) chelators would be more efficient. It is, therefore, likely that PC involvement in heavy metal chelation would be more pronounced in non-tolerant non-hyperaccumulator species, although in this case the techniques described are not sensitive enough for the amount of heavy metals accumulated in the cells.

In conclusion, the role of phytochelatins in heavy metal immobilization, particularly Cd and As, is now becoming obvious, either by genetic and analytical approaches. However, the relative importance of PCs in heavy metal chelation and immobilization will greatly depend on the developmental stage and the physiology of the plant towards tolerance and hyperaccumulation. It is still difficult to imagine that plant have developed such molecules only for Cd and As, the general role of PCs in heavy metal homeostasis remains unclear.

3.2 The elusive role of metallothioneins

As opposed to PCs, metallothioneins (MTs) are proteins obtained by gene expression. Their main biochemical characteristics are a low molecular weight (4-8 kD) and a cystein enrichment, conferring metal-binding properties. Plant MTs contain generally two cystein-rich domains that can bind metal ions through thiolate bonds. Metallothioneins can be classified in four classes, based on the primary amino acid sequence (Cobbett and Goldsbrough 2002). Type 1 MTs contain six Cys-Xaa-Cys motifs (Xaa = another amino acid) distributed equally within the two metal binding domains. These two domains are separated by a stretch of approximately 40 amino acids. In type 2 MTs, a Cys-Cys pair is always present in positions 3 and 4 as well as a Cys-Gly-Gly-Cys motif at the end of the first domain. The C-terminal domain contains three Cys-Xaa-Cys motifs and the two domains are separated by a much more variable spacer. Type 3 MTs have four Cys residues in the N-terminal domain with a consensus sequence containing three Cys as follows: Cys-Gly-Asn-Cys-Asp-Cys. As for type 1 and 2, the C-terminal domain has three Cys-Xaa-Cys motifs and both domains are separated by a 40 amino acid spacer. Type 4 MTs have and additional Cys-rich domain. In this sub-class the Cys residues are found as Cys-Xaa-Cys motifs. Although this classification is based on the primary amino acid sequence, the expression profiles of MT genes also have a tendency to share common patterns within the four MT types. For ex-

ample, type 1 MTs are generally more expressed in the roots than in aerial parts whereas it is the contrary for type 2 MTs. Genes from the type 3 sub-family are highly expressed in fruits during ripening and type 4 MTs expression is generally restricted to the seeds (for a review see Cobbett and Goldsbrough 2002).

The expression of metallothionein genes can be modulated by a wide range of treatments or stresses, both biotic and abiotic (reviewed by Rauser 1999). Although the effects of metals on MT expression are depending on the species, the organ and the MT type, overall, copper is, among heavy metals, the most frequent and potent inducer of MT expression (Zhou and Goldsbrough 1994; Snowden et al. 1995; Garcia-Hernandez et al. 1998; van Hoof et al. 2001; Roosens et al. 2004). *In situ* hybridization experiments have shown that in *A. thaliana* and in *Vicia faba* some MTs are highly expressed in the trichomes (Foley and Singh 1994; Garcia-Hernandez et al. 1998). The role of MTs in foliar trichomes is unknown although it is tempting to involve MTs in heavy metal sequestration as these particular differentiated cells can accumulate very high amounts of metals in some conditions (see Section 3.1). Another possible role could be to deliver metals to metal-binding enzymes, highly expressed in trichomes (Foley and Singh 1994). Beside this particular expression pattern, MT gene expression has been found in all vegetative and reproductive organs although when studied more precisely, the expression was actually mainly located in the vascular tissues (Garcia-Hernandez et al. 1998). In conclusion from expression data, three situations can be proposed: (*i*) the MT expression overlaps metal accumulation and/or storage, for example in the trichomes or in the seeds, (*ii*) MTs are expressed in meristematic and embryonic cells and in this situation MTs could be involved in the protection against metal toxicity, for example in root tips or in the developing embryos, (*iii*) MTs are expressed in vascular tissues, illustrating a role in metal translocation and this hypothesis is strengthened by the identification of MT proteins in the phloem of *Ricinus communis* by quadruple time of flight mass spectrometry (Barnes et al. 2004).

At the protein level, several recombinant MTs have been shown to co-purify with, and bind *in vitro*, metal ions such as Cu, Cd, and Zn, in the decreasing order of affinity (Tommey et al. 1991; Morris et al. 1999). Furthermore, the *Arabidopsis thaliana* MT2 gene can restore Zn tolerance in a MT-deficient *Synechococcus* strain (Robinson et al. 1996) and *A. thaliana* and *Thlaspi caerulescens* MTs are able to functionally complement MT-deficient yeast strains, based on restoration of tolerance on media containing high Cu and Cd concentrations (Zhou and Goldsbrough 1994; van Hoof et al. 2001; Roosens et al. 2004). Taken together, these results indicate that these plant MTs are functional and that the tolerance towards heavy metals was achieved probably through direct metal binding.

From a more physiological point of view, several reports have illustrated links between MTs and copper homeostasis and tolerance. First, the expression of a type 2 MT is statistically highly correlated to the Cu tolerance of ten *Arabidopsis thaliana* ecotypes (Murphy and Taiz 1995). Second, in the Cu-sensitive *Arabidopsis* mutant cup1-1 which accumulates higher amounts of Cu, the MT2a gene is specifically induced in the roots whereas in wild type plants this gene is not expressed (van Vliet et al. 1995). Third, in Cu-tolerant populations of *Silene vulgaris*

isolated from copper mines, a type 2 MT, SvMT2b, is highly expressed, most likely due to a higher number of gene copies and this character is partially inheritable (van Hoof et al. 2001). Fourth, in the metal hyperaccumulator *Thlaspi caerulescens*, a type 3 MT (TcMT3) with higher Cu binding capabilities has been isolated and may reflect a specific adaptation to metal polluted conditions (Roosens et al. 2004).

Having said so, it is currently not possible to conclude definitely on the role of MTs in metal tolerance and sequestration, for two main reasons. First, the high instability of the proteins in aerobic conditions results in very few reports based on the protein characterisation. As a consequence, databases contain a high proportion of articles dealing with gene and cDNA isolation and expression analyses and only in very limited cases the variations in gene expression are completed by the analysis at the protein level. Second, the lack of genetic data is likely due to the difficulty to generate MT-null mutants. Indeed, given the size of MT genes (less than 1 kb), the probability of finding T-DNA insertion mutants is very low (Cobbett and Goldsbrough 2002) and, thus, alternative strategies (RNA interference, transposon tagging...) should be developed and tested, in order to provide unambiguous answers about the function of plant metallothioneins.

3.3 The role of the cell wall

The root cell wall is the first plant structure that is experienced by any metal before its entry inside root cells. In roots, metals might be either immobilized in apoplasmic space and vacuoles or move radially to reach the xylem and then the shoot (Fig. 1). At the level of wall of epidermal and cortical cells, pectin component of the cell wall provides a negatively charged surface that leads to accumulation of cations and repulsion of anion due to electrostatic interactions (Clarkson 1993). In normal conditions of metal supply, apoplastic accumulation does not seem to represent a limiting step for the acquisition of metals inside roots (reviewed in Sattelmacher 2001). In some cases, selectivity for the binding of cations could be correlated with charge surface characteristics. For example, the copper and manganese tolerance of two tobacco genotypes might be paralleled with the charge surface properties of the cell wall due to the balance of $RCCO^-$/RCOOH composition (Wang et al. 1992). Nickel and cadmium distribution between apoplastic and symplastic compartments has been analysed in hairy-roots of *Thlaspi caerulescens*, *Alyssum bertolonii*, a nickel hyperaccumulator and *Nicotiana tabaccum*, a non-metallophyte non-accumulator plant (Boominathan and Doran 2003). Interestingly, the behaviour of the metals was quite different: cell wall accumulation accounted for 75-78% for cadmium in both *T. caerulescens* and N. *tabaccum*, whilst 85-95% of the nickel was associated with the symplasm in *A. bertolonii* and *N. tabaccum*. The Zn distribution in *T. caerulescens* analyzed by XAS has revealed that only 30% of root Zn is bound to the cell wall, the remaining 70% being associated to His, most likely intracellularly, in the cytoplasm (Salt et al. 1999). Several ultrastructural studies (reviewed by Barcelo and Poschenrieder 1999) have also described association of Pb, Al, Zn, and Cd to the cell wall.

More recently, Liu and Kottke (2004) have shown that in *Allium sativum* roots, Cu deposits on the cell wall are only visible, and in limited extend, in cortical cells under severe exposure. The conflicting results reported in the literature concerning the relative role of the cell wall in metal binding may actually reflect the specificity of plants towards different metals. Indeed, cell wall immobilization of metals can be characteristic and dependent of both metal and plant, as exemplified in this section.

3.4 The vacuolar storage

When part of the metal ions have been chelated by the cell wall, the remaining move inside cells where active mechanisms are set up for transient or permanent immobilization. Increasing the vacuolar volume at the root tip, particularly in meristematic cells, by an increased vacuolisation seems to be a rather common feature in plants (Fig. 1). This cellular response has been reported in several ultrastructural studies for Al (Schier and McQuattie 1995), Ni and Zn (Sresty and Madhava Rao 1999), Cd (Liu and Kottke 2003), and Cu (Liu and Kottke 2004) and is considered as a mechanism of tolerance (Hall 2002). In the case of Cu, electron energy loss spectroscopy (EELS) measurements have shown that the increased vacuolisation indeed leads to Cu storage as electron dense Cu precipitates. Although these techniques are not the best suited for precise localization, EXAFS and particularly μEXAFS can provide sufficiently accurate data concerning the ligands of heavy metals to have a deduced picture of their respective localization. This is very well exemplified by the work of Sarret et al. (2002) on the Zn speciation in the Zn/Cd hyperaccumulator *Arabidopsis halleri*. In roots from Zn-contaminated soils, Zn is distributed as follows: Zn-malate 29%, Zn-citrate 39%, and Zn-phosphate 32%. Zinc coordination by organic acids being several orders of magnitude more favourable in acidic pH, it can be concluded that *ca* 68% of root Zn is localized in the vacuolar compartment. The Zn coordination to phosphate is presumably due to phytate complexation, which has already been shown in roots of several crop species (Van Steveninck et al. 1994). Although there is no precise localization of phytates in roots, *in situ* hybridisations of phytase genes in maize have shown that these enzymes are located in the endodermis/pericycle zone, most likely for the regulation of metal ion release to the stelle (Maugenest et al. 1999). The presence of Zn-phosphate/phytate in the roots of *A. halleri* may reflect the capacity of this plant to translocate Zn efficiently and at high rates, through the root cells, to the xylem vessels.

In conclusion, for obvious reasons of accessibility and handling, the analysis of metal chelation in the roots by spectroscopical, ultrastructural and related techniques is much less documented than the corresponding aerial parts (Table 1). Furthermore, these studies are principally realized with hydroponically cultivated plants, with the risk of modifying the amounts and type of speciation of the metal ions in the roots, given the higher availability and different coordination of metals in hydroponic solutions, versus naturally contaminated soils. For instance, growing *A. halleri* plants on hydroponics leads to the precipitation and deposition of

Zn-phosphate at the root surface, limiting the formation and/or masking the detection of the other Zn complexes identified above (Sarret et al. 2002). Nevertheless, in all cases, two main metal immobilization mechanisms occur: chelation by the cell wall and increased vacuolisation for a higher vacuolar storage (Fig. 1). Depending on the metal ion and the plant, the relative importance of each of these two mechanisms will be different, increasing the difficulty to draw a general picture for each heavy metal ion.

4 Immobilization in the aerial parts

Plant hyperaccumulators, with their ability to efficiently tolerate high concentration of metals within plant tissue and cells, have received considerable interest as model system to analyze metal speciation, for two main reasons: (*i*) due to the high level of metal accumulation in shoots that could overcome the fairly high threshold detection limits of most of the *in situ* analytical techniques, (*ii*) for their potential to provide clues on original or differently regulated mechanisms that could allow these plants to tolerate such normally toxic metal concentrations. Metal concentrations in hyperaccumulators are in general in the range of 100-fold the non-toxic concentration in normal plants and account, depending of the metal, for 0.1-1% of dry weight leaf tissue, including a shoot to root ratio accumulation of above one, both in native habitat and in hydroponic conditions (reviewed in Reeves and Baker 2000). Natural occurrence of metal hyperaccumulation (nickel, zinc, cadmium, manganese, cobalt, copper, chromium, selenium) has been reported so far in more than 400 plant species (Baker et al. 1999). Among these metallophytes important insights have been obtained from the study of a limited subset of plants, mainly on the basis of the existing closely related non-accumulating non-metallophyte counterpart in the genus *Thlaspi* and *Arabidopsis* that accumulates cadmium, zinc, and nickel for the former and cadmium and zinc for the latter. The close phylogenetic relationship with *Arabidopsis thaliana* has been one of the strong commitments to focus the efforts on these plants (see for example Becher et al. 2004; Weber et al. 2004). More specifically *Thlaspi caerulescens* and *Arabidopsis halleri* have been widely used to unravel some mechanisms potentially associated with metal hyperaccumulation (reviewed in Salt and Krämer 2000; Clemens et al. 2002).

4.1 Metal accumulation in the trichomes

Trichomes have multiple roles in plants: protection against insects, herbivores, secretion of odours or liquids, mechanic sensing... The key features of these specialized cells or group of cells, regarding heavy metal accumulation, are: (*i*) a high density at the leaf surface, (*ii*) a high differentiation for storage that, coupled to a high density can represent an important accumulation volume, (*iii*) a localization "outside of the leaf" that decreases the potential toxicity in case of decompartmen-

tation (Fig. 1). In 1974, Martell already reported on the accumulation of Pb in tobacco trichomes. Since this initial observation, several authors have described the accumulation of metal ions in trichomes of several non-tolerant species: cadmium in *Brassica juncea* (Salt et al. 1995) *Nympheae* (Lavid et al. 2000) and tobacco (Choi et al. 2001), Mn in *Helianthus annus* (Blamey et al. 1986). In these cases, the metal ions are quite restricted to the trichomes, suggesting a role in tolerance. Metal accumulation in trichomes has also been reported for Ni hyperaccumulator species such as *Alyssum bertolonii* and *A. lesbiacum* (Küpper et al. 2001) and *A. halleri* for Zn and Cd (Küpper et al. 2000; Zhao et al. 2000; Sarret et al. 2002). Interestingly, in both cases, the metals are located in the basal cells and not in the trichome top cell. Nevertheless, the speciation of metal ions has never been precisely established, although co-accumulation, and possibly co-crystallisation, has been described by EDX measurements for Cd and Ca in *Nympheae* and tobacco (Lavid et al. 2000; Choi et al. 2001) and Zn and oxygen (probably from citrate or Zn oxides) in *A. halleri* (Küpper et al. 2000). However, the relative importance of trichomes in heavy metal accumulation has not been assessed. In tobacco, the active excretion of Cd-Ca crystals and the increased tolerance efficiency with increased Ca are good arguments in favour of an active role of trichomes (Choi et al. 2001), whereas in *A. halleri*, increasing the concentration of metals in the culture medium does not change the concentration in the trichome but dramatically in the mesophyll cells (Küpper et al. 2000).

4.2 Epidermal cells and/or mesophyll

The relatively low detection threshold of the X-ray based techniques (electron dispersive and absorption) has been a general constraint, the consequence being that the analyses of metal ions localization and speciation has been performed almost exclusively on plant hyperaccumulators which can concentrate sufficient levels of metals in their tissues and cells. In these particular plant species, two different strategies have evolved, in terms of storage localization (Fig. 1). While in *A. halleri* Zn and Cd accumulate massively in the mesophyll cells and to a lesser extend at the trichome base (Küpper et al. 2000; Zhao et al. 2000; Sarret et al. 2002), final immobilization in the epidermal cells is a common feature of the *Thlaspi* and *Alyssum* genus (Küpper et al. 1999; Frey et al. 2000; Psaras et al. 2000; Küpper et al. 2001).

In *A. halleri*, two complementary works by Küpper et al. (2000) and Sarret et al. (2002) have established the precise and quantitative localization of Zn for the former, and the speciation for the latter. The coupling of scanning electron microscopy to energy dispersive X-ray analysis developed by Küpper et al. (2000) has allowed the *in situ* quantification of Cd and Zn in the leaves. Although trichomes accumulate high concentrations of Zn, the main sink for Cd and Zn is the mesophyll cells, either spongy or palisade. In these cells, EXAFS analyses have shown that Zn is complexed to malate (Fig. 2), whereas in the non-tolerant non-accumulator related species *A. lyrata*, Zn is bound to phosphate (Sarret et al.

2002), illustrating that vacuolar storage as Zn-malate is the key feature of Zn, and most likely Cd, tolerance and hyperaccumulation.

Several reports based on EDX, and particularly microanalyses, have illustrated unambiguously that species from *Thlaspi* and *Alyssum* genus accumulate metal ions specifically in the epidermal cells, excepted in guard cells and cells surrounding the stomatal complexes (Küpper et al. 1999, 2001; Frey et al. 2000; Psaras et al. 2000). At the subcellular level, *ca* 83% of epidermal Zn is located in the vacuole whereas only 13% in bound to the cell wall in *T. caerulescens* (calculated from the values of Frey et al. 2000). These values fit perfectly with the distribution of Zn speciation established earlier (Salt et al. 1999) with 12% Zn associated to the cell wall, 26% to water molecules, 38% to citrate and 9% to oxalate. Mesophyll cells accumulate tenfold less Zn, 96% being associated to the cell wall (Frey et al. 2000). These results are quantitatively and qualitatively similar for the subcellular localization and speciation of Ni in *T. goesingense*. By comparisons of total leaf Ni with protoplast and vacuolar Ni content, Krämer et al. (2000) showed that more than 65% of Ni is bound to the cell wall in mesophyll cells. In the vacuoles, Ni is complexed with citrate whereas in the cytoplasm histidine is the main chelator (see Fig. 2), in good agreement with the 16% Zn-His measured in *T. caerulescens* (Salt et al. 1999). The presence of Zn-His and Ni-His complexes is though to represent the cytoplasmics forms of metals being shuttled to the vacuole. In the case of Ni, the main difference between *T. goesingense* and the relative non-tolerant species *T. arvense* in mesophyll protoplasts is the twofold increase in vacuolar storage in the hyperaccumulator (Krämer et al. 2000). A more dynamic study realized by Küpper et al. (2004) on Cd and Zn complexation in *T. caerulescens* has given a much better and integrated view of the mechanisms involved in Cd and Zn immobilization. Extended X-ray absorption fine structure measurements were performed on different organs at different ages: young, mature, senescent, and dead leaves, mature and senescent petioles, and mature and senescent stems. The first general observation is the relative importance of high affinity ligands in young leaves (His for Zn and S>His for Cd) with a tendency to decrease in favour of O ligands (e.g. organic acids) in mature leaves. Second, is the higher proportion of S-Cd and His-Zn coordinations in stems than in the leaves of the same age, that could represent the forms of metal being transported to the leaves. During senescence cell death causes decompartmentation with the release of Cd and Zn from the vacuole, causing an increased complexation to high affinity ligands, His and S for Cd and His for Zn. For the first time, different types of ligands, with different metabolic origins, are integrated in a general mechanism of heavy metal tolerance and accumulation that can, at least partially, reconciliate conflicting and fragmented observations. Young, metabolically active, less vacuolized tissues or organs will synthesize high affinity-high energy cost ligands (histidine, S-containing ligands like phytochelatins) and with aging and vacuolisation metals accumulate in the vacuole with low affinity, low energy cost ligands such as organic acids that also provide a counter electric charge and can be accumulated at high concentrations.

4.3 Nicotianamine, a potential new actor in heavy metal sequestration

Although no definitive evidence has been provided for the role of nicotianamine in final immobilization of metals, this metabolite is clearly involved in metal transport. However, on the basis of its biochemical properties (high affinity for a wide range of metals, high solubility, and mobility), NA is likely to have a role in final sequestration.

Nicotianamine (NA) is a ubiquitous non-proteinogenic amino acid synthesized from S-adenosyl-methionine by nicotianamine synthase (Higuchi et al. 1999). Nicotianamine is made by all plants and is present in various plant organs and displays a high affinity for several metal ions *in vitro* (Stephan et al. 1996), although its precise mode of action and biological role has not been elucidated yet. From the analysis of the tomato mutant *chloronerva* that lacks the nicotianamine synthase activity, several potential roles of nicotianamine in the cell-to-cell transport of iron and root-to-shoot translocation of Cu have been proposed (Stephan and Scholz 1993; Pich and Scholz 1996). Nicotianamine has been shown to stably chelate Fe3+ and Fe2+, the latter having unusual kinetic stability under aerobic conditions. These complexes are poor Fenton reagents suggesting that nicotianamine may have an important role in scavenging iron and protecting the cell from oxidative damages. Although complexes of nicotianamine with iron, copper, and zinc are stable over a large scale of pH values (Fig. 2), they are preferentially stabilized at pH above 6 for all metal analyzed, *i.e.*, at pH value of the cytosol and the phloem sap (von Wirén et al. 1999). Expression of the *Thlaspi caerulescens* nicotianamine synthase in the yeast *S. cerevisiae* led to the accumulation of nicotianamine and nickel tolerance in yeast. Coupled techniques of size exclusion chromatography followed by ICP-MS or electrospray MS/MS analyses have allowed the characterization of nicotianamine-nickel complexes *in vivo* (Vacchina et al. 2003). Moreover, the same approach has shown that such complexes were present in the xylem sap of *T. caerulescens* (Mari S, Gendre D, Pianelli K, Lebrun M, and Czernic P, unpublished results). In addition, a 100-fold NA accumulation in transgenic *A. thaliana* lines expressing the *T. caerulescens* enzyme is quantitatively correlated with increased tolerance to nickel (Piannelli et al. 2005). Similar results were obtained with the overexpression of AtNAS genes in tobacco, although in this case an increased iron accumulation was also observed (Douchkov et al. 2005). Thus, nicotianamine may play an important role in chelating extravacuolar nickel in hyperaccumulators, based on the preferential stability of the complexes at pH values close to cytosolic pH (von Wirén et al. 1999). Nevertheless, in an attempt to localize nicotianamine in tomato subcellular compartments by classical immunocytochemical techniques, most of the signal produced by the antibodies raised against nicotianamine-keyhole limpet haemocyanin conjugate selectively labelled cells of the stele of the root tips, with the signal mostly confined to vacuoles (Pich et al. 1997). Furthermore, iron overaccumulation in plant cells, either by increasing Fe nutrition or using Fe-overaccumulating pea mutants, brz and dgl, resulted in parallel NA accumulation and immuno-localization in the vacuoles (Pich et al. 2001). The potential importance of the role of nicotianamine in metal homeostasis has been further strengthened by the observation of the con-

stitutive overexpression of the mRNA for nicotianamine synthase gene *AhNAS2* and *AhNAS3* in the zinc/cadmium hyperaccumulator *Arabidopsis halleri*. Ectopic expression of *AhNAS2* and *AhNAS3* in a zinc-hypersensitive *Schizosaccharomyces pombe* strain induces the tolerance to toxic concentrations of zinc (Becher et al. 2004; Weber et al. 2004). Nevertheless, in *S. cerevisiae* cells with a wild type zinc sensitive phenotype, we did not observe any zinc tolerance conferred by the ectopic expression of the nicotianamine synthase gene of *T. caerulescens*, although a high level of resistance to nickel was achieved (Pianelli et al. 2005). In addition, colocalization of a QTL explaining 10,7% of the variance for zinc tolerance in recombinant inbred population issued from ecotypes Landsberg erecta (Ler) x Cape Verde Island (Cvi) accessions of *A. thaliana* supports the proposed role of nicotianamine in metal homeostasis, whatever the respective role in sequestration and transport might be (Vreugdenhil et al. 2004).

5 Concluding remarks

Immobilization of metals is not a firm unequivocal concept. It relates practically on the measure of the accumulation of metals in various organs and compartments of the cell. In plants, most of the metal is, thus, stored in vacuoles and cell wall (Fig. 1). This observation is consistent with the common thought that the critical physiological mechanism is the homeostasis of free metal in the cytoplasm, involving the uptake (Cosio et al. 2004), the intracellular distribution (Krämer et al. 2000) and a high cellular tolerance to metals, illustrated for example for protoplasts of *A. halleri* and *T. caerulescens* (Marquès et al. 2004). This involves the avoidance of free metal increase in the cytoplasm of the cell in conditions where organisms are challenged with excess metal. In general, free metal ion remains almost constant as total metal concentration changes. In other words, buffering cell mechanisms allow the set up of a biologically available metal pool, from metals complexed with abundant small molecules in the cytoplasm. Practically, this view lead to the acceptance that the free metal ion pool does not exist in cytoplasm and shed light on the absolute necessity to have access to the *in vivo* speciation of metals (reviewed in Finney and O'Halloran 2003).

Evolving techniques allowed getting more reliable *in situ* profiling of metal accumulation at different levels of an organ, up to the subcellular level. Secondary ion mass spectrometer (SIMS) has seldom been used to investigate *in vivo* localization of metals, mainly owning to the complex embedding fixation step that should avoid mixing of the compartments and the mobility of the diffusible ions. High spatial resolution have recently been obtained (100-200nm), allowing to identify various ions in phytate granules of the aleurone of wheat grain, and might be extended to improve the spatial localization of a larger range of metal in plants (Heard et al. 2002). Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is a recent technique that allows quantitative elemental depth profiling on fresh material, making possible the *in situ* depth analysis of metal distribu-

tion at selected points around 100 of micrometer on a leaf surface (for example, Punshon et al. 2004).

There is a clear lack of reliable well-established methodology for the exhaustive analysis of the coordination and *in situ* speciation and localization of metal-ligands complexes inside plant cells, and especially other than cadmium. This analysis has not deserved sufficient attention, partly due to the difficulty to access routinely to reliable techniques, such as EXAFS analysis. EXAFS is particularly well suited for analyzing the *in vivo* ligand environment of metals and is prone to be used with intact frozen tissue, thus, considerably minimizing the potential risk of ligands swapping associated with extraction procedure and cell compartments mixing (Küpper et al. 2004). Unfortunately, although biologically relevant, this approach does not allow identifying the definitive structure of an unknown ligand and is dependent on comparisons with known reconstituted metal-ligand complexes.

Careful fractionation of organ and cell, followed by sensitive detection of both metal and associated ligand remains the first committed step to have access to the characterization of any metal ligand. The parallel use of chromatographic (HPLC, CZE) separation and detection by ICP-MS of the metal-complex followed by ES MS-MS to determine the structure of the bioligand offers an elegant and efficient methodology (Schaumlöffel et al. 2003; Vacchina et al. 2003). It opens the way to a generalized analysis of metal speciation, assuming the current improvement both to maintain the interaction formed *in vivo* along the separation process, and to improve the sensitivity of the detection, hopefully at the nanoliter-cell level (for example: nanoHPLC-ICP MS/ES QqTOF MS).

Combined approach using: *(i)* thorough characterization of metal content in the various territories of the organ and cell, *(ii)* fractionation-ICP-MS/ES MS-MS analysis of the various bioligands of metal complexes, *(iii)* EXAFS to definitively confirms the speciation *in vivo*, assuming an appropriate detection level (cell, compartment) is reached, is prone to end up with a unified methodological approach and give a reasonably reliable picture of metal speciation both in the plant and also the evolution of speciation during plant development and upon environmental constraints (Küpper et al. 2004).

References

Barcelo J, Poschenrieder C (1999) Structural and ultrastructural changes in heavy metal exposed plants. In: Prasad MNV, Hagemeyr J (Eds) Heavy metal stress in plants. Springer-Verlag, Berlin, Heidelberg, pp 183-205

Barnes A, Constantinidou C, Ashton P, Jones A, Pritchard J (2004) Determining protein identity from sieve element sap in *Ricinus communis* L. by quadrupole time of flight (Q-TOF) mass spectrometry. J Exp Bot 55:1473-1481

Becher M, Talke IN, Krall L, Krämer U (2003) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J 37:251-268

Blamey FPC, Joyce DC, Edwards DG, Asher CJ (1986) Role of trichomes in sunflower tolerance to manganese toxicity. Plant Soil 91:171-180

Boominathan R, Doran PM (2003) Organic acid complexation, heavy metal distribution and the effect of ATPase inhibition in hairy roots of hyperaccumulator plant species. J Biotechnol 101:131-146

Chen JJ, Zhou JM, Goldsbrough PB (1997) Characterization of phytochelatin synthase from tomato. Physiol Plant 101:165-172

Choi Y-E, Harada E, Wada M, Tsuboi H, Morita Y, Kusano T, Sano H (2001) Detoxification of cadmium in tobacco plants: formation and active excretion of crystals containing cadmium and calcium through trichomes. Planta 213:45-50

Clarkson DT (1993) Roots and the delivery of solutes to the xylem. Phil Trans Royal Soc London, series B 341:5-17

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 18:3325-3333

Clemens S, Palmgren M, Krämer U (2002) A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci 7:309-315

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:159-182

Cosio C, Martinoia E, Keller C (2004) Hyperaccumulation of cadmium and zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. Plant Physiol 134:716-723

De la Fuente JM, Ramirez-Rodriguez V, Cabrera-Ponce JL, Herrera-Estrella L (1997) Aluminum tolerance in transgenic plant by alteration of citrate synthesis. Science 276:1566-1568

de la Rosa G, Peralta-Videa JR, Montes M, Parsons JG, Cano-Aguilera I, Gardea-Torresdey JL (2004) Cadmium uptake and translocation in tumbleweed (*Salsola kali*), a potential Cd-hyperaccumulator desert plant species: ICP/OES and XAS studies. Chemosphere 55:1159-1168

Delhaize E, Craig S, Beaton CD, Bennet RJ, Jagadish VC, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.) (I. Uptake and distribution of aluminum in root apices). Plant Physiol 103:685-693

Delhaize E, Hebb DM, Ryan PR (2001) Expression of a *Pseudomonas aeruginosa* citrate synthase gene in tobacco is not associated with either enhanced citrate accumulation or efflux. Plant Physiol 125:2059-2067

Delhaize E, Ryan PR, Hebb DM, Yamamoto Y, Sasaki T, Matsumoto H (2004) Engineering high-level aluminum tolerance in barley with the *ALMT1* gene. Proc Natl Acad Sci USA 101:15249-15254

Delhaize E, Ryan PR, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.) (II. Aluminum-stimulated excretion of malic acid from root apices). Plant Physiol 103:695-702

Douchkov D, Gryczka C, Stephan UW, Hell R, Bäumlein H (2005) Ectopic expression of nicotianamine synthase genes results in improved iron accumulation and increased nickel tolerance in transgenic tobacco. Plant Cell Env 28:365-374

Finney LA, O'Halloran TV (2003) Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300:931-936

Foley RC, Singh KB (1994) Isolation of a *Vicia faba* metallothionein-like gene: expression in foliar trichomes. Plant Mol Biol 26:435-444

Frey B, Keller C, Zierold K, Schulin R (2000) Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 23:675-687

Garcia-Hernandez M, Murphy A, Taiz L (1998) Metallothioneins 1 and 2 have distinct but overlapping expression patterns in *Arabidopsis*. Plant Physiol 118:387-397

Gisbert C, Ros R, De Haro E, Walker DJ, Bernal MP, Serrano R, Navarro-Aviño (2003) A plant genetically modified that accumulates PBb is especially promising for phytoremediation. Biochem Biophys Res Commun 303:440-445

Grill E, Winnacker E-L, Zenk MH (1985) Phytochelatins: the principal heavy-metal complexing peptides of higher plants. Science 230:674-676

Grill E, Loffler S, Winnacker E-L, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific g-glutamylcysteine dipeptidyl transpeptidase (phytochelation synthase). Proc Natl Acad Sci USA 86:6838-6842

Ha S-B, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett C (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. Plant Cell 11:1-12

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1-11

Heard PJ, Feeney KA, Allen GC, Shewry PR (2002) Determination of the elemental composition of mature wheat grain using a modified secondary ion mass spectrometer (SIMS). Plant J 30:237-245

Higuchi K, Suzuki K, Nakanishi H, Yamaguchi H, Nishizawa N-K, Mori S (1999) Cloning of nicotianamine synthase genes, novel genes involved in the biosynthesis of phytosiderophores. Plant Physiol 119:471-479

Hill KA, Lion LW, Ahner BA (2002) Reduced Cd accumulation in *Zea mays*: a protective role for phytosiderophores? Environ Sci Technol 36:5363-5368

Howden R, Goldsbrough PB, Andersen CR, Cobbett C (1995) Cadmium sensitiven cad1, mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol 107:1059-1066

Kerkeb L, Krämer U (2003) The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. Plant Physiol 131:716-724

Koyama H, Kawamura A, Kihara T, Hara T, Takita E, Shibata D (2000) Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* improved growth on a phosphorus-limited soil. Plant Cell Physiol 41:1030-1037

Krämer U, Pickering IJ, Prince RC, Raskin I, Salt DE (2000) Subcellular localisation and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. Plant Physiol 122:1343-1353

Küpper H, Lombi E, Zhao F-J, McGrath S (2000) Cellular compartmentation of cadmium and zinc in t-relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212:75-84

Küpper H, Lombi E, Zhao F-J, Wieshammer G, McGrath S (2001) Cellular compartmentation of nickel in the hyperaccumlators *Alyssum lesbiacum*, *Alyssum bertolonii* and *Thlaspi goesingense*. J Exp Bot 52:2291-2300

Küpper H, Mijovilovich A, Meyer-Klaucke W, Kroneck P (2004 Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by X-ray absorption spectroscopy. Plant Physiol 134: 748-757

Lavid N, Barkay Z, Tel-Or E (2000) Accumulation of heavy metals in epidermal glands of the waterlily (*Nymphaeaceae*). Planta 212:323-322

Lee S Moon JS, Petros D, Goldsbrough PB, Korban SS (2003) Overexpression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. Plant Physiol 131:656-663

Lee S, Korban SS (2002) Transcriptional regulation of *Arabidopsis thaliana* phytochelatin synthase (*AtPCS1*) by cadmium during early stages of plant development. Planta 215:689-693

Li Y, Dhankher P, Carreira L, Lee D, Chen A, Schroeder JI, Balish RS, Meagher RB (2004) Overexpression of phytochelatin synthase in *Arabidopsis* leads to enhanced arsenic tolerance and cadmium hypersensitivity. Plant Cell Physiol 45:1787-1797

Li Z-S, Lu Y-P, Zhen R-G, Szczypka M, Thiele DJ, Rea PA (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc Natl Acad Sci USA 94:42-47

Liu D, Kottke I (2003) Subcellular localisation of cadmium in the root cells of *Allium sativum* by electron energy loss spectroscopy. J Biosci 28:471-478

Liu D, Kottke I (2004) Subcellular localization of copper in the root cells of *Allium sativum* by electron energy loss spectroscoy (EELS). Biores Technol 94:153-158

Loeffler S, Hochberger A, Gril E, Winnacker E-L, Zenk M-H (1989) Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction products. FEBS Lett 258:42-46

Ma JF, Furukawa J (2003) recent progress in the research of external Al detoxification in higher plants: a mini-review. J Inorganic Biochem 97:46-51

Maitani T, Kubota H, Sato K, Yamada T (1996) The composition of metals bound to class III metallothionein (phytochelatin and its desglycin peptide) induced by various metals in root cultures of *Rubia tinctorum*. Plant Physiol 110:1145-1150

Marquès L, Cossegal M, Bodin S, Czernic P, Lebrun M (2004) Heavy metal specificity of cellular tolerance in two hyperaccumulating plants, *Arabidopsis halleri* and *Thlaspi caerulescens*. New Phytol 164:286-295

Martell EA (1974) Radioactivity of tobacco trichomes and insoluble cigarette smoke particles. Nature 249:215-217

Mehra RK, Kodati R, Abdullah R (1995) Chain lenght-dependent Pb(II)-coordination in phytochelatins. Biochem Biophys Res Commun 215:730-736

Mehra RK, Miclat J, Kodati R, Abdullah R, Hunter TC, Mulchandani P (1996) Optical spectroscopic and reverse-phase HPLC analyses of Hg(II) binding to phytochelatins. Biochem J 314:73-82

Mehra RK, Mulchandani P, Hunter TC (1994) Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*. Biochem Biophys Res Commun 200:1193-1200

Maugenest S, Martinez I, Godin B, Perez P, Lescure AM (1999) Structure of two maize phytase genes and their spatio-temporal expression during seedling development. Plant Mol Biol 39:503-514

Murphy A, Taiz L (1995) Comparison of metallothionein gene expression and nonprotein thiols in ten *Arabidopsis* ecotypes. Plant Physiol 109:945-954

Morris CA, Nicolaus B, Sampson V, Harwood JL, Kille P (1999) Identification and characterization of a recombinant metallothionein protein from a marine alga, *Fucus vesiculosus.* Biochem J 338:553-560

Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J 11:3491-3499

Ortiz DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270:4721-4728

Pianelli K, Mari S, Marquès L, Lebrun M, Czernic P (2005) Nicotianamine over-accumulation confers resistance to nickel in *Arabidopsis thaliana*. Trans Res, in press

Pich A, Scholz G (1996) Translocation of copper and other micronutrients in tomato plants (*Lycopersicon esculentum* Mill): nicotianamine-stimulated copper transport in the xylem. J Exp Bot 47:41-47

Pich A, Hillmer S, Manteuffel R, Scholz G (1997) First immuno-histochemical localization of the endogenous Fe2+ -chelator nicotianamine. J Exp Bot 48:759-767

Psaras GK, Constantinidis TH, Cotsopoulos B, Manetas Y (2000) Relative abundance of nickel in the leaf epidermis of eight hyperaccumulators: evidence that the metal is excluded from both guard cells and trichomes. Annals Bot 86:73-78

Punshon T, Jackson BP, Bertsch PM, Burger J (2004) Mass loading of nickel and uranium on plant surfaces: application of laser ablation-ICP-MS. J Environ Monit 6:153-159

Raab A, Feldmann J, Meharg AA (2004) The nature of arsenic-phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. Plant Physiol 134:1113-1122

Rauser WE (1995) Phytochelatins and related peptides: structure, biosynthesis, and function. Plant Physiol 109:1141-1149

Rauser WE (1999) Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin and metallothioneins. Cell Biochem Biophys 31:19-48

Reeves RD, Baker AJM (2000) Metal-accumulating plants. In: Raskin I, Ensley BD (eds) Phytoremediation of toxic metals: using plants to clean up the environment. John Wiley & Sons, New York, pp 193-219

Robinson NJ, Wilson JR, Turner JS (1996) Expression of the type 2 metallothionein-like gene MT2 from *Arabidopsis thaliana* in Zn^{2+} -metallothionein-deficient *Synechococcus* PCC7942: putative role for MT2 in Zn^{2+} metabolism. Plant Mol Biol 30:1169-1179

Roosens NH, Bernard C, Leplae R, Verbruggen N (2004) Evidence for copper homeostasis function of metallothionein (MT3) in the hyperaccumulator *Thlaspi caerulescens*. FEBS Letters 577:9-16

Ryan PR, Delhaize E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annual Rev Plant Physiol Plant Mol Biol 52:527-560

Salt DE, Krämer U (2000) Mechanisms of metal hyperaccumulation in plants. In: Raskin I, Ensley BD (eds) Phytoremediation of toxic metals: using plants to clean up the environment. John Wiley & Sons, New York, pp 231-246

Salt DE, Prince RC, Baker AJM, Raskin Y, Pickering IJ (1999) Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. Environ Sci Technol 33:713-717

Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanisms of cadmium mobility and accumulation in indian mustard. Plant Physiol 109:1427-1433

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann J-L Traverse A, Marcus MA, Manceau A (2002) Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiol 130:1815-1828

Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J 37:645-653

Sattelmacher B (2001) The apoplast and its significance for plant mineral nutrition. New Phytol 149:167-192

Schaumlöffel D, Ouerdane L, Bouyssiere B, Lobinski R (2003) Speciation analysis of nickel in the latex of the tree *Sebertia acuminata* by HPLC and CZE with ICP MS and electrospray MS-MS detection. J Anal At Spectrom 18:120-127

Schier GA, McQuattie CJ (1995) Effect of aluminum on the growth, anatomy, and nutrient content of ectomycorrhizal and non-mycorrhizal eastern white pine seedlings. Can J For Res 25:1252-1262

Snowden KC, Richards KD, Gardner RC (1995) Aluminum-induced genes. Induction by toxic metals, low calcium, and wounding and pattern of expression in root tips. Plant Physiol 107:341-348

Stephan UW, Scholz G (1993) Nicotianamine: mediator of transport of iron and other heavy metals in the phloem? Physiol Plant 88:522-529

Stephan UW, Schmidke I, Stephan VW, Scholz G (1996) The nicotianamine molecule is made-to-measure for complexation of metal micronutrients in plants. Biometals 9:84-90

Sresty TV, Madhava Rao KV (1999) Ultrastructural alterations in response to zinc and nickel stress in the root cells of pigeonpea. Envir Experi Bot 41:3-13

Tesfaye M, Temple SJ, Allan DL, Vance CP, Samac DA (2001) Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. Plant Physiol 127:1836-1844

Toaspern M, Lasat M, Kochian L, Smolenski K, Bilderback D, Fontes E, Finkelstein K (2000) X-ray imaging of zinc accumulation in bioremediators. CHESS Newsletter 2000:44-45

Tommey AM, Shi J, Lindsay WP, Urwin PE, Ronbinson NJ (1991) Expression of the pea gene PsMTa in *E. coli*-metal-binding properties of the expressed protein. FEBS Lett 292:48-52

Vacchina V, Mari S, Czernic P, Marques L, Pianelli K, Schaumlöffel D, Lebrun M, Lobinski R (2003) Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray ms/ms assisted by cloning using yeast complementation. Analytical Chem 75:2740-2745

Van Hoof NALM, Hassinen VH, Hakvoort HWJ, Ballintijn KF, Schat H, Verkleij JAC, Ernst WHO, Karenlampi SO, Tervahauta AI (2001) Enhanced copper tolerance in *Silene vulgaris* (Moench) Garke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. Plant Physiol 126:1519-1526

Van Steveninck RFM, Barbare A, Fernando DR, Van Steveninck ME (1994) The binding of zinc, but not cadmium, by phytic acid in roots of crop plants. Plant Soil 167:157-164

Van Vliet C, Andersen CR, Cobbett CS (1995) Copper-sensitive mutant of *Arabidopsis thaliana*. Plant Physiol 109:871-878

Vatamaniuk OK, Mari S, Lang A, Chalasani S, Demkiv LO, Rea PA (2004) Phytochelatin synthase, a dipeptidyltransferase that undergoes multisite acylation with gamma-glutamylcysteine during catalysis: stoichiometric and site-directed mutagenic analysis

of *Arabidopsis thaliana* PCS1-catalyzed phytochelatin synthesis. J Biol Chem 279:22449-22460

Vatamaniuk OK, Mari S, Lu Y-P, Rea PA (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis:* isolation and *in vitro* reconstitution. Proc Natl Acad Sci USA 96:7110-7115

Vatamaniuk OK, Mari S, Lu Y-P, Rea PA (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase-blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. J Biol Chem 275:31451-31459

Vogeli-Lange R, Wagner GJ (1990) Subcellular localisation of cadmium and cadmium-binding peptides in tobacco leaves. Implication of a transport function for cadmium-binding peptides. Plant Physiol 92:1086-1093

Von Wirén N, Klair S, Bansal S, Briat JF, Khodr H, Shioiri T, Leigh RA, Hider RC (1999) Nicotianamine chelates both FeIII and FeII. Implications for metal transport in plants. Plant Physiol 119:1107-1114

Vreugdenhil D, Aarts MGM, Koornneef M, Nelissen H, Ernst WHO (2004) Natural variation and QTL analysis for cationic mineral content in seeds of *Arabidopsis thaliana*. Plant Cell Envi 27:829-839

Wang J, Evangelou BP, Nielsen MT (1992) Surface chemical properties of purified root cell walls from two tobacco genotypes exhibiting different tolerance to manganese toxicity. Plant Physiol 100:496-501

Weber M, Harada E, Vess C, Roepenack-Lahaye E, Clemens S (2003) Comparative microarray analysis of *Arabidopsis thaliana and Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. Plant J 37:269-281

Yang YY, Jung J-Y, Song W-Y, Suh H-S, Lee Y (2000) Identification of rice varieties with high tolerance or sensitivity to lead and characterization of the mechanism of tolerance. Plant Physiol 124:1019-1026

Zenk MH (1996) Heavy metal detoxification in higher plants-a review. Gene 179:21-30

Zhao FJ, Lombi E, Breedon T, McGrath SP (2000) Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. Plant Cell Environ 23:507-514

Zhou J, Goldsbrough PB (1994) Functional homologs of functional metallothionein genes from *Arabidopsis*. Plant Cell 6:875-884

Zhu Y, Pilon-Smits EA, Jouanin L, Terry N (1999) Overexpression of glutathione synthetase in indian mustard enhances cadmium accumulation and tolerance. Plant Physiol 119:73-80

Zhu YL, Pilon-Smits EA, Tarun AS, Weber SU, Jouanin L, Terry N (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. Plant Physiol 121:1169-1178

Lebrun, Michel
Unité Mixte de Recherche, Biochimie et Physiologie Moléculaire des Plantes, INRA, CNRS, Université Montpellier II, Agro-M, Place Viala, 34060 Montpellier.
lebrun@univ-montp2.fr

Mari, Stéphane
Unité Mixte de Recherche, Biochimie et Physiologie Moléculaire des Plantes, INRA, CNRS, Université Montpellier II, Agro-M, Place Viala, 34060 Montpellier.

Phytoremediation and hyperaccumulator plants

Wendy Ann Peer, Ivan R. Baxter, Elizabeth L. Richards, John L. Freeman, Angus S. Murphy

Abstract

Phytoremediation is a group of technologies that use plants to reduce, remove, degrade, or immobilize environmental toxins, primarily those of anthropogenic origin, with the aim of restoring area sites to a condition useable for private or public applications. Phytoremediation efforts have largely focused on the use of plants to accelerate degradation of organic contaminants, usually in concert with root rhizosphere microorganisms, or remove hazardous heavy metals from soils or water. Phytoremediation of contaminated sites is a relatively inexpensive and aesthetically pleasing to the public compared to alternate remediation strategies involving excavation/removal or chemical *in situ* stabilization/conversion. Many phytoremediation plans have multi-year timetables, but since most sites in need of remediatrion have been contaminated for more than ten years, as such a ten year remediation plan does not seem excessive. Seven aspects of phytoremediation are described in this chapter: phytoextraction, phytodegradation, rhizosphere degradation, rhizofiltration, phytostabilization, phytovolatization, and phytorestoration. Combining technologies offer the greatest potential to efficiently phytoremediate contaminated sites. The major focus of this chapter is phytoextraction of arsenic, cadmium, chromium, copper, mercury, nickel, lead, selenium, and zinc.

1 Introduction to phytoremediation

Phytoremediation is a term applied to a group of technologies that use plants to reduce, remove, degrade, or immobilize environmental toxins, primarily those of anthropogenic origin, with the aim of restoring area sites to a condition useable for private or public applications. To date, phytoremediation efforts have focused on the use of plants to accelerate degradation of organic contaminants, usually in concert with root rhizosphere microorganisms, or remove hazardous heavy metals from soils or water. Phytoremediation of contaminated sites is appealing because it is relatively inexpensive and aesthetically pleasing to the public compared to alternate remediation strategies involving excavation/removal or chemical *in situ* stabilization/conversion. Seven aspects of phytoremediation are described in this chapter: phytoextraction, phytodegradation, rhizosphere degradation, rhizofiltration, phytostabilization, phytovolatization, and phytorestoration. However, the major focus is on phytoextraction.

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_100 / Published online: 11 August 2005

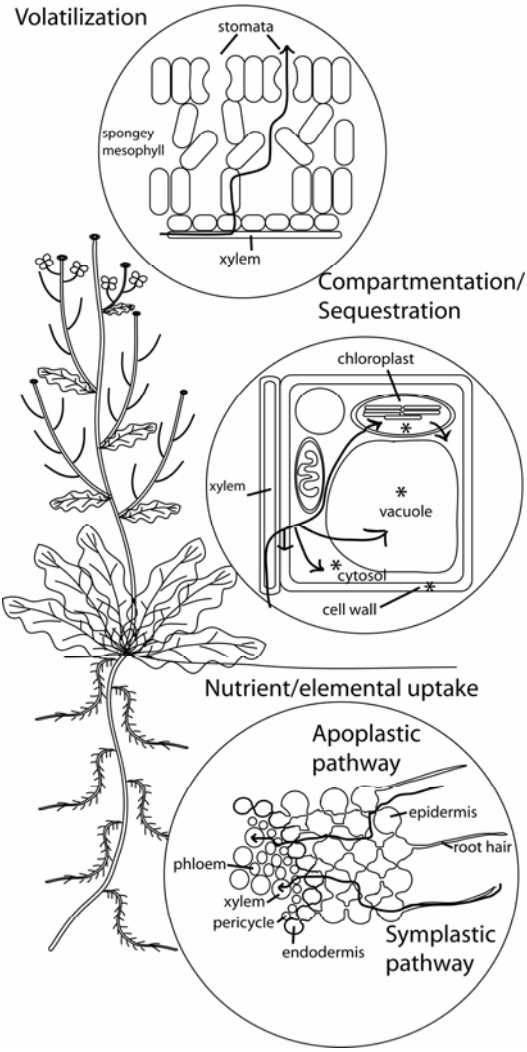


Fig. 1. Pathway of metal/nutrient uptake in plants. Soluble metals can enter into the root symplast by crossing the plasma membrane of the root endodermal cells or they can enter the root apoplast through the space between cells. If the metal is translocated to aerial tissues, then it must enter the xylem. To enter the xylem, solutes must cross the Casparian strip, a waxy coating which is impermeable to solutes, unless they pass through the cells of the endodermis probably through the action of a membrane pump or channel. Once loaded into the xylem, the flow of the xylem sap will transport the metal to the leaves, where it must be loaded into the cells of the leaf, again crossing a membrane. Once in the shoot or leaf tissues, metals can be stored in various cell types, depending on the species and the form of the metal, since it can be converted into less toxic forms (to the plant) through chemical conversion or complexation. The metal can be sequestered in several subcellular compartments (cell wall, cytosol, vacuole) or volatilized through the stomata.

1.1 Phytoextraction

Phytoextraction involves the removal of toxins, especially heavy metals and metalloids, by the roots of the plants with subsequent transport to aerial plant organs (Salt et al. 1998; Lombi et al. 2001a) (Fig. 1). Pollutants accumulated in stems and leaves are harvested with accumulating plants and removed from the site. Phytoextraction can be divided into two categories: continuous and induced (Salt et al. 1998). Continuous phytoextraction requires the use of plants that accumulate particularly high levels of the toxic contaminants throughout their lifetime (hyperaccumulators), while induced phytoextraction approaches enhance toxin accumulation at a single time point by addition of accelerants or chelators to the soil. In the case of heavy metals, chelators like EDTA assist in mobilization and subsequent accumulation of soil contaminants such as lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), and zinc (Zn) in *Brassica juncea* (Indian mustard) and *Helianthus anuus* (sunflower) (Blaylock et al. 1997; Turgut et al. 2004). The ability of other metal chelators such as CDTA, DTPA, EGTA, EDDHA, and NTA to enhance metal accumulation has also been assessed in various plant species (Huang et al. 1997; Lombi et al. 2001b). However, there may be risks associated with using certain chelators considering the high water solubility of some chelator-toxin complexes which could result in movement of the complexes to deeper soil layers (Wu et al. 1999; Lombi et al. 2001b) and potential ground water and estuarian contamination.

1.2 Phytodegradation

In phytodegredation, organic pollutants are converted by internal or secreted enzymes into compounds with reduced toxicity (Schnoor 1997; Salt et al. 1998; Suresh and Ravishankar 2004). For instance, the major water and soil contaminant trichloroethylene (TCE) was found to be taken up by hybrid poplar trees, *Populus deltoides x nigra*, which break down the contaminant into its metabolic components (Newman et al. 1997). TCE and other chlorinated solvents can be degraded to form carbon dioxide, chloride ion and water (Schnoor et al. 1995). Poplars have also been shown to take up the ammunition wastes 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5 triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7 tetrazocine (HMX) and partially transform them (Thompson et al. 1998; Yoon et al. 2002). Root exudates from *Datura innoxia* and *Lycopersicon peruvianum* containing peroxidase, laccase, and nitrilase have been shown to degrade soil pollutants (Schnoor et al. 1995; Lucero et al. 1999) and nitroreductase and laccase together can break down TNT, RDX, and HMX (Schnoor et al. 1995). The plants are then able to incorporate the broken ring structures into new plant material or organic soil components that are thought to be non-hazardous.

1.3 Phytovolatilization

Plants can also remove toxic substances, such as organics, from the soil through phytovolatization. In this process, the soluble contaminants are taken up with water by the roots, transported to the leaves, and volatized into the atmosphere through the stomata (Tollsten and Muller 1996; Newman et al. 1997; Davis 1998) (Fig. 1). The best example of this is the volatilization of mercury (Hg) by conversion to the elemental form in transgenic *Arabidopsis* and yellow poplars containing bacterial mercuric reductase (*merA*) (Rugh et al. 1996, 1998) (see Section 3.5). In a study where the movement of volatile organics was monitored by Fourier transform infrared spectrometry (FT-IR) in hybrid poplars (*Populus deltoides x nigra*), *Tamarix parviflora* (saltcedar), and *Medicago sativa* (alfalfa), chlorinated hydrocarbons were found to move readily through the plants, but less polar compounds like gasoline constituents did not (Davis et al. 1998). However, amounts of the contaminant transpired are in proportion to water flow and are relatively low, especially in the field. Rubin and Ramaswami (2001) found that poplar saplings can concentrate (100 ppb) and transpire methyl tertiary-butyl ether (MTBE), a compound added to gasoline which is commonly found as a groundwater pollutant. In a one week time period, they observed a 30% reduction in MTBE mass in hydroponic solution by saplings at both high (1600 ppb) and low (300 ppb) MTBE concentrations, which suggested that these plants could be successful in the phytoremediation of this toxin from groundwater (Rubin and Ramaswami 2001). Selenium (Se) is a special case of a metal that is taken up by plants and volatilized (see Section 3.8). Se can also be volatilized following conversion to dimethylselenide by microbes and algae (Neumann et al. 2003) (see Chapter 13).

1.4 Rhizosphere degradation

Like phytodegradation, rhizosphere degradation involves the enzymatic breakdown of organic pollutants, but through microbial enzymatic activity. These breakdown products are either volatilized or incorporated into the microorganisms and soil matrix of the rhizosphere. The types of plants growing in the contaminated area influence the amount, diversity, and activity of microbial populations (Jones et al. 2004; Kirk et al. 2005). Grasses with high root density, legumes that fix nitrogen, and alfalfa that fix nitrogen and have high evapotranspiration rates are associated with different microbial populations. These plants create a more aerobic environment in the soil that stimulates microbial activity that enhances oxidation of organic chemical residues (Anderson 1993; Schnoor et al. 1995; Narayanan 1998; Jones et al. 2004; Kirk et al. 2005). Secondary metabolites and other components of root exudates also stimulate microbial activity, a byproduct of which may be degradation of organic pollutants (Pieper et al. 2004).

1.5 Rhizofiltration

Rhizofiltration removes contaminants from water and aqueous waste streams, such as agricultural run off, industrial discharges, and nuclear material processing wastes (Salt et al. 1998; Suresh and Ravishankar 2004). Absorption and adsorption by plant roots play a key role in this technique, and consequently large root surface areas are usually required. In research associated with Epcot Center, closed systems with recirculating nutrients have exhibited the benefits of rhizofiltration and biofiltration using a variety of species (such as mosses and scented geraniums) (Negri and Hinchman 1996). Rhizofiltration was also shown to be useful in the San Francisco Bay study directed by Norman Terry (University of California, Berkely) and supported by Chevron (Hansen et al. 1998). A wetland constructed next to the bay was shown to remove 89% of the Se from selenite-contaminated wastewater released from various oil refineries. The water flowing into the wetland was measured to have 20–30 μg L^{-1}selenite, while the outflow from the wetland had less than 5 μg L^{-1} selenite (Hansen et al. 1998). In a study of Se removal from agricultural subsoil drainage in the San Joaquin Valley (Gao et al. 2003), a flow-through wetland system was constructed with cells containing either a single species, or a combination of species [e.g. *Schoenoplectus robustus* (sturdy bulrush), *Juncus balticus* (baltic rush), *Spartina alterniflora* (smooth cordgrass), *Polypogon monspeliensis* (rabbit's foot grass), *Distichlis spicata* (saltgrass), *Typha latifolia* (cattail), *Schoenoplectus acutus* (Tule grass), and *Ruppia maritima* (widgeon grass)]. Four years after planting, comprehensive analysis showed that 59% of the Se remained in the wetland, mostly in the organic detrital layer and surface sediment, 35% in the outflow, 4% in seepage and 2% to volatilization. Wetland plant uptake of Se varies with species type, and parrot's feather (*Myriophyllum aquaticum*), iris-leaved rush (*Juncus xiphioides*), cattail, and sturdy bulrush were particularly noted for high Se uptake potential (Gao et al. 2003).

1.6 Phytostabilization

Erosion and leaching can mobilize soil contaminants resulting in aerial or waterborne pollution of additional sites. In phytostabilization, accumulation by plant roots or precipitation in the soil by root exudates immobilizes and reduces the availability of soil contaminants. Plants growing on polluted sites also stabilize the soil and can serve as a groundcover thereby reducing wind and water erosion and direct contact of the contaminants with animals. Significant phytostabilization projects have been employed in France and the Netherlands (Ernst et al. 1996; Bouwman et al. 2001; Marseille et al. 2000). A 2005-2010 superfund basic research program (Maier 2004) is developing a phytostabilization revegetation strategy to remediate mine tailings in arid and semi-arid ecosystems. The researchers will monitor the bioavailability of metals for the native metal- and drought-tolerant plant species used, and determine the permanence of expected toxicity reductions. Plants with high transpiration rates, such as grasses, sedges, forage

Table 1. Summary of elemental levels in background soil, metalliferous (or contaminated) soil, critical load in soil above which biodiversity and ecosystem function are adversely affected, and Commission des Communautés Européennes (EU) and Environmental Protection Agency (USA) permissible limits.

Element	Background soil levels (ppm)[a]	Metalliferous soil levels (ppm)[c]	Critical load in loam/silt (ppm)	CCE limits (ppm)[h]	EPA limits[k,i]
As	2.2 – 25	1 510	–	–	0.01 ppm
Cd	0.06 – 1.1	317	1.10	0.5[i]	5 ppb
Cr	7 – 221	3 450[d]	64.41	1.5[i]	100 ppb
Cu	6 – 80	3 783	48.78	50[j]	1.3 ppm
Hg	0.02 – 0.41	12 000[e]	0.56	1.5	2 ppb
Ni	4 – 55	11 260	54.64	1[i]	0.7 ppm
Pb	10 – 84	49 910	75.68	5[i]	1.5 ppb
Se	0.01 – 0.09[b]	>50[f]	–	–	0.05 ppm
Zn	17 – 125	7 480	207.32	150[j]	5 ppm

[a]http://www.sandia.gov
[b]Lakin 1972
[c]Reeves and Baker 2000
[d]http://www.dtsc.ca.gov
[e]http://www.deq.state.or.us
[f]http://www.nwo.usace.army.mil
[g]Bannick et al. 2002; Sand ~50% of loam/silt levels; clay ~1.5X loam/silt levels
[h]http://europa.eu.int
[i]H_2O
[j]Soil
[k]http://www.epa.gov

plants, and reeds are useful for phytostabilization by decreasing the amount of ground water migrating away from the site carrying contaminants (Suresh and Ravishankar 2004). Combining these plants with hardy, perennial, dense rooted or deep rooting trees (poplar, cottonwoods) can be an effective combination (Berti and Cuningham 2000).

1.7 Phytorestoration

Phytorestoration involves the complete remediation of contaminated soils to fully functioning soils (Bradshaw 1997). In particular, this subdivision of phytoremediation uses plants that are native to the particular area, in an attempt to return the land to its natural state. An examination of phytorestoration compared to the other forms of phytoremediation brings to light an important issue: what degree of decontamination do phytoremediation projects aim to achieve? There is a vast difference between removing just enough soil pollutants to reach legally defined levels of compliance, remediating soils to a level at which they can be used again, and completely restoring land from its contaminated state to an environmentally

uncontaminated state (Table 1). The objective of many phytoremediation projects is to restore the land to a legally acceptable level of contamination.

Lastly, a combination of phytoremediation approaches can be used for more effective environmental restoration. This may help to simultaneously remove different types of wastes from the same site. For example, a remediation system could include plants that hyperaccumulate toxic metals and plants that stimulate the activity of microbes that specialize in organic contaminant degradation.

2 Definitions of tolerant, indicator, and hyperaccumulator species

When categorizing plants that can grow in the presence of toxic elements, the terms "tolerant," "indicator", and "hyperaccumulator" are used. A tolerant species is one that can grow on soil with concentrations of a particular element that are toxic to most other plants. While both indicator species and hyperaccumulators are also tolerant, studies have shown the genetic distinction of the mechanisms involved (Assunção et al. 2001; Bert et al. 2003;Macnair et al. 1999). However, tolerant species are not necessarily indicators or hyperaccumulators, as tolerant non-accumulators can exclude metals from entering the root tissue. Examples of tolerant excluders include *Holcus lanatus* (Meharg and Macnair 1992a, 1992b), *Agrostis capillaris*, *Mimulus guttatus,* and *Silene vulgaris* (Pollard et al. 2002).

Indicator plants have been employed in biogeochemical prospecting. As early as 1865, F. Risse observed Zn accumulating plants, now known as *Thlaspi caerulescens*, growing near the German-Belgium border (Sachs 1865). This observation led others to associate the sites where these plants grew with soil containing elevated Zn. In the 1950s and 1960s, Helen Cannon and other members of the United Sates Geological Society cataloged indicator plants that were potentially important for "bioprospecting" for ore (Cannon 1960). Examples include mosses, which have been recognized as bioindicators of high metal concentrations in water (Gstoettner and Fisher 1995) and *Stanleya pinnata* (Prince's Plume), which is a recognized Se indicator.

Hyperaccumulators take up particularly high amounts of a toxic substance, usually a metal or metalloid, in their shoots during normal growth and reproduction (Reeves 1992; Baker and Whiting 2002). Hyperaccumulation reported in senescing plants generally represents a breakdown of homeostatic mechanisms and is clearly not a function of normal growth processes, although such accumulations could be technologically useful. The metal/metalloid concentration that must be accumulated by the plant before it is designated a "hyperaccumulator" depends upon the particular metal or metalloid in question. In early hyperaccumulator studies, Brooks and coworkers (1977) defined nickel (Ni) hyperaccumulators as those accumulating greater than 1000 μg Ni g^{-1} dry weight in their leaves. Subsequently, Baker and Brooks (1989) defined threshold concentrations for other metals hyperaccumulated in plants as 100 μg g^{-1} dry weight for Cd, 1,000 μg g^{-1} dry weight for Ni, Cu, Co, Pb, and 10,000 μg g^{-1} dry weight for Zn and Mn. The defined levels of

these elements are typically at a concentration of one order of magnitude greater that those found in non-accumulator species (Salt and Kramer 2000). Hyperaccumulators are found in 45 different families, with the highest occurrence among the Brassicaceae (Reeves and Baker 2000). These plants are quite varied, from perennial shrubs and trees to small annual herbs.

While tolerance is necessary for accumulation, evidence suggests that tolerance and accumulation are independent traits. There is a strong positive correlation between glutathione (GSH), cysteine (Cys), O-acetyl-L-serine (OAS) levels, compounds associated with tolerance, and Ni accumulation in shoot tissues of *Thlaspi* species from serpentine soils (Freeman et al. 2004). *Thlaspi goesingense* has constitutively high levels of serine acetyltransferase (SAT) and glutathione reductase activity associated with resistance to Ni-induced oxidative stress in this plant (Freeman et al. 2004). Elevated levels of reduced GSH contribute to a decrease in Ni-induced lipid peroxidation in *T. goesingense* shoots and lower levels of Ni-induced reactive oxygen species in roots. Salicylic acid (SA) was observed to both regulate glutathione accumulation through post-translational activation of SAT, and to increase glutathione reductase activity in *A. thaliana* (Freeman et al. 2005). Changes in the levels of these compounds and enzymes appear to be associated with Ni tolerance required for hyperaccumulation (For additional details about Ni tolerance in plants, see Chapter 9; for additional details about heavy metal chelation, see Chapter 10).

Overexpression of *T. goesingense* mitrochondrial SAT (TgSAT-m) in *A. thaliana* was found to confer increased Ni tolerance compared to controls (Freeman et al. 2004). These overexpressors also showed increased OAS, Cys and GSH biosynthesis (Freeman et al. 2004). However, *A. thaliana* SAT overexpressors and controls had the same shoot Ni content indicating that the SAT overexpressors neither hyperaccumulated nor excluded Ni, and chemical speciation showed that the Ni was not bound to thiols in these plants (Freeman et al. 2004). It is noteworthy that this demonstration of Ni tolerance without accumulation represents an example of the distinction between metal tolerance mechanisms and metal hyperaccumulation mechanisms and supports previous findings showing partial independent genetic control of hyperaccumulation and tolerance (Assunção et al. 2001; Bert et al. 2003; Macnair et al. 1999).

2.1 How do plants take up and transport metal?

The process of metal accumulation involves several steps, outlined in Fig. 1, one or more of which are enhanced in hyperaccumulators.

2.1.1 Solubilization of the metal from the soil matrix

Many metals are found in soil-insoluble forms. Plants use two methods to desorb metals from the soil matrix: acidification of the rhizosphere through the action of plasma membrane proton pumps and secretion of ligands capable of chelating the metal. Plants have evolved these processes to liberate essential metals from the

soil, but soils with high concentrations of toxic metals will release both essential and toxic metals to solution. To our knowledge, there are no reports of plants with the ability to solublize Pb from the soil matrix, where most of soil Pb exists in an insoluble form (Blaylock and Huang 2000). Experiments demonstrating Pb hyperaccumulation have used $Pb(NO_3)_2$, a soluble form of Pb, though it must be questioned whether this is the most appropriate form of Pb for analysis. Aside from Pb, the solubilization mechanisms for hyperaccumulators are similar for metals discussed, and therefore will not be addressed independently for each metal. While no hyperaccumulators have evolved to handle high concentrations of toxic metals if they are present in solution, phytoremediator plants could be modified to solubilize contaminants that are bound to the soil.

2.1.2 Uptake into the root

Soluble metals can enter into the root symplast by crossing the plasma membrane of the root endodermal cells or they can enter the root apoplast through the space between cells (Fig. 1). While it is possible for solutes to travel up through the plant by apoplastic flow, the more efficient method of moving up the plant is through the vasculature of the plant, called the xylem. To enter the xylem, solutes must cross the Casparian strip, a waxy coating, which is impermeable to solutes, unless they pass through the cells of the endodermis (Fig. 1). Therefore, to enter the xylem, metals must cross a membrane, probably through the action of a membrane pump or channel. Most toxic metals are thought to cross these membranes through pumps and channels intended to transport essential elements. Excluder plants survive by enhancing specificity for the essential element or pumping the toxic metal back out of the plant (Hall 2002; Meharg and Macnair 1992a, 1992b).

2.1.3 Transport to the leaves

Once loaded into the xylem, the flow of the xylem sap will transport the metal to the leaves, where it must be loaded into the cells of the leaf, again crossing a membrane (Fig. 1). The cell types where the metals are deposited vary between hyperaccumulator species. For example, *T. caerulescens* was found to have more Zn in its epidermis than in its mesophyll (Kupper et al. 1999), while *A. halleri* preferentially accumulates its Zn in its mesophyll cells instead of its epidermal cells (Kupper et al. 2000).

2.1.4 Detoxification/Chelation

At any point along the pathway, the metal could be converted to a less toxic form through chemical conversion or by complexation. Various oxidation states of toxic elements have very different uptake, transport, sequestration or toxicity characteristics in plants. Chelation of toxins by endogenous plant compounds can have similar effects on all of these properties as well. As many chelators use thiol groups as ligands, the sulfur (S) biosynthetic pathways have been shown to be

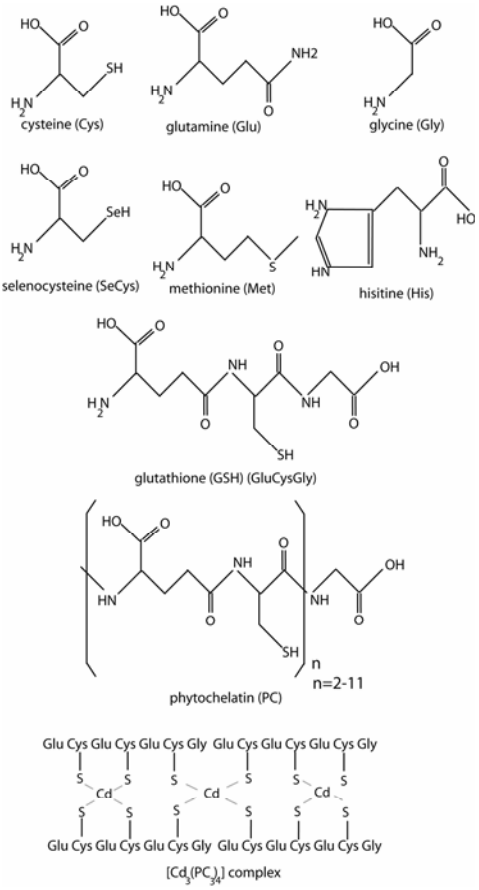


Fig. 2. Structures of some compounds that can bind metals: amino acids, glutathione, and phytochelatin.

Table 2. Summary of elements and the element-organic complexes that may be formed within plants.

Element	Analogue	Organic ligand
Arsenic	Phosphate	Phytochelatin, thiol, glutathione, ADP-As, ascorbic acid
Cadmium	Zn, Fe	Phytochelatin, glutathione, γ-glutamylcysteine, thiols
Chromium	Mn	Thiols
Copper	Cu	Citrate, metallothioniens, phytochelatin 2, phytochelatin 3
Mercury	Unknown[a]	Thiols
Nickel	Fe	Nicotianamine, histidine, thiols, citrate
Lead	Zn, Fe	Glutathione
Selenium	S	Cystiene, methionine, with and without methylation
Zinc	Zn	Phytochelatin, glutathione, γ-glutamylcysteine, thiols, citrate, malate

[a]Enters cell through passive diffusion.

critical for hyperaccumulator function (Ng and Anderson 1979; Pickering et al. 2003; Van Huysen et al. 2004) and for possible phytoremediation strategies (Figs. 2 and 3; Table 2). Oxidative stress is one of the most common effects of heavy metal accumulation in plants, and the increased anti-oxidant capabilities of hyperaccumulators allow tolerance of higher concentrations of metals (Freeman et al. 2004).

2.1.5 Sequestration/Volatilization

The final step for the accumulation of most metals is the sequestration of the metal away from any cellular processes it might disrupt. Sequestration usually occurs in the plant vacuole, where the metal/metal-ligand must be transported across the vacuolar membrane. Metals may also remain in the cell wall instead of crossing the plasma membrane into the cell, as the negative charge sites on the cell walls may interact with polyvalent cations (Wang and Evangelou 1994). Selenium may also be volatized through the stomata.

2.2 Strategies for phytoremediation using hyperaccumulators

The effectiveness of a phytoremediation plan is dependent on the selection of the appropriate plant or plants. Plants native to the target area should be considered since they are adapted to the local climate, insects, and diseases. Any plant used as a phytoremediator must be able to tolerate high concentrations of the toxic substance of interest, in addition to any other pollutants found at the particular site, as candidate sites for phytoremediation usually have multiple contaminants. In the United States, more than 80% of the metal contaminated Superfund, Department of Defense, and Department of Energy sites are also contaminated by organic pollutants (Ensley 2000).

Plants used for phytoextraction should develop a large amount of biomass quickly and be easy to cultivate and harvest, preferably multiple times per year (Newman 1997; Tong et al. 2004). For example, Mcgrath and Zhao (2003) have calculated that a plant with a bioconcentration factor of 40 can halve the concentration of metal in the top 20 cm of soil in 10 crops if it produces 5 tonnes ha^{-1} $crop^{-1}$, but a plant with a bioconcentration factor of 20 must produce at least 10 tonnes ha^{-1} $crop^{-1}$ to have the same effect. Since most metal hyperaccumualtors are small plants with low biomass, efforts are being made to locate new hyperaccumulators, selectively breed for promising plant traits, and create transgenic phytoremediators. For example, hyperaccumulation of arsenic in the fern *Pteris vittata* was recently discovered (Ma et al. 2001). Plant breeding approaches used to increase crop plant biomass and improved nutrient compositions can be potentially used to increase hyperaccumulator biomass.

Numerous research groups have emphasized transgenic solutions to obtaining appropriate plants for phytoremediation. The general strategy underlying this approach is to introduce genes conferring the ability to tolerate and hyperaccumulate

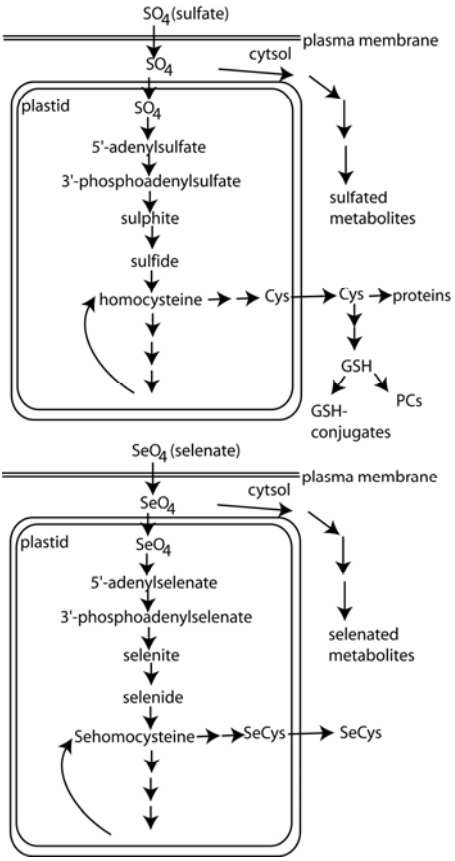


Fig. 3. Sulfur and selenium assimilation pathways in plants

toxic metals into larger plants capable of rapid development (reviewed in Salt et al. 1998; Clemens et al. 2002). Ideally, the genes could be introduced into plants which are accustomed to growing in the climate where they will be used. Plants could also be engineered to hyperaccumulate multiple different metals for sites with numerous contaminants. Existing hyperaccumulators could be engineered with increased biomass and metal storage capacity. Many studies have begun to elucidate the molecular mechanisms underlying metal hyperaccumulation and tolerance in plants (Ellis et al. 2004; Freeman et al. 2004; Pence et al. 2000). While it is possible that these discoveries mark the approaching widespread implementation of transgenic hyperaccumulators at phytoremediation sites, there is still much controversy surrounding the agricultural use of genetically modified organisms. Opposition to the deployment of transgenic plants in the field, especially in the promiscuous cruciferous species most closely related to the majority of hyperaccumulators, is potentially strong enough to prevent this type of phytoremediation technique from being widely used.

If it were possible to deploy genetically engineered phytoremediators, several issues must be considered before transgenes are introduced into the plants. Tissue-specific gene promoters that optimize transgene expression in tissues of interest must be tested for efficacy. The introduction of expression microarray techniques (Atgenex; Birnbaum et al. 2003) and activation tagged promoter lines (Alvarado et al. 2003) to profile gene expression in different tissues should accelerate this process. If transgenes from non-plant organisms were to be introduced, the proteins encoded by these transgenes must often be engineered to include signal sequences to correctly target the new proteins to the appropriate cellular compartment. Bizily et al. (2003) and Pilon et al. (2003) have recently shown that proper targeting of trangenically expressed proteins in phytoremediator plants can increase efficiency and remediation capacity.

3 Common elemental contaminants

Elements naturally occur in the earth's crust in a range of background levels that are generally below the critical load, i.e., the amount of the element above which there is a negative effect on biodiversity and ecosystem function (Table 1). However, the concentrations of elements in localized, naturally occurring metalliferous soils or in depositions from anthropogenic activity (e.g. mining, waste disposal, etc.) are considerably higher (Table 1). In the following section, plant mechanisms of tolerance and/or hyperaccumulation of common elemental contaminants are discussed.

3.1 Arsenic (As)

As is a naturally occurring metalloid, which has been used in pesticides and wood preservatives, leading to As contaminated sites (Meharg and Hartley-Whitaker 2002). For example, a Canberra, Australia suburb has As contamination from a pesticide spill (Ng et al. 1998), and localized soil contaminations resulting from use of As in pressure-treated lumber have been widely reported. In the alluvial planes of Bangladesh and West Bengal, India, As contamination of ground water from microbial degradation of peat has resulted in widespread well contamination and health risk. Irrigation has dispersed As contamination to surrounding soils, resulting in As poisoning of humans and other animals (McArthur et al. 2001). Similar contamination is seen in regions with As in subsoils worldwide.

Arsenite [AsO_2^- or As(III)] and arsenate [AsO_4^{-3} or As(V)] are the dominant inorganic arsenic moieties found in terrestrial plants. Both forms are phytotoxic, although via different mechanisms. Arsenate, the predominant form found in aerobic soils, is a phosphate analog. Formation of ADP-As complexes instead of ATP leads to cell death; arsenite can cause cell death by binding to and inhibiting enzymes with sulfhydryl groups. Arsenate is often designated as the more phytotoxic of the two arsenic species (Quaghebeur and Rengel 2003; Wang et al. 2002) but

the relative toxicities are species-specific (Wang et al. 2002a). As tolerant clones of the grass *H. lanatus* have a smaller proportion of their total As as arsenite compared to non-tolerant clones (Quaghebeur and Rengel 2003), while the As tolerant fern *Pteris vittata* (Chinese brake fern) almost exclusively accumulates arsenite in its fronds (Webb et al. 2003). While non-accumulators have a phytotoxic threshold at approximately 5-100 mg kg^{-1} As dry weight, *H. lanatus* can accumulate up to 560 mg kg^{-1} As (Porter 1975), and *P. vittata* can accumulate up to 27,000 mg kg^{-1} As dry weight, with phytotoxic symptoms appearing around 10,000 mg kg^{-1} As dry weight (Gumaelius et al. 2004). *Pteris cretica, Pteris longifolia*, and *Pteris umbrosa* are fern species that also hyperaccumulate As.

3.1.1 Uptake into the root

Pteris vittata accumulates As in contaminated and non-contaminated soils suggesting that hyperaccumulation is a constitutive trait (Wang et al. 2002). Arsenic accumulation is correlated with the phosphorus concentration of the media surrounding *P. vittata*. Wang et al. (2002) found that phosphate starvation resulted in a 2.5 fold increase in As net uptake, while the presence of phosphate in the media decreased arsenate influx. This increase in P/As uptake under starvation conditions is mediated by an increase in phosphate transporter gene expression and a consequent increase in the amount of protein (Liu et al. 1998; Muchhal and Raghothama 1999; Poynton et al. 2004) (For an in depth description of metal-dependent transcriptional regulation, see Chapter 12). Arsenite does not compete with phosphate for uptake into *P. vittata* roots, suggesting that there is another mechanism for arsenite uptake (Wang et al. 2002).

3.1.2 Transport to the leaves

In *Pteris vittata*, arsenite is more efficiently translocated from roots to fronds than arsenate (Wang et al. 2002). In *B. juncea,* addition of the dithiol As chelator dimercaptosuccinate to nutrient solution increased arsenite transport from roots to shoots (Pickering et al. 2000), but it is not yet clear whether arsenite is complexed before xylem loading and transport in *P. vittata* (Wang et al. 2002). Consistent with arsenate competition for phosphate transport sites, high phosphorus levels were shown to result in significantly decreased As concentrations in *P. vittata* fronds (Wang et al. 2002).

3.1.3 Detoxification/Chelation

A detoxification pathway for arsenate (AsO_4^{-3}) by conversion to arsenite (AsO_2^-) upon its uptake into roots has been proposed (Meharg and Hartley-Whitaker 2002). Arsenate can be reduced to arsenite enzymatically by arsenate reductase as shown *in vitro* (Delnomdedieu et al. 1994) and non-enzymatically by glutathione (GSH) or ascorbic acid as in yeast (Mukhopadhyay et al. 2000) followed by the formation of an arsenite-thiol (AsO_2^--SH) complex. Phytochelatins (PCs) have also been proposed as As chelators in *H. lanatus* (Raab et al. 2004), and it has

been suggested that arsenite-PC complexes are stored in the vacuole (Meharg and Hartley-Whitaker 2002). It does not appear that arsenite-PC complexes are the dominant form of arsenite in *P. vittata* as neither PC nor total S are present in sufficient quantities in *P. vittata* for the expected As:thiol ratio which is found in populations of As tolerant non-accumulators (Zhao et al. 2002, 2003). Therefore, if PCs are important for *Pteris* hyperaccumulation, they may function as a cytoplasmic shuttle and not a storage complex (Raab et al. 2004). Furthermore, X-ray spectroscopy showed that, at most, 20% of the As in the fronds is coordinated to S suggesting that most of the As stored in the vacuole is aqueous, but uncomplexed with thiols (Lombi et al. 2002b).

3.1.4 Sequestration

Arsenic species are thought to be sequestered in extra- or sub-cellular compartments in *P. vittata* to prevent interaction between As species and cellular components. X-ray spectroscopy detected the majority of As intracellularlly in the frond epidermal cells, probably in the vacuole (Lombi et al. 2002b). Arsenite-PC complexes in *H. lanatus* are most likely vacuolar (Meharg and Hartley-Whitaker 2002; Quaghebeur et al. 2003).

3.1.5 Phytoremediation of As

Various studies propose the use of *P. vittata* for phytoremediation of soil and water. Field experiments have indicated that *P. vittata* could remediate contaminated soil sites in 10 years or less (Salido et al. 2003) and can reduce arsenic levels in water to less than 10 μg L^{-1} (ppb) (Blaylock et al. 2001). In Albuquerque, New Mexico, a study was conducted where the ferns significantly decreased the level of arsenic in samples of the city's drinking water. While a study of the *Pteris* species could be beneficial in lending insight into hyperaccumulation mechanisms, using these plants for phytoremediation is not suggested. This is due to aforementioned evidence that the plants convert arsenate to arsenite. Although this conversion could make the arsenic less harmful for the plants, it is more harmful to animals and other organisms that might be exposed to the arsenite through plant contact. The ideal phytoremediator would accumulate arsenic at levels similar to *P. vittata*, but store it in a less toxic form. Arsenobetaine and arsenocholine, which are the major forms of arsenic found in fish (Lopez 2004), have low toxicity to humans and are readily excreted in urine. Transgenics with the ability to convert the inorganic forms of arsenic to these or similar compounds could be viable phytormediators.

Dhankar et al. (2002) were able to greatly increase the arsenic tolerance and accumulation of *Arabidopsis* with only two genes. Constitutive overexpression of γ-glutamylcysteine synthetase (γ-ECS) from the glutathione biosynthesis pathway coupled with the leaf specific expression of arsenate reductase (*arsC*) from *E. coli* increased the fresh weight of arsenate challenged plants by ~5-fold and the shoot accumulation ~3-fold. While these significant improvements were not enough to make *Arabidopsis* into a viable phytoremediator, this shows promise for adding

arsenic tolerance and extraction capabilities to other hyperaccumulator species (Dhankher et al. 2002).

3.2 Cadmium (Cd)

Cd is a toxic metal and probable carcinogen associated with Zn mining and industrial operations where Cd has been used to prevent corrosion of machinery. Resulting air-borne Cd dust presents a significant health hazard. Ecotypes of *T. caerulescens* accumulate a wide range of Cd levels. The Ganges and Vivez ecotypes can accumulate up to 10,000 mg kg^{-1} Cd dry weight and 12, 500 mg kg^{-1} Cd dry weight, respectively, without showing signs of toxicity; however, the Puy de Wolf and Prayon ecotypes only accumulate 2,300 mg kg^{-1} Cd dry weight and 4,800 mg kg^{-1} Cd dry weight, respectively (Lombi et al. 2000, 2001a, 2001b; Peer et al. 2003). Hyperaccumulation of Cd in *Arabidopsis hallerii* has also been reported (Cosio et al. 2004; Kupper et al. 2000). However, reports of hyperaccumulation of Cd in *B. juncea* are questionable, although some Cd accumulation in this species is evident (Salt et al. 1997).

3.2.1 Uptake into the root

Cd uptake is likely mediated through transporters or channels for other divalent ions (Cosio et al. 2004). Several of the Zn and Fe transporting ZIP genes in plants have been shown to transport Cd, although with a wide range of affinities (Grotz et al. 1998; Pence et al. 2000; Ramesh et al. 2003; Vert et al. 2001). Excess divalent cations in the media, such as Zn, can reduce Cd uptake in many plant species, including *T. caerulescens* Prayon (Lombi et al. 2002a, 2001a). Significantly, divalent cations and Ca channel blockers had no effect on the Cd uptake of *T. caerulescens* Ganges, suggesting that this ecotype may have developed a novel Cd uptake system (Lombi et al. 2001a, 2002a).

3.2.2 Transport to the leaves

Piñeros and Kochian (2003) demonstrated that *T. caerulescens* and *T. arvense* mesophyl cells exhibit different plasma membrane ion transport properties, but the differences cannot be directly linked to the differences in Zn and Cd accumulation. Analysis of Cd/Zn transport capacity in leaf mesophyll protoplasts demonstrated that the constitutive transport capacity and affinity for these metals were indistinguishable in *T. caerulescens* Ganges, *A. halleri*, and *T. caerulescens* Prayon; however, Cd accumulation increased in Ganges protoplasts but decreased in *A. halleri* protoplasts in conjunction with Cd pre-exposure (Cosio et al. 2004). Therefore, there may be multiple Cd transport systems in leaves. This suggests that in addition to its novel Cd root uptake pathway, Ganges has developed mechanisms in leaves to facilitate hyperaccumulation.

3.2.3 Detoxification/Chelation

Upon Cd exposure, *Nicotiana tabaccum* hairy roots had 5 times more reactive oxygen species (ROS) than *T. caerulescens* hairy roots (Boominathan and Doran 2003a). As GSH has been shown to act as an antioxidant in other species, it was hypothesized that increased GSH synthesis might account for increased tolerance in *T. caerulescens*. However, exposure to the GSH synthesis inhibitor buthionine sulfoximine (BSO) did not significantly affect ROS levels in *T. caerulescens* compared to controls, suggesting that GSH was not required for Cd tolerance in *T. caerulescens* (Boominathan and Doran 2003). *Thlaspi caerulescens* has constitutively high levels of antioxidant enzyme activity like catalase, 300-fold higher than *N. tabaccum* (Boominathan and Doran 2003), therefore, this may contribute to Cd tolerance. While Cd treatment does not induce phytochelatin (PC) synthesis in non-tolerant plants like *A. thaliana*, most metal tolerant plants do not accumulate phytochelatin-metal complexes in response to metal toxicity (Cobbett and Goldsbrough 2002). Although *T. caerulescens* and *T. arvense* had increased PCs following Cd treatment, total PCs were lower in the hyperaccumulator *T. caerulescens*, and PC levels did not correlate with increased tolerance in this plant (Ebbs et al. 2002) (For a detailed discussion of phytochelatins, see Chapter 10).

3.2.4 Sequestration

Few studies have addressed the sequestration of Cd. Vázques et al. (1992) found Cd in the apoplast and vacuoles of *T. caerulescens*, and most Cd in *T. caerulescens* hairy roots appears to be localized in the cell walls (Boominathan and Doran 2003). More recently, Cosio et al. (2005) demonstrated that ~35% Cd taken up accumulates in the cell wall/apoplast in *T. caerulescens* leaves.

3.2.5 Phytoremediation of Cd

Pilot studies of Cd and Zn phytoremediation have been attempted with contaminated soils collected from a Zn smelter site in Palmerton, PA (Brown et al. 1994), a Zn smelter in France, sewer sludge contaminated agricultural soil from the UK (Lombi et al. 2001b), and a site contaminated by mine tailings in Silver Bow Creek, MT (Ebbs et al. 1997). In each case, *T. caerulescens* removed Cd and Zn from the soils, but at rates that would require more than 15 years to remove most of the metals, and only from a narrow soil horizon. In the UK, agricultural soil, Cu toxicity limited the growth of *T. caerulescens*, demonstrating the need for phytoremediator plants that can tolerate toxic concentrations of multiple pollutants (Lombi et al. 2001b). The remediation potential of *T. caerulescens* is also limited by its small stature and biomass. In one study, even though *T. caerulescens* accumulated the highest concentrations of Cd and Zn in its leaves, *B. juncea* removed more Zn and equivalent amounts Cd due to its larger size (Ebbs et al. 1997). Transgenic approaches to either make *T. caerulescens* grow larger or to make *B. juncea* accumulate more Cd and Zn could make Cd phytoextraction feasible.

Brassica juncea plants genetically modified with bacterial genes to overproduce γ-glutamylcysteine synthetase (ECS) or glutathione synthetase (GS) were found to accumulate 1.5 times more Cd and Zn compared to wild type *B. juncea* growing on metal-contaminated soil from a USEPA Superfund site near Leadville, CO (Bennett et al. 2003). Both ECS and GS overexpressors were able to remove up to 25% of the soil Cd, and Bennet et al. (2003) predict that such transgenic plants should be 1.5 to 3 fold more efficient in phytoextraction than wild type plants.

Zhu et al. (1999) overexpressed the *E. coli* gene *gshI* (with a chloroplast targeting sequence) in *B. juncea*, which resulted in five times the ECS activity in the transgenic plants compared to wild type. Transgenic seedlings also had increased Cd tolerance, PCs, γ-glutamylcysteine (γ-GluCys), GSH, total non-protein thiols, and Cd accumulation (40-90%). These results indicate that ECS is important in Cd accumulation and tolerance and that overexpressing ECS could potentially be an effective phytoremediation strategy (Zhu et al. 1999).

Lee et al. (2003) overexpressed an *Arabidopsis* PC synthase (*AtPCS1*) in *Arabidopsis* resulting in 1.3 to 2.1-fold increase PCs; however, the transgenic lines were hypersensitive to Cd stress as measured by root growth and this hypersensitivity could be alleviated by the addition of glutathione. This suggests that the regulation of glutathione levels and perhaps the entire S assimilation pathway is important for Cd tolerance and accumulation. In a contrasting study, PC-deficient *Arabidopsis* (*cad1-3*) were transformed with wheat *TaPCS1* cDNA (Gong et al. 2003). While the Cd sensitivity of the transgenic *cad1-3* plants was complemented by *TaPCS1* expression, these *TaPCS1* expressing plants accumulated *less* Cd than the *cad1-3* mutants but increased Cd transport to leaves (Gong et al. 2003). Finally, Song et al. (2003) report that expression of the yeast vacuolar glutathione-Cd transporter YCF1 in *Arabidopsis* increased biomass and Cd uptake was ~2-fold greater than wild type. In addition, preliminary data suggest that expressing YCF1 in poplar also increases biomass (Song et al. 2003). Thus far, transgenic plants do not have sufficiently high levels of accumulation needed for significant Cd phytoremediation; however, a combined approach could result in an effective Cd phytoremediation technology.

3.3 Chromium (Cr)

Cr(III) is an essential nutrient for animals and is present in many oxidation states in the environment [Cr(II) to Cr(VI)], including the most common forms Cr(0), Cr(III), and Cr(VI). Cr(VI) and the steel component Cr(0) are usually byproducts of industrial processes. Cr(VI) is considered to be 1,000 times more toxic than Cr(III), and the World Health Agency and EPA has determined that Cr(IV) is a carcinogen. Cr(VI) contamination in the soil and groundwater has been detected in various parts of southern California and in Tennessee (EPA 2000; EPA 2004), where wells were closed because Cr(IV) levels exceeded EPA limits (Table 1). Areas in and around Glasgow, Scotland are also heavily contaminated by Cr used

for ore processing in the 19th century which subsequently leached into the ground water supply and accumulated at toxic levels (Farmer et al. 1999).

3.3.1 Phytoremediation of Cr

Although Cr(IV) is oxidized to the relatively non-hazardous Cr(III) in soil (ATSDR, 2001), Cr(IV), and Cr(VI) in groundwater and estuaries can pose health hazards and disrupt ecosystems. *Betula* and *Salix* trees are able to take up Cr, and therefore, would be useful for phytoremediation of Cr(IV) contaminated groundwater (Pulford et al. 2001) while Cr(VI) in estuaries can be absorbed by coconut husks and bagasse (Krishnani et al. 2004; Parimala et al. 2004). A recent report of *Salsola kali* (tumbleweed) accumulating Cr(VI) may prove to be useful for phytoremediation of Cr(VI) in the soil (Gardea-Torresdey et al. 2005).

3.4 Copper (Cu)

Cu is an essential element and enzyme co-factor for oxidases (cytochrome c oxidase, superoxide dismutase) and tyrosinases; however, animals and plants can accumulate toxic levels of Cu. Cu contamination for soil and groundwater usually results from mine sites like Blackbird Creek, ID (Mebane 1997) or munitions research or disposal sites like the Picatinny Arsenal, in northern New Jersey (EPA 1989). The Deer Lodge Valley and Anaconda areas of the Beaverell pedon in Montana, a region affected by long-term Cu smelting, has soil polluted with high levels of Cu and lower concentrations of As, Zn, and Pb (Burt et al. 2000, 2003). Cu contamination is also problematic in European soils exposed to historical mining and smelting activity. During the 19th century, the Devon Great Consols Mine in the Tamar Valley of Devon, UK, was the world's largest producer of Cu. As a result, Cu in the form of chalcopyrite has permeated the soil in this region (Lombi et al. 2004). In France, due to the continuous treatment of vine downy mildew with Bordeaux mix since the end of the 19th century, extensive deposits of Cu have accumulated in many vineyard soils (Brun et al. 2001). In Kayseri, Anatolia, high Cu concentrations were found in Zn-producing industrial sites (Aksoy et al. 2000), while a study in Denizli found that high levels of Cu characterize urban roadsides associated with road traffic (Celik et al. 2005). Cyprus (Kupros) has an extensive history of Cu mining extending back over 2,000 years. Cu mining wastes from this prolonged activity have had a significant impact on the environment and biota of this island (Pyatt 2001). Greece is also afflicted by Cu contamination: lake ecosystems, such as Lake Pamvotis in north-western Greece, exhibit high concentrations of this metal (Papagiannis et al. 2004), and Cu poisoning from high Cu content in local food has occurred in areas such as Veria county (Zantopoulos et al. 1999).

3.4.1 Phytoremediation of Cu

At superoptimal levels, Cu is highly toxic to plants and Cu ligands in plants are citrate, PC_2, PC_3, and metallothioniens (Murphy et al. 1999; Rauser 1999). Correspondingly, most Cu-tolerant plants are excluders, and no confirmed Cu accumulators have been identified. It was originally thought that *Elsholtzia splendens* was a Cu hyperaccumulator, but after further investigation it was concluded to be a tolerant excluder like *Elsholtzia argyi* (Jiang et al. 2004b), *Silene vulgaris* (Song et al. 2004) and *Mimiulus guttatus* (Harper et al. 1998). While 37 taxa of Cu hyperaccumulators have been reported, mainly from the Shaban Copper Arc of Congo, more research is needed to determine if the high Cu levels are due to hyperaccumulation or depositions of Cu dust on leaves (Song et al. 2004). *Salix nigra* was shown to accumulate more Cd and Cu than other *Salix* species, and field studies should determine the feasibility of this species for phytoremediation (Kuzovlina et al. 2004). In addition, soil amendments, like phosphate, increase Cu uptake, and therefore, may further phytoremediation efforts (Wu et al. 2004) (For a detailed discussion of Cu in plants, see Chapter 9). For aqueous Cu contamination, *Eichhornia crassipes*, the water hyacinth, is estimated to absorb 21.62 kg Cu ha^{-1}, and could potentially be used for phytoremediation of low level Cu contamination in waste water (Liao and Chang 2004).

3.5 Mercury (Hg)

Mercury is toxic to humans, and depending on the form it takes can cause severe neurological disorders (Carty and Malone 1979). Mercury exposure to humans is mostly through ingestion of fish, as Hg is biomagnified through the aquatic food chain, and amalgam dental fillings. Over the past century, several thousand tons of Hg have been released to the environment by human activity (Andren and Nriagu 1979). Many developing countries use elemental Hg-Au amalgamation mining practices, which results in significant Hg contamination of surrounding soil and water. Examples include artisanal Au mines in Suriname and the Amazon basin (Gray et al. 2002). Mercury in soil can be converted to cinnabar (HgS) as a result of sulfate reduction after the deposition and burial of mercury-contaminated soils. However, mercury release from solid forms, such as cinnabar, can also create environmental hazards. Studies of contaminated soil from industrial mercury dumping at the headwaters of East Fork Poplar Creek in Oak Ridge, TN, and in a Florida Everglades study indicate that organic matter could increase mercury mobilization from cinnabar and affect mercury bioavailability (Barnett et al. 1997; Ravichandran et al. 1998). These are candidate areas for phytoremediation.

The most toxic forms of mercury are organomercurials like methyl-Hg and phenyl mercuric acetate, followed by ionic Hg(II), with elemental Hg(0) as the least toxic form. Organomercurials and ionic Hg are toxic to plants, and to date Hg hyperaccumulating plants have not been identified. However, a Hg hyperaccumulating mushroom *Amanita muscaria* has been found that accumulates 96-1900 ng g^{-1} dry wt in the caps and 61-920 ng g^{-1} dry wt in the stalks depending on the col-

lection site (Falandysz et al. 2003). A recent study investigating Hg accumulation among *Salix* spp. found that the majority of the Hg is accumulated and retained in the cell wall of the roots and only 0.45-0.65% was translocated to the shoots (Wang and Greger 2004).

3.5.1 Phytoremediation of Hg

Instead of using plants to phytoextract mercury, several studies have focused on converting the organomercurials to Hg(0), which is volatile and is released into the atmosphere. The Meagher group at the University of Georgia has accomplished this transformation by expressing the bacterial genes *merB*, which encodes organomercury lyase (Bizily et al. 1999), and *merA*, encoding mercuric reductase in plants (Rugh et al. 1996). MerB severs the mercury-carbon bond and MerA reduces ionic mercury to elemental mercury. Transgenic poplar and cottonwood trees expressing *merA* and/or *merB* could be used as phytoremediators which do not require harvesting or replanting each season (Rugh et al. 1998; Che et al. 2004). In an elegant demonstration of the importance of proper subcellular targeting, Bizily et al. (2003) created ER and cell wall targeted versions of MerB. This appears to have targeted the MerB activity to the secretory pathway, which is thought to be the main location of hydrophobic organomercurials within the cells. Even though the plants produced tenfold or less targeted MerB than the untargeted MerB, Bizily et al. (2003) were able to identify lines that converted equivalent amounts of elemental Hg(0). Ruiz et al. (2003) were able to express *merA* and *merB* in chloroplasts which allows for high levels of protein production as well as other possible advantages. While these approaches show great promise from a scientific and technical perspective, there is a great deal of public resistance to a technology which volatilizes mercury, even if it is in a form that is 200 times less toxic than the form present in soil and water.

3.6 Nickel (Ni)

Ni is an essential element that can be toxic and possibly carcinogenic in high concentrations (ATSDR 2003). Ni toxicity in humans usually results from repeated occupational exposure resulting in dermatitis, asthma or headaches (Davies 1986; Akeeson and Skerfing 1985), but Ni contamination of soils is primarily restricted to regions surrounding Ni smelting operations such as Sudbury, Ontario, and Harare, Zimbabwe (Johnson and Hale 2004; Lupankwa et al. 2004). As, Cd, and Pb are often present in Ni mining and smelting wastes, and soil and water heavy metal concentrations from Ni mining operations can exceed governmental safety limits (Lupankwa et al. 2004). Serpentine and ultramafic soils are naturally occurring regions of high Ni concentrations characterized by unique Ni-tolerant flora. The majority of Ni hyperaccumulators have been collected from these soils.

Alyssum lesbiacum and *Thlaspi goesingense* are both Ni hyperaccumulating plants in the Brassicaceae family. In the genus *Alyssum* alone, 48 different speices have been discovered containing between 1,000 μg g^{-1} and 30,000 μg g^{-1} Ni in leaf

dry biomass (Baker and Brooks 1989; Kerkeb and Kramer 2003). *Thlaspi goesingense* has been reported to accumulate 9,490 mg Ni g^{-1} dry weight. (Freeman et al. 2004; Kramer et al. 1997; Reeves and Brooks 1983) (For a detailed description of Ni in plants, see Chapter 9). Ni phytoextraction using hyperaccumulators has been patented (Chaney et al. 1999).

3.6.1 Uptake into the root

Little is known about Ni uptake into roots. Evidence that histidine (His) chelates Ni suggests that His might assist root uptake of Ni. *Alyssum lesbiacum* has constitutively high free His levels, and when *Salmonella typhimurium* ATP phosphoribosyl transferase enzyme (StHisG) was expressed in *A. thaliana*, the His increased twofold and biomass increased 14-40-fold when grown on Ni (Wycisk et al. 2004). But, a comparison of the uptake mechanisms of *A. lesbiacum* and *B. juncea*, a non-accumulator, indicated that Ni and His are taken up independently, as His uptake inhibitors had no effect on the Ni uptake, and Ni was taken up as a free cation (Kerkeb and Kramer 2003). Furthermore, Salt et al. (1999) found that, while root exudation of histidine and citrate may help reduce Ni uptake for the nonaccumulator *T. arvense*, these exudates did not appear to be involved in the hyperaccumulation of Ni by *T. goesingense*.

3.6.2 Transport to the leaves

Ni and His loading into the xylem appear to be correlated (Kerkeb and Kramer 2003), and nicotianamine-Ni complexes have been shown to be transported from the roots to the shoots and across plant membranes in a manner similar to nicotianamine-Fe complexes (Becher et al. 2004; DiDonato et al. 2004; Koike et al. 2004; Vacchina et al. 2003; Weber et al. 2004).

3.6.3 Detoxification/Chelation

Nicotianamine is thought to be involved in Ni detoxification in *T. caerulescens* (Vacchina et al. 2003). Nicotianamine synthase (NAS) is constitutively expressed at high levels in both *T. caerulescens* and *A. halleri* which strongly suggests a role for nicotianamine in Ni/Zn hyperaccumulation (Becher et al. 2004; Vacchina et al. 2003; Weber et al. 2004). Kramer and coworkers found that much of the intracellular Ni of *T. goesingense* associated with citrate, and it is likely that citrate-Ni association is vacuolar (Kramer et al. 2000). His (or a His-like ligand) could facilitate shuttling Ni across the cytoplasm in *T. goesingense* (Kramer et al. 2000). Ni has a higher affinity for both nitrogen and oxygen ligands than S ligands, and the observed absence of Ni-S ligands indicates a lack of PC binding (Freeman et al. 2004; Kramer et al. 2000).

3.6.4 Sequestration

Ni is sequestered in multiple locations in *T. goesingese*. When cell walls from *T. arvense* and *T. goesingense* were incubated in levels of Ni normally toxic to *T. arvense* plants, more Ni was found bound to *T. goesingense* cell walls compared to *T. arvense,* suggesting cell wall modifications in *T. goesingense* might be conducive to enhanced Ni binding (Kramer et al. 2000). Reduced cell wall binding in *T. arvense* might alternatively be explained by pH changes resulting from exposure to a toxic concentration of Ni. Cell fractionation analyses also indicate that the majority of intracellular Ni is localized in the vacuole, and *T. goesingense* accumulates twice as much as *T. arvense* even though there was no observed difference in the vacuole sizes of the two species (Kramer et al. 2000; Salt and Persans 2000). Overexpression of *T. goesingense* metal-tolerance proteins (MTPs), members of the cation diffusion facilitator (CDF) family, conferred resistance to Ni, Cd, Co, and Zn in yeast (Persans et al. 2001). *TgMTP1* (*T. goesingense MTP1*) was expressed in yeast, and *in vivo* and *in vitro* staining with hemagglutinin (HA)-tagged TgMTP::1HA showed that TgMTP1 is localized to both the vacuole and the plasma membrane (Kim et al. 2004). Furthermore, TgMTP1 is constitutively expressed at high levels in *T. goesingense* (Persans et al. 2001). TgMTP1 may enhance vacuolar sequestration and act as a metal efflux pump at the plasma membrane (Kim et al. 2004). The CDF homologs *ZTP1* in *T. caerulescens* and *AhMTP1* in *A. halleri* are also constitutively expressed at elevated levels (Assunção et al. 2001; Becher et al. 2004).

3.6.5 Phytoremediation of Ni

Different species of *Alyssum* Ni hyperaccumulators have been evaluated for phytoremediation of mine sites (McGrath and Zhao 2003), and *Alyssum* hybrids have been bred with suitable traits for phytomining Ni on serpentine soils in Oregon and Washington (Chaney et al. 1999; Li et al. 2003b), and phytomining technology has been commercialized (Li et al. 2003a). Other metal hyperaccumulators are being investigated for their uses in the phytomining of Ni, thallium, and gold from soils (Anderson et al. 1999; Boominathan et al. 2004).

The overexpression of genes from different hyperaccumulators, like the MTPs, could elevate shoot Ni accumulation. In addition, overexpression of multiple MTP genes in conjunction with SAT metal tolerance genes could enhance both metal accumulation and tolerance, thereby improving Ni phytomining and phytoremediation technologies.

3.7 Lead (Pb)

Pb is an extremely toxic heavy metal which is a serious threat to the health of children and wildlife (EPA 2005a). The main sources of Pb poisoning include lead paint and old gasoline spills (PbBrCl, $2PbBrCl \cdot NH_4Cl$) resulting in dust and soil contamination of food and water (Xintaras 1992). Other areas with high Pb con-

centrations include Pb mines and smelters ($PbSO_4$, $PbO{\cdot}PbSO_4$, and PbS), such as the Leadington mine in Leadington, MO (Tom and Miles 1935), shooting ranges, and disposal sites for old batteries. A shooting range in Cortland, NY was estimated to have accumulated 500 tons of Pb after 30 years of use. The New York Department of Environmental Conservation found that the Pb concentrations at this site posed a health threat to people and wildlife, and the site received clean-up order from the EPA (Cape Cod Times 1997). College Grove, TN, has been identified as an area of concern due to Pb contamination from old battery cases on railroad property, with Pb levels ranging from 2,700 to 5,500 ppm (Chavez 1999).

Elemental Pb is insoluble and the most water soluble forms of Pb compounds are lead acetate (2 mg ml^{-1}), lead chloride (0.009 mg ml^{-1}), and lead nitrate (5 mg ml^{-1}) (Xintaras 1992). Atmospheric Pb mostly exists as $PbSO_4$ and $PbCO_3$. Although many plants may have a strategy of Pb exclusion as *Thlaspi praecox,* which hyperaccumulates Cd and Zn but excludes Pb (Vogel-Mikus et al. 2005), several plant species can hyperaccumulate soluble Pb in the soil. It has been reported that *Sesbania drummondii*, a leguminous shrub, and several *Brassica* species can accumulate significant amounts of Pb in their roots (Blaylock et al. 1997; Sahi et al. 2002; Wong et al. 2001), and *Piptathertan miliacetall*, a grass, accumulates Pb directly correlating to soil concentrations without symptoms of toxicity for 3 weeks (Garcia et al. 2004). Sahi et al. (2002) have noted that *S. drummondii* can tolerate Pb levels up to 1500mg L^{-1} and accumulate ~40g kg^{-1} shoot dry weight. *Brassica juncea* shows reduced growth at 645 ug g^{-1} Pb in the soil substrate, but can accumulate 34.5 g kg^{-1} shoot dry weight, although significant shoot accumulation is not observed until Pb reaches saturation levels in the roots. Most of the shoot accumulation was found in stems and not leaves suggesting that Pb is relatively insoluble (Kumar et al. 1995). Microanalysis spectra data through *S. drummondii* root sections show a decreasing gradient of Pb contents from the epidermis to the root central axis, and electron microscopy of *S. drummondii* roots revealed Pb deposition in the cell membrane and cell wall (Sahi et al. 2002).

3.7.1 Phytoremediation of Pb

The biggest challenge to effective phytoremediation of Pb is its extremely low solubility, as only ~0.1% of soil Pb is available for extraction (Huang et al. 1997). Efforts at phytoremediation of Pb have concentrated on using soil amendments like EDTA to increase the available Pb uptake (Blaylock et al. 1997; Huang et al. 1997; Wu et al. 1999). Addition of chelators does increase the solubility and uptake, but the amount Pb transferred to shoots is still low in comparison to the amount of Pb in the soil, and increases the likelihood that the mobilized Pb-EDTA will leach out of the soil and contaminate groundwater (Wu et al. 1999). The prospects for phytoremediation of Pb will depend on the development of novel systems for solublizing Pb and for transporting it to the leaves. Lastly, the expression of the glutahione-Cd vacuolar transporter YCF-1 in *Arabidopsis* has been found to increase the tolerance and slightly increases the accumulation of Pb (Song et al. 2003).

3.8 Selenium (Se)

Se is an essential element for animals, but so far has not been demonstrated essential for plants; mammalian Se-glutathione peroxidase protects against oxidative stress (Michiels et al. 1994), and Se also has anti-cancer/cancer preventative activities when present in compounds like methylselenocysteine (MeSeCys) (Ellis et al. 2004). Livestock in the southeastern United States with low-Se soils exhibit nutrition-related deformities, while livestock grazing on high-Se soils in Western states exhibit toxicity symptoms (Cosgrove 2001). Se naturally leaches from the soil, but Se becomes concentrated where leachates from highly irrigated soils or waste releases from petroleum refining accumulate to toxic levels in shallow groundwater regions or wetlands. Kesterson National Wildlife Refuge in California is a notable example of toxic Se accumulation that resulted in deformities in water fowl and other wildlife. In 1971 a manmade reservoir was built to catch agricultural run-off as part of a plan to conduct the run-off to the Pacific Ocean, but the conduit was never completed, and the Kesterson Reservoir became a dead-end holding area instead of a flow-through-regulating reservoir. In 1978, the reservoir began to receive drainage water, and by 1982, the ecological disaster was evident: the salt and pesticide accumulations were expected, but the Se accumulations were not, and Se remediation came to the forefront of public and scientific concern (US DOI 2004). Other Se remediation sites are the Stillwater Wildlife Management Area in Nevada, the Salton Sea irrigation area in California, the Middle Green River Basin Area in Utah, the Kendrick Reclamation Project in Wyoming, and the Gunnison/Grand Valley area in Colorado.

Se hyperaccumulators such as *Astragulus bisulcatus*, the two-grooved milkvetch, have been shown to accumulate Se up to 0.65% (w/w) (Pickering et al. 2003), and *B. juncea* accumulated 50 mg kg^{-1} dry mass in the field (Banuelos et al. 1997). *A. bisulcatus* accumulates high concentrations of Se-methylseleno-Cysteine (Se-MeSeCys) in young leaves, while mature leaves have predominately selenate and 40 to 60-fold less Se-MeSeCys. Seleno-Cys methyl transferase (SMT1), which catalyzes Se-MeSeCys from seleno-Cys (SeCys) and S-methyl-transferase, is present in leaves of all ages. This suggests that the synthesis of Se-MeSeCys in older leaves must be blocked at an earlier metabolic step and that mature leaves cannot reduce selenate (SeO_4^{-2}) to selenite (SeO_3^{-2}) (Pickering et al. 2003).

3.8.1 Uptake into the root

Shibagaki et al. (2002) have demonstrated that selenate is taken up through the sulfate transporter *Sultr1;2* (Fig. 3). *Arabidopsis* mutants in the sulfate transporter gene *Sultr1;2* were resistant to selenate, and *Sultr1;2* expression is localized in the root tip, root cortex, and lateral roots (Shibagaki et al. 2002).

3.8.2 Transport to the leaves

Biochemical forms of Se isolated from plants suggests that Se metabolism is similar to the S metabolic pathway and that Se analogs of S assimilated into S path-

ways (Leustek 2002), and selenate (SeO_4^{-2}), like sulfate (SO_4^{-2}), is transported to the chloroplasts after uptake into the roots (Ellis et al. 2003; Leustek 2002) (Fig. 3).

3.8.3 Detoxification/Chelation

Selenate (SeO_4^{-2}) and sulfate (SO_4^{-2}) metabolism in plants parallel selenate and sulfate accumulations in mature *A. bisulcatus* leaves and non-accumulators (Ellis et al. 2003; Pickering et al. 2003) (Fig. 3). While there is no direct evidence, selenate reduction in *A. bisulcatus* most likely occurs via the ATP sulfurylase/APS reductase pathway, as a Se-specific selenate reductase has not been identified.

Se non-accumulating species accumulate seleno-Methionine (SeMet) and Se-methylseleno-Met (Se-MeSeMet) (McCluskey et al. 1986; Virupaksha and Shrift 1965) while MeSeCys accumulates *A. bisulcatus*. If SeMet or Se-MeSeMet is incorporated into proteins, the seleno-protein is non-functional resulting in cellular toxicity. In contrast, *A. bisulcatus* forms MeSeCys from the methylation of SeCys by SMT (Wang et al. 1999). MeSeCys and proteins incorporating it are not toxic to the plant, and therefore, accumulate to high concentrations. Pickering et al. (2003) have suggested that mature leaves of *A. bisulcatus* could export Se-MeSeCys to younger tissues, and Se-MeSeCys is likely incorporated in seeds.

MeSeCys is an intermediate in the formation of dimethyl diselenide, a volatile form of Se. Dimethyl diselenide is the primary volatile of *A. bisulcatus*, the distinctive malodorous, signature smell of the plants (Pickering et al. 2003). Identification of all of the enzymes involved in the metabolic pathway of Se-MeSeCys in *A. bisulcatus* will clarify this pathway, and further elucidate the mechanisms whereby this plant establishes its hyperaccumulation capabilities.

3.8.4 Phytoremediation of Se

When ATP sulfurylase, an enzyme that reduces selenate to selenite, was overexpressed in *B. juncea*, Se accumulation in shoots was twofold greater and greater biomass than the Se hyperaccumulator *Stanleya pinnata* (Van Huysen et al. 2004) indicating that *B. juncea* ATP sulfurylase overexpressors have the potential to successfully phytoremediate Se contaminated sites. As *B. juncea* plants overexpressing ATP sulfurylase already have a bioconcentration factor of ~10, any improvement in accumulation or volatization could make these plants suitable for efficient phytoremediation.

Other studies have focused on overexpressing enzymes in Se metabolism. Overexpression of the mammalian selenocysteine lyase, which converts SeCys to elemental Se and alanine, in *Arabidopsis* slightly increases the amount of Se accumulated and slightly reduces the amount of Se incorporated into proteins (Pilon et al. 2003). Interestingly, cytosolic versions of selenocysteine lyase increased tolerance to Se while chloroplastic versions decreased tolerance, illustrating the importance of proper sub-cellular localization of novel proteins in transgenic plants. Two biochemical pathways can convert SeCys to a volatile compound, either to dimethyl selenide or dimethyl diselenide. Cystathionine-γ-synthase catalyzes Se-

Cys to dimethyl selenide, and overexpression of cystathionine-γ-synthase in *B. juncea* increased Se tolerance and enhanced Se volatization (Van Huysen et al. 2004). Overexpression of SMT from *A. bisulcatus* in *Arabidopsis* and *B. juncea* increased Se tolerance, accumulation of MeSeCys and volatization of Se (Ellis et al. 2004; LeDuc et al. 2004). These transgenic plants were more tolerant to selenite than to selenate, indicating that the reduction of selenate to selenite is limiting. Overexpression of ATP sulfurylase with selenocysteine lyase, cystathionine-γ-synthase or SMT could therefore have synergistic effects.

3.9 Zinc (Zn)

Zn is an essential microelement, but is toxic to animals and plants at high concentrations (Cobbett and Goldsbrough 2002; Gupta and Gupta 1998). The first Zn hyperaccumualtor identified was *T. caerulescens*. This plant was reported to accumulate between 25,000 and 30,000 μg g^{-1} total Zn before exhibiting symptoms of toxicity, although *T. caerulescens* can accumulate a maximum dry weight of 40,000 ug g^{-1} Zn in its shoots (Pence et al. 2000). *Arabidopsis halleri* has also been found to increase in its shoot Zn concentration from 300 ug g^{-1} dry wt at 1 μM Zn to 32 000 ug g^{-1} at 1000 μM Zn without phytototoxicity (Zhao et al. 2000). *Arabidopsis lyrata* ssp. Friedensville accumulates high leaf concentrations of Zn in the field (Cannon 1960), but exhibits variable accumulation in the axenic culture.

3.9.1 Uptake into the root

The ZIP family of proteins (ZRT/IRT-like proteins) transport Zn into the plants (Grotz et al. 1998; Ramesh et al. 2003). ZNT1 from *T. caerulescens* mediates low affinity Zn uptake as expected for a plant that grows on high concentrations of Zn (Pence et al. 2000). ZNT1 expression is higher in the hyperaccumulator *T. caerulescens* than in the non-accumulator *T. arvense*, possibly leading to a higher density of Zn transporters in the root-cell plasma membrane (Pence et al. 2000). This difference in transporter concentration could account for the observation that the hyperaccumulator and the nonaccumulator have the same affinity for Zn, but the hyperaccumulator has a higher rate of uptake (Lasat et al. 1996).

3.9.2 Transport to the leaves

Despite lower rates of uptake**,** the roots of *T. arvense* were found to accumulate substantially more Zn than in *T. caerulescens* (Lasat et al. 1996). This difference is likely due to better transport to the leaves in the hyperaccumulator. *T. caerulescens* had five times more xylem sap Zn (Lasat et al. 1998) and ten times more Zn was translocated to the shoots in *T. caerulescens* than in *T. arvense* (Lasat et al. 1996). The leaf cells of the hyperaccumulator are able to accumulate more Zn when leaf sections are subjected to high Zn (1mM) conditions (Lasat et al. 1998).

The molecular mechanisms of this increased uptake are unknown. Additional studies of leaf Zn and Cd uptake are included in Section 3.2.

3.9.3 Detoxification/Chelation

Mechanisms of Zn detoxification, chelation, and sequestration are species-specific. Zn was mostly found coordinated to malate in *A. halleri* leaves (Sarret et al. 2002). Although malate is the most common organic acid in *T. caerulescens* shoots (Tolra et al. 1996) no Zn-malate complexes were detected with X-ray absorption spectroscopy (Salt et al.1999). Instead, the predominant form of Zn in the roots was Zn-histidine with the remaining 30% bound to the cell wall. In the xylem sap, most of the Zn exists as the free hydrated Zn^{2+}cation with ~20% as Zn-citrate, while in the leaves, all four forms are found with citrate being the most common (For detailed description of heavy metal chelation, see Chapter 10).

3.9.4 Sequestration

Both *T. caerulescens* and *T. arvense* store similar amounts of Zn in their root apoplasts, indicating that cell wall compartmentation is not a tolerance mechanism. The higher concentration of Zn in the root vacuoles of the non-accumulator noted above suggests that root vacuole accumulation is a tolerance mechanism for non accumulators which lack a mechanism to transport to the leaves.

Leaf vacuoles are the primary site of Zn sequestration in *T. caerulescens* (Kupper et al. 1999). X-ray microanalysis of shoot tissue indicated that Zn is sequestered in the vacuoles of epidermal and sub-epidermal leaf cells in *T. caerulescens* (Frey et al. 2000; Vazquez et al. 1992), but in the mesophyll cells of *A. halleri* (Kupper et al. 2000).

3.9.5 Phytoremediation of Zn

See Section 3.2 (Cd) for discussion of Zn and Cd phytoremediators.

4 Future outlook

Phytoremediation is a technology with great potential. Phytoextraction using a combination of high-biomass with hyperaccumulator mechanisms will successfully remove heavy metal contaminants from the environment. The underlying mechanisms of hyperaccumulation can be applied to many different technologies. For example, trace metal deficiency could be reduced or eliminated in humans and animals if essential mineral nutrient levels were elevated in food crops (Gstoettner and Fisher 1995). Phytomining with hyperaccumulating plants selected to uptake high levels of valuable metals, such as gold and Ni, would eliminate the need for traditional mining technologies which have heavy metal contamination as a by-product.

Among the limitations of phytoremediation strategies has been that *in situ* remediation often takes many years to accomplish compared to traditional decontamination approaches to substantially restore a polluted area (Suresh and Ravishankar 2004; Schnoor et al. 1995, 1997). However, phytoremediation costs at least ten times less than traditional methods of excavation and removal (Schnoor 1997), and if an additional economic incentive were present (not only an environmental benefit) such as phytomining or forestry, then phytoremediation would be viewed as economically viable (Robinson et al. 2003).

Another limitation to consider is the availability of the contaminants in question to the plants. Schnoor et al. (1995) note that areas where contamination is less than 5 m in depth are the best suited sites for phytoremediation. Furthermore, the solubility of the contaminant in the soil determines whether phytoextraction is possible: in the case of metals, only metals found as free metal ions, soil soluble metal complexes, or metals adsorbed to inorganic soil constituents at ion exchange sites are readily available for uptake by the plants (Lasat et al. 2000). Metals that are bound to soil organic matter, precipitated (oxides, hydroxides, carbonates), or embedded in the structure of silicate minerals are not available to the plants. It has been suggested that phytoremediation is best suited for removing moderately hydrophobic pollutants such as BTEX compounds (benzene, toluene, ethylbenzene, and xylenes), chlorinated solvents, or nitrotoluene ammunition wastes; or excess nutrients (nitrate, ammonium, and phosphate) (Schnoor et al. 1995). The concentrations of soil pollutants should also be considered, as at some contaminated sites such as the Iron Mountain superfund site in Redding, CA, high levels of contaminants are toxic to plants and prevents successful phytoremediation (Schroder et al. 2002).

While many examples in both literature and patents propose the potential of different plants to remove soil heavy metals, McGrath and Zhao (2003) explain how the bioconcentration factor of many of these plants is not conducive to actual phytoremediation. The bioconcentration factor is the ratio of the plants shoot metal concentration to the soil metal concentration, which can be interpreted as the ability of a plant to take up the metal and transport it to its shoots. While most plants have a bioconcentration factor for heavy metals and metalloids of less than one (including many reported putative phytoremediators), a much greater value is required for phytoremediation. McGrath and Zhao note that even if one assumes a high biomass production of twenty tones per hectare per crop, a bioconcentration factor of greater than ten is required to reduce soil metals by half in less than ten crops. Ten tonnes per hectare per crop is possible for many agricultural crops, and the bioconcentration factor would need to be twenty or greater to reduce soil metals by half in less than ten crops. Many of these sites have been contaminated for more than ten years, as such a ten year remediation plan does not seem excessive. In general, the benefits and disadvantages of phytoremediation must be accessed for a particular project to determine whether this kind of remediation is the most appropriate for the task. Combining technologies described above (1) offer the greatest potential to efficiently phytoremediate contaminated sites.

References

(2000) Rapport de la commission au conseil et au parlement européen sur la mise en oeuvre de la législation communautaire en matière de déchets. Bruxelles, La Commission Au Conseil Et Au Parlement Européen. http://europa.eu.int/comm/environment/waste/reporting/fr.pdf

(2001) Former badlands bombing range restoration advisory board meeting minutes, Former Badlands Bombing Range Restoration Advisory Board. http://www.nwo.usace.army.mil/html/pm-hc/RAB_Minutes/29-mar-2001.htm

(2005) Sandia National Laboratories. http://www.sandia.gov/

Akeeson B, Skerfing S (1985) Exposure in welding high nickel alloy. Int Arch Occup Environ Health 56:111-117

Aksoy A, Sahün U, Duman F (2000) *Robinia pseudo-acacia* L. as a posssible biomonitor of heavy metal pollution in Kayseri. Turk J Bot 24:279-284

Alvarado MC, Zsigmond LM, Kovács I, Csépl A, Koncz C, Szabados LM (2003) Gene trapping with firefly luciferase in *Arabidopsis*:tagging of stress-responsive genes. Plant Physiol 134:18-27

Anderson CWN, Brooks RR, Chiarucci A, Lacoste CJ, Leblanc M, Robinson BH, Simcock R, Stewart RB (1999) Phytomining for nickel, thallium and gold. J Geochem Explor 67:407-415

Anderson TA, Guthrie EA, Walton BT (1993) Bioremediation. Environ Sci Technol 27:2630-2636

Andren AW, Nriagu JO (1979) The Global Cycle of Mercury. In: Nriagu J (ed) The Biogeochemistry of mercury in the environment. ElsevierBiomedical, Amsterdam, pp 1-21

Assunção A, Martins P, De Folter S, Vooijs R, Schat H, Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 24:217-226

ATSDR (2001) ToxFAQs™ for Chromium, Agency for Toxic Substances and Disease Registry. http://atsdr1.atsdr.cdc.gov/tfacts7.html

ATSDR (2003) ToxFAQs™ for Nickel; Agency for Toxic Substances and Disease Registry http://www.atsdr.cdc.gov/tfacts15.html

Atgenex Microarrays. http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm

Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metallic elements- a review of their distribution, ecology and phytochemistry. Biorecovery 1:81-126

Baker AJM, Whiting SN (2002) In search of the Holy Grail- a further step in understanding metal hyperaccumulation? New Phytol 155:1-4

Baker R, Simpson FS (1998) Cleanup Order Issued to Chrome Crankshaft. Sacramento, CA, California Environmental Protection Agency, Department of Toxic Substances Control:1-2, http://www.dtsc.ca.gov/SiteCleanup/Chrome_Crankshaft/NEWS_1998_Chrome_Crankshaft_Cleanup_Order_t-21-98.pdf

Bannick CG, Hahn J, Penning J (2002) Zur einheitlichen Ableitung von Schwermetallgrenzwerten bei Düngemitteln. Müll und Abfall 8:424-430

Banuelos GS, Ajwa HA, Terry N, Zayed A (1997) Phytoremediation of selenium laden soils:A new technology. J Soil Water Conserv 52:426-430

Barnett M, Harris L, Turner RR, Stevenson RJ, Henson TJ, Melton RC, Hoffman DP (1997) Formation of mercuric sulfide in soil. Environ Sci Technol 31:3037-3043

Becher M, Talke I, Krall L, Kramer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J 37:251-268

Bennett LE, Burkhead JL, Hale KL, Terry N, Pilon M, Pilon-Smits EA (2003) Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. J Environ Qual 32:432-40

Bert V, Meerts P, Saumitou-Laprade P, Salis P, Gruber W, Verbruggen N (2003) Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. Plant Soil 249:9-18

Berti WR, Cunningham SD (2000) Phytostabilization of metals. In: Raskin I, Ensley B (eds) Phytoremediation of toxic metals: Using plants to clean up the environment. Wiley Interscience, New York, pp 71-88

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the *Arabidopsis* root. Science 302:1956-60

Bizily SP, Kim T, Kandasamy MK, Meagher RB (2003) Subcellular targeting of methylmercury lyase enhances its specific activity for organic mercury detoxification in plants. Plant Physiol 131:463-471

Bizily SP, Rugh CL, Summers AO, Meagher RB (1999) Phytoremediation of methylmercury pollution:merB expression in *Arabidopsis thaliana* confers resistance to organomercurials. Proc Natl Acad Sci USA 96:808-813

Blaylock M, Huang J (2000) Phytoextraction of Metals. In: Raskin I, Ensley B (eds) Phytoremediation of toxic metals: Using plants to clean up the environment. Wiley Interscience, New York. pp 53-70

Blaylock M, Salt D, Dushenkov S, Zakharova O, Gussman C, Kapulnik Y, Ensley B, Raskin I (1997) Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. Environ Sci Technol 31:860-865

Blaylock MJ, Huang JW, Elless MP, Edenspace Systems Corporation (2001) Phytoremediation of Arsenic in Soil. Annual International Conference on Soils, Sediments and Waters

Boominathan R, Doran PM (2003) Cadmium tolerance and antioxidative defenses in hairy roots of the cadmium hyperaccumulator, *Thlaspi caerulescens*. Biotechnol Bioeng 83:158-167

Boominathan RR, Saha-Chaudhury NM, Sahajwalla V, Doran PM (2004) Production of nickel bio-ore from hyperaccumulator plant biomass: applications in phytomining. Biotechnol Bioeng 86:243-250

Bouwman LA, Bloem J, Romkens PFAM, Boon GT, Vangronsveld J (2001) Beneficial effects of the growth of metal tolerant grass on biological and chemical parameters in copper- and zinc-contaminated sandy soils. Minerva Biotechnologica 13:19-26

Bradshaw A (1997) Restoration of mined lands - using natural processes. Ecol Eng 8:255-269

Brooks RR, Lee J, Reeves RD, Jaffré T (1977) Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. J Geochem Explor 7:49-77

Brown S, Chaney R, Angle JS, Baker AJM (1994) Phytoremediation potential of *Thlaspi caerulescens* and bladder campion for zinc-contaminated and cadmium-contaminated soil. J Environ Qual 23:1151-1157

Brun LA, Maillet J, Hinsinger P, Pepin M (2001) Evaluation of copper availability to plants in copper-contaminated vineyard soils. Environ Pollut 111:293-302

Burt R, Wilson M, Mays MD, Keck TJ, Fillmore M, Rodman AW, Alexander EB, Hernandez L (2000) Trace and major elemental analysis applications in the US Cooperative Soil Survey program. Commun Soil Sci Plant Anal 31:1757-1771

Burt R, Wilson M, Mays MD, Lee CW (2003) Major and trace elements of selected pedons in the USA. J Environ Qual 32:2109-2121

Cannon H (1960) Botanical prospecting for ore deposits. Science 132:591-598Cape Cod Times (1997) http://www.capecodonline.com/base/bullets.htm

Carty A, Malone S (1979) The Chemistry of Mercury in Biological Systems. In: Nriagu J (ed) The Biogeochemistry of mercury in the environment. ElsevierBiomedical, Amsterdam, pp 433-479

Celik A, Kartal AA, Akdogan A, Kaka Y (2005) Determining the heavy metal pollution in Denizli (Turkey) by using *Robinio pseudo-acacia* L. Environ Int 31:105-112

Chaney RL, Angle JS Li YM, Baker AJM (1999) Method for phytomining of nickel, cobalt and other metals from soil. U.S. Patent No. 5944872 (continuation in-part of US Patent 5711784 issued Jan. 27, 1998)

Chavez K (1999) Lead contamination spurs public meeting. Tennessean. http://www.tennessean.com/sii/99/08/14/lead14.shtml

Che D, Meagher R, Heaton ACP, Lima A, Rugh CL, Merkle SA (2003) Expression of mercuric ion reductase in Eastern cottonwood (*Populus deltoides*) confers mercuric ion reduction and resistance. Plant Biotech J 1:311-319

Clemens S, Palmgren MG, Kramer U (2002) A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci 7:309-315

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. Annu Rev Plant Bio 53:159-182

Cosgrove J (2001) Selenium and Livestock Metabolism, Toxicity, and Deficiency. Cornell University, http://www.ansci.cornell.edu/plants/toxicagents/selenium/selenium.html

Cosio C, DeSantis L, Frey B, Diallo S, Keller C (2005) Distribution of cadmium in leaves of *Thlaspi caerulescens*. J Exp Bot doi:10.1093/jxb/eri062

Cosio C, Keller C (2004) Hyperaccumulation of cadmium and zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. Plant Physiol 134:716-725

Cosio C, Martinoia E, Keller C (2004) Hyperaccumulation of cadmium and zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. Plant Physiol 134:716-725

Davies J (1986). Occupational asthma caused by nickel salts. J Soc Occ Med 36:29-31

Davis LC, Vanderhoof S, Dana J, Selk K, Smith K, Goplen B, Erickson LE (1998) Movement of chlorinated solvents and other volatile organics through plants monitored by Fourier transform infrared (FT-IR) spectrometry. J Hazardous Subst Research 1:4-1, 4-26

Delnomdedieu M, Basti M, Otvos JD, Thomas DJ (1994) reduction and binding of arsenate and dimethylarsinate by glutathione - a magnetic-resonance study. Chem Biol Interact 90:139-155

Dhankher OP, Li Y, Rosen BP, Shi J, Salt D, Senecoff JF, Sashti NA, Meagher RB (2002) Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. Nat Biotechnol 20:1140-1145

DiDonato RL, Roberts LA, Sanderson T, Eisley RB, Walker EL (2004) *Arabidopsis* Yellow Stripe-Like2 (YSL2): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine-metal complexes. Plant J 39:403-414

Ebbs S, Lasat M, Brady DJ, Cornish J, Gordon R, Kochian LV (1997) Phytoextraction of cadmium and zinc from a contaminated soil. J Environ Qual 26:1424-1430

Ebbs S, Lau I, Ahner B, Kochian L (2002) Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescenes* (J. and C. Presl). Planta 214:635-640

Ellis DR, Salt DE (2003) Plants, selenium and human health. Curr Opin Plant Biol 6:273-279

Ellis DR, Sors TG, Brunk DG, Albrecht C, Orser C, Lahner B, Wood KV, Harris HH, Pickering IJ, Salt DE (2004) Production of Se-methylselenocysteine in transgenic plants expressing selenocysteine methyltransferase. BMC Plant Biol 4:1

Ensley B (2000) Rationale for use of phytoremediation. In: Raskin I, Ensley B (eds) Phytoremediation of toxic metals: Using plants to clean up the environment. Wiley Interscience, NewYork, pp 3-12

EPA (1989) EPA Superfund Record of Decision: Picatinny Arsenal (US Army). Rockaway Township, NJ, U.S. Environmental Protection Agency Superfund. http://www.epa.gov/superfund/sites/rods/fulltext/r0289093.pdf

EPA (2000) Chromium contamination in the San Fernando Valley (SFV), California Environmental Protection Agency, Los Angeles Regional Water Quality Control Board. http://www.swrcb.ca.gov/rwqcb4/html/water_qty/chromium_S1.html

EPA (2004) National priorities list for Smalley-Piper. Collierville, Tennessee, U.S. Environmental Protection Agency http://www.epa.gov/superfund/sites/npl/nar1727.htm

EPA (2005) Lead in paint, dust, and soil, U.S. Environmental Protection Agency. http://www.epa.gov/lead/

Ernst WHO (1996) Bioavailability of heavy metals and decontamination of soils by plants. Appl Geochem 11:163-167

Falandysz J, Lipka K, Kawano M, Brzostowski A, Dadej M, Jedrusiak A, Puzyn T (2003) Mercury content and its bioconcentration factors in wild mushrooms at Lukta and Morag, northeastern Poland. J Agric Food Chem 51:2832-2836

Farmer JG, Graham MC, Thomas RP, Licona-Manzur C, Paterson E, Campbell CD, Geelhoed JS, Lumsdon DG, Meeussen LCL, Roe MJ, Conner A, Fallick AE, Bewley RJF (1999) Assessment and modelling of the environmental chemistry and potential for remediative treatment of chromium-contaminated land. Environ Geochem Health 21:331-337

Freeman JL, Garcia D, Kim D, Hopf A, Salt DE (2005) Constitutively elevated salicylic acid signals glutathione mediated nickel tolerance in *Thlaspi* Nickel hyperaccumulators. Plant Physiol 137 (3), in press

Freeman JL, Persans MW, Nieman K, Albrecht C, Peer WA, Pickering IJ, Salt DE (2004) Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. Plant Cell 16:2176-2191

Frey B, Keller C, Zierold K, Schulin R (2000) Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 23:675-687

Gao S, Tanji K, Peters D, Lin Z, Terry N (2003) Selenium removal from irrigation drainage water flowing through constructed wetland cells with special attention to accumulation in sediments. Water Air Soil Pollut 144:263-284

Garcia G, Faz A, Cunha M (2004) Performance of *Piptatherum miliaceum* (Smilo grass) in edaphic Pb and Zn phytoremediation over a short growth period. Int Biodeter & Biodeg 54:245-250

Gardea-Torresdey JL, de la Rosa G, Peralta-Videa JR, Montes M, Cruz-Jimenez G, Cano-Aguilera I (2005) Differential uptake and transport of trivalent and hexavalent chromium by tumbleweed (*Salsola kali*). Arch Environ Contam Toxicol. PMID:15696348

Gong J, Lee D, Schroeder JI (2003) Long-distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*. Proc Natl Acad Sci USA 100:10118-10123

Gray JE, Labson VF, Weaver JN, Krabbenhoft DP (2002) Mercury and methylmercury contamination related to artisanal gold mining, Surinam. Geophys Res Lett 29:2105

Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. Proc Natl Acad Sci USA 95:7220-7224

Gstoettner EM, Fisher NS (1995) Accumulation of cadmium, chromium and zinc by the moss S*phagnum papillosum* Lindle. Water Air Soil Pollut 93:321-330

Gumaelius L, Lahner B, Salt DE, Banks JA (2004) Arsenic hyperaccumulation in gametophytes of *Pteris vittata*. A new model system for analysis of arsenic hyperaccumulation. Plant Physiol 136:3198-3208

Gupta UC, Gupta SC (1998) Trace element toxicity relationships to crop production and livestock and human health: Implications for management. Commun Soil Sci Plant Anal 29:1491-1522

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1-11

Hansen D, Duda PJ, Zayed A, Terry N (1998) Selenium removal by constructed wetlands: role of biological volatilization. Environ Sci Technol 32:591-597

Harper FA, Smith S, Macnair MR (1998) Can an increased copper requirement in copper-tolerant *Mimulus guttatus* explain the cost of tolerance? II. Reproductive phase. New Phytol 140:637-654

Huang J, Chen J, Berti W, Cunningham S (1997) Phytoremediation of lead-contaminated soils: Role of synthetic chelates in lead phytoextraction. Environ Sci Technol 31:800-805

Jiang LY, Yang XE, He ZL (2004) Growth response and phytoextraction of copper at different levels in soils by *Elsholtzia splendens*. Chemosphere 55:1179-1187

Johnson D, Hale B (2004) White birch (*Betula papyrifera* Marshall) foliar litter decomposition in relation to trace metal atmospheric inputs at metal-contaminated and uncontaminated sites near Sudbury, Ontario and Rouyn-Noranda, Quebec, Canada. Environ Pollut 127:65-72

Jones R, Sun W, Tang, CS, Robert, FM (2004) Phytoremediation of petroleum hydrocarbons in tropical coastal soils. II. Microbial response to plant roots and contaminant. Environ Sci Pollut Research 11:340-346

Kerkeb L, Kramer U (2003) The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. Plant Physiol 131:716-24

Kim D, Gustin J, Lahner B, Persans MW, Baek D, Yun DJ, Salt DE (2004) The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. Plant J 39:237-251

Kirk J, Klironomos J, Lee H, Trevors JT (2005) The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. Environ Pollut 133:455-465

Koike S, Inoue H, Mizuno, D, Takahashi, M, Nakanishi, H, Mori, S, Nishizawa, NK(2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant J 39:415-424

Kramer U, Pickering IJ, Prince RC, Raskin I, Salt DE (2000) Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. Plant Physiol 122:1343-1353

Kramer U, Smith RD, Wenzel WW, Raskin I, Salt DE (1997) The role of metal transport and tolerance in nickel nyperaccumulation by *Thlaspi goesingense* halacsy. Plant Physiol 115:1641-1650

Krishnani K, Parimala V, Meng, XG (2004) Detoxification of chromium(VI) in coastal water using lignocellulosic agricultural waste. Water SA 30:541-545

Kumar P, Dushenkov V, Motto H, Raskin I (1995) Phytoextraction - the use of plants to remove heavy-metals from soils. Environ Sci Technol 29:1232-1238

Kupper H, Lombi E, Zhao FJ, McGrath SP (2000) Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212:75-84

Kupper H, Zhao F, McGrath SP (1999) Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. Plant Physiol 119:305-311

Kuzovkina YA, Knee M, Quigley MF (2004) Cadmium and copper uptake and translocation in five willow (*Salix* L.) species. Int J Phytoremediation 6:269-287

Lasat MM, Baker A, Kochian LV (1996) Physiological characterization of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of *Thlaspi*. Plant Physiol 112:1715-1722

Lasat MM, Baker AJ, Kochian LV (1998) Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. Plant Physiol 118:875-883

Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV (2000) Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. J Exp Bot 51:71-79

LeDuc DL, Tarun AS, Montes-Bayon M, Meija J, Malit MF, Wu CP, AbdelSamie M, Chiang CY, Tagmount A, deSouza M, Neuhierl B, Bock A, Caruso J, Terry N (2004) Overexpression of selenocysteine methyltransferase in *Arabidopsis* and Indian mustard increases selenium tolerance and accumulation. Plant Physiol 135:377-383

Lee S, Moon J, Ko TS, Petros D, Goldsbrough PB, Korban SS (2003) Overexpression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. Plant Physiol 131:656-663

Leustek T (2002) Sulfate Metabolism. In: Somerville C, Meyerowitz E (eds) The Arabidopsis Book, American Society of Plant Biologists, Rockville, MD, pp 1-16

Li YM, Chaney RL, Brewer EP, Angle JS, Nelkin J (2003a) Phytoextraction of nickel and cobalt by hyperaccumulator *Alyssum* species grown on nickel-contaminated soils. Environ Sci Tech 37:1463-1468

Li Y, Chaney R, Brewer EP, Roseberg R, Angle JS, Baker A, Reeves R, Nelkin J (2003b) Development of a technology for commercial phytoextraction of nickel: economic and technical considerations. Plant Soil 249:107-115

Liao S, Chang N (2004) Heavy metal phytoremediation by water hyacinth at constructed wetlands in Taiwan. J Aquatic Plant Manag 42:60-68

Liu C, Muchhal U, Uthappa M, Kononowicz AK, Raghothama KG (1998) Tomato phosphate transporter genes are differentially regulated in plant tissues by phosphorus. Plant Physiol 116:91-99

Lombi E, Hamon RE, Wieshammer G, McLaughlin MJ, McGrath SP (2004) Assessment of the use of industrial by-products to remediate a copper- and arsenic-contaminated soil. J Environ Qual 33:902-910

Lombi E, Zhao FJ, Dunham SJ, McGrath SP (2000) Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. New Phytol 145:11-20

Lombi E, Tearall KL, Howarth JR, Zhao FJ, Hawesford MJ, McGrath SP (2002a) Influence of iron status on cadmium and zinc uptake by different ecotypes of the hyperaccumulator Thlaspi caerulescens. Plant Phyiol 128:1359-1367

Lombi E, Zhao F, Fuhrmann M, Ma LQ, McGrath SP (2002b) Arsenic distribution and speciation in the fronds of the hyperaccumulator *Pteris vittata*. New Phytol 156:195-203

Lombi E, Zhao F, McGrath S, Young S, Sacchi G (2001a) Physiological evidence for a high-affinity cadmium transporter highly expressed in a *Thlaspi caerulescens* ecotype. New Phytol 149:53-60

Lombi E, Zhao FJ, Dunham SJ, McGrath SP (2001b) Phytoremediation of heavy metal-contaminated soils: natural hyperaccumulation versus chemically enhanced phytoextraction. J Environ Qual 30:1919-1926

Lopez W (2004) Case Studies in Environmental Medicine- Arsenic Toxicity, Agency for Toxic Substances and Disease Registry

Lucero ME, Mueller W, Hubstenberger J, Phillips GC, O'Connell MA (1999) Tolerance to nitrogenous explosives and metabolism of TNT by cell suspensions of *Datura innoxia*. In Vitro Cell Dev Biol-Plant 35:480-486

Lupankwa K, Love D, Mapani, BS, Mseka, S (2004) Impact of a base metal slimes dam on water systems, Madziwa Mine, Zimbabwe. Physics Chem Earth 29:1145-1151

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED (2001) A fern that hyperaccumulates arsenic. Nature 409:579

Macnair M, Bert V, Huitson SB, Saumitou-Laprade P, Petit D (1999) Zinc tolerance and hyperaccumulation are genetically independent characters. Proc Royal Soc of London Series B-Biol Sci 266:2175-2179

Maier R (2004) Phytostabilization of mine tailings in the southwestern United States: plant-soil-microbe interactions and metal speciation dynamics, superfund project. Superfund Basic Research Program, University of Arizona

Marseille F, Tiffreau C, Laboudigue A, Lecomte P (2000) Impact of vegetation on the mobility and bioavailability of trace elements in a dredged sediment deposit: a greenhouse study. Agronomie 20:547–556

McArthur J, Ravenscroft P, Safiulla S, Thirlwall MF (2001) Arsenic in groundwater: Testing pollution mechanisms for sedimentary aquifers in Bangladesh. Water Resources Research 37:109-117

Mccluskey T, Scarf A, Anderson JW (1986) Enzyme catalyzed alpha,beta-elimination of selenocystathionine and selenocystine and their sulfur isologs by plant-extracts. Phytochemistry 25:2063-2068

McGrath SP, Zhao FJ (2003) Phytoextraction of metals and metalloids from contaminated soils. Curr Opin Biotechnol 14:277-282

Mebane C (1997) Use Attainability Analysis Blackbird Creek Lemhi County Idaho, Idaho Division of Environmental Quality, Water Quality Assessment and Standards Bureau and U.S. Environmental Protection Agency Region 10

Meharg A, Macnair M (1992a) Genetic correlation between arsenate tolerance and the rate of influx of arsenate and phosphate in *Holcus lanatus* L. Heredity 69:336-341

Meharg A, Macnair M (1992b) Suppression of the high-affinity phosphate-uptake system - a mechanism of arsenate tolerance in *Holcus lanatus* L. J Exp Botany 43:519-524

Meharg AA, Hartley-Whitaker J (2002) Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. New Phytol 154:29-43

Michiels C, Raes M, Toussaint O, Remacle J (1994) Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell-survival against oxidative stress." Free Rad Biol Medicine 17:235-248

Muchhal U, Raghothama K (1999) Transcriptional regulation of plant phosphate transporters. Proc Natl Acad Sci USA 96:5868-5872

Mukhopadhyay R, Shi J, Rosen BP (2000) Purification and characterization of Acr2p, the *Saccharomyces cerevisiae* arsenate reductase. J Biol Chem 275:21149-21157

Murphy AS, Eisinger WR, Shaff JE, Kochian LV, Taiz L (1999) Early copper-induced leakage of K(+) from *Arabidopsis* seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiol 121:1375-1382

Narayanan MT, Tracy JC, Davis LC, Erickson LE (1998) Modeling the fate of toluene in a chamber with alfalfa plants 2. Numerical results and comparison study. J of Hazardous Substance Research 1:5b-1 - 5b-28

Negri C, Hinchman R (1996) Plants that remove contaminants from the environment. Lab Med 27:36-40

Neumann PM, De Souza MP, Pickering IJ, Terry N (2003) Rapid microalgal metabolism of selenate to volatile dimethylselenide. Plant Cell Environ 26:897-905

Newman L, Strand S, Choe N, Duffy J, Ekuan G, Ruszaj M, Shurtleff B, Wilmoth J, Heilman P, Gordon M (1997) Uptake and biotransformation of trichloroethylene by hybrid poplars. Environ Sci Tech 31:1062-1067

Ng BH, Anderson JW (1979) Light-dependent incorporation of selenite and sulphite into selenocysteine and cysteine by isolated pea chloroplasts. Phytochemistry 18:573-580

Ng J, Kratzmann SM, Qi LX, Crawley H, Chiswell B, Moore MR (1998) Speciation and absolute bioavailability: risk assessment of arsenic-contaminated sites in a residential suburb in Canberra. Analyst 123:889-892

Papagiannis I, Kagalou I, Leonardos, J, Petridis, D, Kalfakakou, V (2004) Copper and zinc in four freshwater fish species from Lake Pamvotis (Greece). Environ Int 30:357-362

Parimala V, Krishnani K, Gupta, BP, Jayanthi, M, Abraham, M (2004) Phytoremediation of chromium from seawater using five different products from coconut husk. Bull Environ Contam Tox 73:31-37

Peer WA, Mamoudian M, Lahner B, Reeves, RD, Murphy AS, Salt DE (2003) Identifying model metal hyperaccumulating plants: germplasm analysis of 20 Brassicaceae accessions from a wide geographic area. New Phytol 159:421-430

Pence NS, Larsen PB, Ebbs SD, Letham DL, Lasat MM, Garvin DF, Eide D, Kochian LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proc Natl Acad Sci USA 97:4956-4960

Persans M, Nieman K, Salt DE (2001) Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. Proc Natl Acad Sci USA 98:9995-10000

Pickering IJ, Prince RC, George MJ, Smith RD, George GN, Salt DE (2000) Reduction and coordination of arsenic in Indian mustard. Plant Physiol 122:1171-1177

Pickering IJ, Wright C, Bubner B, Ellis D, Persans MW, Yu EY, George GN, Prince RC, Salt DE (2003) Chemical form and distribution of selenium and sulfur in the selenium hyperaccumulator *Astragalus bisulcatus*. Plant Physiol 131:1460-1467

Pieper DH, Martins dos Santos VAP, Golyshin PN (2004) Genomic and mechanistic insights into the biodegredation of organic pollutants. Curr Opin Biotechnol 15:215-224

Pilon M, Owen JD, Garifullina GF, Kurihara T, Mihara H, Esaki N, Pilon-Smits EA (2003). Enhanced selenium tolerance and accumulation in transgenic Arabidopsis expressing a mouse selenocysteine lyase. Plant Physiol 131:1250-1257

Piñeros M, Kochian L (2003) Differences in whole-cell and single-channel ion currents across the plasma membrane of mesophyll cells from two closely related *Thlaspi* species. Plant Physiol 131:583-594

Pollard JA, Powell KD, Harper FA, Smith JAC (2002) The genetic basis of metal hyperaccumulation in plants. CRC Crit Rev Plant Sci 21:539-566

Porter EK, Peterson PJ (1975) Arsenic accumulation by plants on mine waste (United Kingdom). Sci Total Environ 4:365–371

Poynton CY, Huang JW, Blaylock M J, Kochian LV, Elless MP (2004) Mechanisms of arsenic hyperaccumulation in *Pteris* species: root As influx and translocation. Planta 219:1080-1088

Pulford I, Watson C, McGregor, SD (2001) Uptake of chromium by trees: Prospects for phytoremediation. Environ Geochem Health 23:307-311

Pyatt FB (2001) Copper and lead bioaccumulation by *Acacia retinoides* and *Eucalyptus torquata* in sites contaminated as a consequence of extensive ancient mining activities in Cyprus. Ecotoxicol Environ Saf 50:60-64

Quaghebeur M, Rengel Z (2003) The distribution of arsenate and arsenite in shoots and roots of *Holcus lanatus* is influenced by arsenic tolerance and arsenate and phosphate supply. Plant Physiol 132:1600-1609

Raab A, Feldmann J, Meharg AA (2004) The nature of arsenic-phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. Plant Physiol 134:1113-1122

Ramaswami A, Rubin E, Bonola S (2003) Non-significance of rhizosphere degradation during phytoremediation of MTBE. Int J Phytoremediation 5:315-31

Ramesh SA, Shin R, Eide DJ, Schachtman DP (2003) Differential metal selectivity and gene expression of two zinc transporters from rice. Plant Physiol 133:126-134

Rauser WE (1999) Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin, and metallothioneins. Cell Biochem Biophys 31:19-48

Ravichandran M, Aiken G, Reddy, MM, Ryan, JN (1998) Enhanced dissolution of cinnabar (mercuric sulfide) by dissolved organic matter isolated from the Florida Everglades. Environ Sci Tech 32:3305-3311

Reeves R, Brooks R (1983) European species of *Thlaspi* L (Cruciferae) as indicators of nickel and zinc. J Geochem Explor 18:275-283

Reeves RD (1992) The Hyperaccumulation of Ni by serpentine plants. In: Baker AJM et al. (eds) The Vegetation of Ultramafic (Serpentine) Soils. Intercept Ltd, Andover, Hampshire, UK, pp 253-277

Reeves RD, Baker AJM (2000) Metal-accumulating plants. In: Raskin I, Ensley, BD (eds) Phytoremediation of toxic metals: Using plants to clean up the environment. John Wiley & Sons, Inc, New York, pp 193-229

Robinson B, Fernandez J, Madejon P, Maranon T, Murillo JM, Green S, Clothier B (2003) Phytoextraction: an assessment of biogeochemical and economic viability. Plant Soil 249:117-125

Rubin E, Ramaswami A (2001) The potential for phytoremediation of MTBE. Water Res 35:1348-1353

Rugh CL, Senecoff JF, Meagher RB, Merkle SA (1998) Development of transgenic yellow poplar for mercury phytoremediation. Nat Biotechnol 16:925-928

Rugh CL, Wilde HD, Stack NM, Thompson DM, Summers AO, Meagher RB (1996) Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene. Proc Natl Acad Sci USA 93:3182-3187

Ruiz ON, Hussein HS, Terry N, Daniell H (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. Plant Physiol 132:1344-1352

Sachs J (1865) Handbuch der Experimental-Physiologie der Pflanzen. In: Hofmeister W (ed) Handbuch der Physiologischen Botanik. Engelmann, Leipzig, pp 153–154

Sahi SV, Bryant NL, Sharma NC, Singh SR (2002) Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii*. Environ Sci Technol 36:4676-4680

Salido AL, Hasty KL, Lim JM, Butcher DJ (2003) Phytoremediation of arsenic and lead in contaminated soil using Chinese brake ferns (*Pteris vittata*) and Indian mustard (*Brassica juncea*). Int J Phytoremediation 5:89-103

Salt DE, Persans MW (2000) Possible molecular mechanisms involved in nickel, zinc, and selenium hyperaccumulation in plants. Biotechnol Genet Eng Rev 17:389-413

Salt DE, Kramer U (2000) Mechanisms of metal hyperaccumulation in plants. In: Raskin I, Ensley B (eds) Phytoremediation of Toxic Metals. John Wiley and Sons Inc., New York, pp 231-246

Salt DE, Pickering IJ, Prince RC, Gleba D, Dushenkov S, Smith RD, Raskin I (1997) Metal accumulation by aquacultured seedlings of Indian mustard. Environ Sci Technol 31:1635-1644

Salt DE, Prince RC, Baker AJM, Raskin I, Pickering IJ (1999) Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. Environ Sci Tech 33:713-717

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu Rev Plant Physiol Plant Mol Biol 49:643-668

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse AS, Marcus MA, Manceau A (2002) Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiol 130:1815-1826

Schnoor J, Licht L, Mccutcheon S, Wolfe N, Carreira L (1995) Phytoremediation of organic and nutrient contaminants. Environ Sci Technol 29:A318-A323

Schnoor J (1997) Phytoremediation: ground water remediation technologies analysis center evaluation report TE-98-01, 37

Schroder P, Harvey PJ, Schwitzguebel JP (2002) Prospects for the phytoremediation of organic pollutants in Europe. Environ Sci Pollut Res Int 9:1-3

Shibagaki N, Rose A, McDermott JP, Fujiwara T, Hayashi H, YoneyamaT, Davies JP (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots. Plant J 29:475-486

Song J, Zhao FJ, Luo YM, McGrath SP, Zhang H (2004) Copper uptake by *Elsholtzia splendens* and *Silene vulgaris* and assessment of copper phytoavailability in contaminated soils. Environ Pollut 128:307-315

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y (2003) Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nat Biotechnol 21:914-919

Suresh B, Ravishankar G (2004) Phytoremediation - A novel and promising approach for environmental clean-up. Crit Rev Biotech 24:97-124

Thompson P, Ramer L, Guffey AP, Schnoor JL (1998) Decreased transpiration in poplar trees exposed to 2,4,6-trinitrotoluene. Environ Toxicol Chem 17:902-906

Tollsten L, Muller P (1996) Volatile organic compounds emitted from beech leaves. Phytochem 43:759-762

Tolra R, Poschenrieder C, Barcelo J (1996) Zinc hyperaccumulation in *Thlaspi caerulescens*. 2. Influence on organic acids. J Plant Nutr 19:1541-1550

Tom J, Miles AM (1935) Brief authentic history of St. Francois County, Missouri. In: Tom J, Miles AM (eds) The Farmington News, Farmington, MO

Tong YP, Kneer R, Zhu YG (2004) Vacuolar compartmentalization: a second-generation approach to engineering plants for phytoremediation. Trends Plant Sci 9:7-9

Turgut C, Pepe KM, Cutright TJ (2004) The effect of EDTA and citric acid on phytoremediation of Cd, Cr, and Ni from soil using *Helianthus annuus*. Environ Pollution 131:147-154

US DOI (2004) Review of the Department of the Interior's National Irrigation Water Quality Program: Planning and Remediation, U.S. Department of the Interior, Bureau of Reclamation Committee on Planning and Remediation for Irrigation-Induced Water Quality Problems Water Science and Technology Board, Commission on Geosciences Environment, and Resources, National Research Council.http://www.usbr.gov/niwqp/Bibliography/niwqp.abs/html%20abs/Committee1.htm

Vacchina V, Mari S, Czernic P, Marques L, Pianelli K, Schaumloffel D, Lebru, M, Lobinski R (2003) Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray MS/MS assisted by cloning using yeast complementation. Anal Chem 75:2740-2745

Van Huysen T, Terry N, Pilon-Smits EAH (2004) Exploring the selenium phytoremediation potential of transgenic Indian Mustard overexpressing ATP sulfurylase or cystathionine-γ-synthase. Int J Phytoremediation 6:1-8

Vazquez M, Barcelo J, Poschenrieder C, Madico J, Hatton P, Baker AJM, Cope GH (1992) Localization of zinc and cadmium in *Thlaspi caerulescens* (Brassicaceae), a metallophyte that can hyperaccumulate both metals. J Plant Physiol 140:350-355

Vert G, Briat JF, Curie C (2001) *Arabidopsis IRT2* gene encodes a root-periphery iron transporter. Plant J 26:181-189

Virupaksha TK, Shrift A (1965) Biochemical differences between selenium accumulator and non-accumulator *Astragalus* species. Biochim Biophys Acta 107:69-80

Vogel-Mikus K, Drobne D, Regvar M (2005) Zn, Cd and Pb accumulation and arbuscular mycorrhizal colonisation of pennycress *Thlaspi praecox* Wulf. (Brassicaceae) from the vicinity of a lead mine and smelter in Slovenia. Environ Pollut 133:233-242

Wang J, Evangelou VP (1994) Metal tolerance aspects of plant cell walls and vacuoles. In: Pessaraki M (ed) Handbook of Plant and Crop Phyiology. Marcel Dekker, Inc, New York, pp. 695-717

Wang J, Zhao F, Meharg AA, Raab A, Feldmann,J, McGrath SP (2002)Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. Plant Physiol 130:1552-1561

Wang Y, Bock A, Neuhierl B (1999) Acquisition of selenium tolerance by a selenium non-accumulating *Astragalus* species via selection. Biofactors 9:3-10
Wang Y, Greger M (2004) Clonal differences in mercury tolerance, accumulation, and distribution in willow. J Eniron Qual 33(5):1779-1785
Webb SM, Gaillard JF, Ma LQ, Tu C (2003) XAS speciation of arsenic in a hyper-accumulating fern. Environ Sci Technol 37:754-760
Wong JWC, Lai KM, Su DS, Fang M (2001) Availability of heavymetals for *Brassica chinensis* grown in an acidic loamy soil amended with a domestic and an industrial sewage sludge. Water Air Soil Pollut 128:339-353
Wu J, Hsu F, Cunningham S (1999) Chelate-assisted Pb phytoextraction: Pb availability, uptake, and translocation constraints. Environ Sci Tech 33:1898-1904
Wu L, Li H, Luo YM, Christie P (2004) Nutrients can enhance phytoremediation of copper-polluted soil by Indian mustard. Environ Geochem Health 26:331-335
Wycisk K, Kim EJ, Schroeder JL, Kramer U (2004) Enhancing the first enzymatic step in the histidine biosynthesis pathway increases the free histidine pool and nickel tolerance in *Arabidopsis thaliana*. FEBS Lett 578:128-134
Xintaras C (1992) Analysis paper: Impact of lead-contaminated soil on public health. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry. http://www.atsdr.cdc.gov/cxlead.html.
Yoon JM, Oh B-T, Just CL, Schnoor JL (2002) Uptake and leaching of octahydro-1,3,5,7-tetranitro-1,3,5,7- tetrazocine by hybrid poplar trees. Environ Sci Tech 36:4649-4655
Zantopoulos N, Antoniou V, Nikolaidis E (1999) Copper, zinc, cadmium, and lead in sheep grazing in North Greece. Bull Environ Cont Tox 62:691-699
Zhao F, Dunham SJ, McGrath SP (2002) Arsenic hyperaccumulation by different fern species. New Phytol 156:27-31
Zhao F, Lombi E, Breedon T, McGrath SP (2000) Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. Plant Cell Envirion 23:507-514
Zhao F, Wang J, Barker JHA, Schat H, Bleeker PM, McGrath SP (2003) The role of phytochelatins in arsenic tolerance in the hyperaccumulator *Pteris vittata*. New Phytol 159:403-410
Zhu YL, Pilon-Smits EA, Tarun AS, Weber SU, Jouanin L, Terry N (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. Plant Physiol 121:1169-1178

List of abbreviations

EDTA, CDTA, DTPA, EGTA, EDDHA and NTA, metal chelators;
TCE: trichloroethylene
TNT: 2,4,6-trinitrotoluene
RDX: hexahydro-1,3,5-trinitro-1,3,5 triazine
HMX: octahydro-1,3,5,7-tetranitro-1,3,5,7 tetrazocine
MTBE: methyl tertiary-butyl ether
BTEX compounds:benzene, toluene, ethylbenzene, and xylenes; organic pollutants;
ROS: reactive oxygen species

BSO: buthionine sulfoximine
SA: salicylic acid
MTPs: metal-tolerance proteins
CDF: cation diffusion facilitator
SAT: serine acetyltransferase
γ-ECS: γ-glutamylcysteine synthetase
ECS: γ-glutamylcysteine synthetase
GS: glutathione synthetase
γ-GluCys: γ-glutamylcysteine
NAS: nicotianamine synthase
SMT1: seleno-Cys methyl transferase
PC: phytochelatin
Cys: cysteine
His: histidine
Met: methionine
GSH: glutathione
OAS: O-acetyl-L-serine
SeCys: seleno-Cys
SeMet: seleno-Methionine
MeSeCys: methylselenocysteine
Se-MeSeCys: Se-methylseleno-Cysteine
Se-MeSeMet: Se-methylseleno-Met

Baxter, Ivan R.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA

Freeman, John L.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA
Current address: Dept. of Biology, Colorado State University, Fort Collins, CO USA

Murphy, Angus S.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA

Peer, Wendy Ann
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA
peerw@purdue.edu

Richards, Elizabeth L.
Center for Phytoremediation, Purdue Univeristy, West Lafayette, IN 47907 USA

Family matters: gene regulation by metal-dependent transcription factors

Harm van Bakel and Cisca Wijmenga

Abstract

All organisms require trace amounts of metal ions, such as copper, iron, and zinc, since they form an essential component of a number of enzymes. In the past few years many metal-responsive transcriptional regulators have been identified in both prokaryotes and eukaryotes, which can be grouped in distinct families, based on their evolutionary and structural relationships. By regulating systems involved in metal uptake as well as metal efflux and sequestering, these transcription factors help to maintain a delicate balance between necessity and toxicity. Despite the structural similarities within the transcription factor families, individual members can have an affinity for different, and sometimes multiple, metal substrates. The recent availability of crystal structures for key members has led to a detailed understanding of the origins of metal specificity and the mechanisms of transcriptional activation for most of these transcription factor families.

1 Introduction

A number of metals are essential for life because they function as redox-active cofactors in enzymes (Table 1). However, the same properties that make these metals useful to cells can also lead to toxicity, since they can participate in reactions that generate highly reactive free radicals. In addition, some metal ions do not have a known physiological role and are toxic to cells at all concentrations; these include cadmium, lead, mercury, aluminium, and arsenic. Specialized systems have therefore evolved in prokaryotes and eukaryotes to ensure delivery of essential metals to their target sites, while minimizing exposure of sensitive cellular components to metal toxicity. These systems include transporters to import metal ions from the environment and protein chaperones to guide them to their destination. When metal-ion concentrations reach toxic levels, organisms can express a multitude of specialized detoxification systems. These include metal-specific efflux transporters; cytoplasmic or periplasmic carrier proteins and metal-sequestering systems such as glutathione, phytochelatins, and metallothioneins that bind multiple metal ions. An alternative mechanism for protection against metal toxicity in some prokaryotes uses reductases to convert toxic mercury ions to the metallic form, which subsequently leave the cell by diffusion (Schiering et al. 1991).

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_104 / Published online: 15 September 2005

Table 1. Essential metal requirements.

Element	Symbol	Described in	Examples of use
Copper	Cu	Archaea	Respiratory chain
		Bacteria	Iron metabolism; multicopper oxidases
		Eukaryotes	Cu/Zn Superoxide dismutase
Iron	Fe	Archaea	Respiratory chain
		Bacteria	Iron-sulfur cluster proteins
		Eukaryotes	Fe Superoxide dismutase
			Hemoglobin
			Methane monooxygenase
Zinc	Zn	Archaea	Zinc finger proteins
		Bacteria	Zinc hydrolases
		Eukaryotes	Cu/Zn Superoxide dismutase
			Alcolhol dehydrogenases
			Angiotensin-converting enzyme
Manganese	Mn	Bacteria	Serine-Tyrosine-Threonine phosphatases
		Eukaryotes	Mg Superoxide dismutase
Chromium	Cr	Eukaryotes (Human)	Insulin metabolism: chromodulin
Molybdenum	Mb	Bacteria	Sulfite oxidases
		Eukaryotes	Aldehyde oxidases
			Xantine dehydrogynases
			Bacterial nitrogenases
Selenium	Se	Eukaryotes	Glutathione peroxidase
			Thioredoxin reductase
			Formate dehydrogenase
			Selenophosphate synthase
Vanadium	V	Bacteria	Bacterial nitrogenases
		Fungi	Chloroperoxidases
Cobalt	Co	Bacteria	Vitamin B12
		Fungi	Electron carriers
			Hydrolysis enzymes
Nickel	Ni	Bacteria	Ureases
			NiFe hydrogenases
			CO dehydrogenases
			Acetyl-CoA decarboxylase/synthase
			methyl coenzyme M reductase
			Glyoxalases
			aci-reductone dioxygenase
			Ni-dependent superoxide dismutases
			Methylene diurease
Boron	Bo	Bacteria	Cell wall
		Eukaryotes	AI-2 cell communication

Homeostatic balance of metal metabolism is regulated at the protein level, for example, through rapid internalization and degradation of uptake proteins and re-localization of exporters to the plasma membrane upon metal exposure (Petris et al. 1996, 2003; Kim et al. 2004), and at the transcriptional level by controlling expression of genes involved in metal uptake or detoxification. Post-transcriptional

regulation at the level of mRNA stability is also known to play an important role in iron metabolism (Tang and Guest 1999; Dubrac and Touati 2000; Hentze et al. 2004; Puig et al. 2005). The efficiency of these homeostatic systems has been displayed by the observation that some organisms can restrict concentrations of free metal ions to less than 1 atom per cell (Rae et al. 1999; Outten and O'Halloran 2001; Changela et al. 2003).

Prokaryotes and eukaryotes possess transcription regulation systems that are responsive to environmental or intracellular metal concentrations. In many cases these responses are regulated at the level of transcription factors, which contain regulatory binding sites for their appropriate metal ions. Binding of the target metal to the transcription factor induces a conformational change that can either induce or repress activity. In addition, prokaryotic two-component systems exist where transcription factors are indirectly activated by sensor protein kinases when the latter is exposed to metals. Alternative regulation of metal homeostasis genes can occur through connections with other cellular pathways, including the oxidative stress response and metabolic pathways (Zheng et al. 1999).

The large number of metal-homeostasis transcription factors identified to date can be organized in a number of distinct families, according to their structural and evolutionary relationships. The crystal structures of many of the key members of these transcriptional regulator families, most notably from prokaryotes, have now been resolved and provide a wealth of information on how these families regulate gene transcription. Here, we attempt to give an overview of some of the major transcription factor families that have been identified in prokaryotes and eukaryotes, focusing on their regulatory mechanisms, metal specificity and their interactions in transcriptional regulatory networks.

2 Metal-responsive transcription factor families in prokaryotes

The majority of metal-responsive transcription factor families described in this review are found exclusively in the prokaryotic superkingdoms (Table 2). Transcription factors involved in copper and iron homeostasis and zinc uptake have also been described in eukaryotes, but are mainly confined to fungi (Table 3). The shared evolutionary origin and structural similarities within each prokaryotic transcription factor family do not necessarily mean that they bind identical metal ligands, instead individual members of these families can have an affinity for different, and sometimes multiple, metal substrates. The following sections provide an overview of the four main regulator families with the broad metal specificities that have been identified in prokaryotes to date, as well as a number of smaller families that primarily respond to a single metal.

Table 2. Prokaryotic metal-responsive transcription factors

Regulator	Metal	Organism	Targets	Description	References
Fur family					
Fur	Fe/Mn/Co	*Escherichia coli*	*fhuACDB*	Ferrichrome transport	Braun 2003
			sodB	Iron-dependent superoxide dismutase	Braun 2003
			acnA	Aconitase A	Braun 2003
				Metabolic pathways, Oxidative response, Acid shock response, Chemotaxis	Braun 2003
Fur	Fe	*Bacillus subtilis*	*fhuD*	Ferrichrome-binding protein	Schneider and Hantke 1993
Fur	Fe/Mn	*Yersinia pestis*	*yfeABCD*	Iron and manganese ABC transporter	Bearden and Perry 1999
Irr	Fe	*Bradyrhizobium japonicum*	*hemB*	Aminolevulinic acid dehydratse	Hamza et al. 1998
Zur	Zn	*Escherichia coli*	*znuABC*	High-affinity zinc uptake system	Patzer and Hantke 1998
Zur	Zn	*Bacillus subtilis*	*ycdHI yceA*	Zinc ABC transporter	Gaballa and Helmann 1998
PerR	Fe	*Bacillus subtilis*	*katA*	Catalase A	Bsat et al. 1998
DtxR family					
DtxR	Fe	*Corynebacterium diphtheriae*	*tox*	Diphtheria toxin	Boyd et al. 1990
			hmuO	Heme oxygenase	Schmitt 1997a; 1997b
IdeR	Fe	*Mycobacterium smegmatis*	*fxbA*	Putative formyl transferase	Dussurget et al. 1999
TroR	Zn/Mn	*Treponema pallidum*	*troABCD*	ABC transporter for manganese uptake	Posey et al. 1999
MntR	Mn	*Bacillus subtilus*	*mntH*	Manganese NRAMP transporter	Kehres et al. 2000; Que and Helmann 2000
			mntABCD	Manganese ABC transporter	Que and Helmann 2000
MntR	Mn	*Salmonella enterica*	*mntH*	Manganese NRAMP transporter	Kehres et al. 2000
			sitABCD	Manganese ABC transporter	Kehres et al. 2002
MntR	Mn/Fe	*Escherichia coli*	*mntH*	Manganese NRAMP transporter	Kehres et al. 2000
MerR family, metal-regulated					
MerR	Hg	*Pseudomonas aeruginosa*	*merR merTPAD*	Mercury resistance determinant	Lund and Brown 1989
ZntR	Zn	*Staphylocuccus aureus*	*zntA*	Zinc efflux pump	Singh et al. 1999
CoaR	Co	*Synechocystis Sp. PCC 6803*	*coaT*	Cobalt CPx-type ATPase efflux pump	Rutherford et al. 1999
CueR	Cu/Hg/Ag	*Escherichia coli*	*copA cueO*	Copper exporting ATPase	Stoyanov et al. 2001
PbrR	Pb	*Ralstonia metallidurans*	*pbrTR pbrABCD*	Lead resistance determinant	Borremans et al. 2001
ArsR/SmtB family					
ArsR/Ars	As/Sb/Bi	*Escherichia coli*	*arsRDABC*	Arsenic efflux system	Xu et al. 1996
SmtB	Zn	*Synechococcus PCC 7942*	*smtA*	Class II metallothionein	Huckle et al. 1993
NmtR	Ni/Co	*Mycobacterium tuberculosis*	*nmtA*	P1 type ATPase efflux pump	Singh et al. 1999
CrzA	Co/Zn	*Staphylococcus aureus*	*crzAB*	Resistance determinant for zinc	Busenlehner et al. 2002; Kuroda et al. 1999
ZiaR	Zn	*Synechocystis Sp. PCC*	*ziaA*	Zinc ATPase efflux pump	Thelwell et al. 1999

Regulator	Metal	Organism	Targets	Description	References
		6803			
CadC	Cd/Pb/Bi	*Staphylococcus aureus*	*cadCA*	Cadmium ATPase efflux pump	Endo and Silver 1995; Dell et al. 1994
CopY regulators					
CopY	Cu	*Enterococcus hirae*	*copYZAB*	P type ATPase copper transporters	Strausak and Solioz 1997
ModE regulators					
ModE	Mb	*Escherichia coli*	*modABCD modF*	Molybdenum ABC transporter	Grunden et al. 1996
			moaABCDE	Molybdenum cofactor biosynthesis	Rivers et al. 1993
			dmsABC	Mb-dependent DMSO reductase	McNicholas et al. 1996
			nap, nar, hyc	Molybdoenzymes	McNicholas and Gunsalus 2002
ModE	Mb	*Azotobacter vinelandii*	*modEABC modG*	Molybdenum ABC transporter	Mouncey et al. 1996
			anfA	Activator for alternative nitrogenases	Premakumar et al. 1998
MopA/B	Mb	*Rhodobacter capsulatus*	*mopAmodABCD*	Molybdenum ABC transporter	Kutsche et al. 1996; Masepohl and Klipp 1996
			anfA	Activator for alternative nitrogenases	Kutsche et al. 1996
NikR regulators					
NikR	Ni	*Escherichia coli*	*nikABCDE*	ABC transporter for nickel uptake	De Pina et al. 1999
NikR	Ni	*Helicobacter pylori*	*ureAB*	Urease involved in virulence	van Vliet et al. 2002
			nixA	Nickel importer of the hoxA family	Contreras et al. 2003
Two-component systems					
PcoRS	Cu	*Escherichia coli*	*pcoABCDE*	Copper efflux system	Tetaz and Luke 1983
CusRS	Cu	*Escherichia coli*	*cusC pcoE*	Provides increased copper resistance	Munson et al. 2000
CopRS	Cu	*Pseudomonas syringae*	*copABCD*	Copper resistance system	Bender and Cooksey 1987; Mills et al. 1993
ManRS	Mn	*Synechocystis Sp. PCC 6803*	*mntCAB*	Manganese ABC transporter	Ogawa et al. 2002
CzrSR	Zn/Cd	*Pseudomonas aeruginosa*	*czrCBA*	Resistance determinant for Zinc/Cadmium	Hassan et al. 1999
NrsRS	Ni	*Synechocystis Sp. PCC 6803*	*nrsBACD*	Resistance determinant for Nickel	Lopez-Maury et al. 2002

Table 3. Prokaryotic metal-responsive transcription factors

Regulator	Metal	Organism	Type	TargetGenes	References
Copper uptake regulators					
Mac1	Cu	*Saccharomyces cerevisiae*	Activator; metal-unbound	High-affinity copper transport; Ferric/cupric reductases	Jungmann et al. 1993
Cuf1	Cu	*Schizosaccharomyces pombe*	Activator; metal-unbound	High-affinity copper transport; Ferric/cupric reductases; High-affinity iron transport complex	Labbe et al. 1999
GRISEA	Cu	*Podospora anserina*	Activator; metal-unbound	Copper uptake; Oxidative stress response	Borghouts and Osiewacz 1998
Copper resistance regulators					
Ace1	Cu	*Saccharomyces cerevisiae*	Activator; metal-bound	Metallothioneins; Oxidative stress response	Thiele 1988
Crf1	Cu	*Yarrowia lipolytica*	Activator; metal-bound	Metallothioneins	Garcia et al. 2002
Amt1	Cu	*Candida glabrata*	Activator; metal-bound	Metallothioneins	Zhou and Thiele 1991
Crr1 regulator					
Crr1	Cu	*Chlamydomonas reinhardtii*	Unknown	Heme biosynthesis; Photosystem I	Quinn and Merchant 1995 Eriksson et al. 2004
Aft iron uptake regulators					
Aft1, Aft2	Fe	*Saccharomyces cerevisiae*	Activator; metal-bound	High-affinity iron transport complex; Copper chaperone; Siderophore iron uptake; Ferric/cupric reductases	Yamaguchi-Iwai et al. 1995 Yamaguchi-Iwai et al. 1996 Blaiseau et al. 2001 Rutherford et al. 2001
GATA-binding iron uptake regulators					
Fep1	Fe	*Schizosaccharomyces pombe*	Repressor; metal-bound	High-affinity iron transport complex; Siderophore iron uptake	Pelletier et al. 2002
SREA	Fe	*Aspergillus nidulans*	Repressor; metal-bound	Siderophore iron uptake; Siderophore biosynthesis	Haas et al. 1999
SRE	Fe	*Neurospora crassa*	Repressor; metal-bound	Unknown	Zhou et al. 1998
SreP	Fe	*Penicillium chrysogenum*	Repressor; metal-bound	Unknown	Haas et al. 1997
Urbs1	Fe	*Ustilago maydis*	Repressor; metal-bound	Siderophore biosynthesis	Voisard et al. 1993
MTF1 metal resistance regulators					
MTF1	Zn/Cu/Cd	*Mammals*	Activator; metal-bound	Metallothioneins; Oxidative stress response; Zinc transport	Westin and Schaffner 1988 Heuchel et al. 1994 Brugnera et al. 1994
MTF1	Cu/Cd/Zn	*Drosophila melanogaster*	Activator; metal-bound	Metallothioneins	Egli et al. 2003
MTF1	Zn	*Fugu rubripes*	Activator; metal-bound	Unknown	Auf der Maur et al. 1999
Zap1 zinc uptake regulator					
Zap1	Zn	*Saccharomyces cerevisiae*	Activator; metal-unbound	Zinc uptake system; Vacuolar zinc transporters; Phosphate/lipid metabolism; Other metabolism	Zhao and Eide 1997

2.1 MerR family

The MerR family is unique to prokaryotes and is not only involved in metal-dependent gene regulation, but also in responses to cellular stress resulting from exposure to free radicals or toxic compounds (O'Halloran and Walsh 1986; Amabile-Cuevas and Demple 1991; Ahmed et al. 1994; Noll et al. 1998) (Table 2). The MerR family derives its name from the metal-responsive MerR regulator, which controls the mercury resistance (*mer*) system in Gram-negative and Gram-positive bacteria. In *Pseudomonas aeruginosa*, this system consists of the *merR* regulator and the *merTPAD* resistance operon, which are divergently transcribed from a shared regulatory region containing the P_{merR} and $P_{merTPAD}$ promoters (Lund and Brown 1989) (Fig. 1). The resistance operon encodes a transporter (MerT), a periplasmic protein (MerP), the mercuric reductase (MerA) and the putative co-regulatory protein (MerD). MerR was found to drive the expression of the *merTPAD* genes in the presence of Hg(II), while repressing them when Hg(II) was absent (Lund et al. 1986; Lund and Brown 1989). Interestingly, both repression and activation occur with the regulator bound at the same location, between the –35 and –10 regions of the $P_{merTPAD}$ promoter. Binding of MerR to the $P_{merTPAD}$ promoter also results in a block of *merR* transcription, constituting a negative feedback loop that controls MerR levels (Lund and Brown 1989).

Subsequent studies have identified additional metal-responsive transcriptional regulators that activate gene transcription in similar ways, but respond to different metals, such as lead (PbrR), zinc (ZntR), copper (CueR) and cobalt (CoaR). All genes identified as targets of MerR-like regulators thus far function as resistance systems, which is understandable considering that transcriptional activation occurs upon binding of their ligands. Similar to *mer*, PbrR controls expression of the *Ralstonia metallidurans* CH34 lead resistance locus *pbr* by binding the combined O/P region of the divergently transcribed *pbrTR* and *pbrABCD* operons (Borremans et al. 2001). The *pbr* structural resistance genes are thought to encode a Pb(II) uptake protein (PbrT), a P-type efflux ATPase (PbrA), a membrane protein of unknown function (PbrB), a predicted prolipoprotein signal peptidase (PbrC) and a Pb(II) binding protein (PbrD) (Borremans et al. 2001). The cyanobacterial, cobalt-responsive CoaR controls expression of a much smaller cobalt resistance determinant, consisting only of the P-type cobalt efflux ATPase CoaA (Rutherford et al. 1999; Garcia-Dominguez et al. 2000). Unlike *merR*, *pbrR*, and *coaR* that are divergently transcribed from their target promoters, both *zntR* and *cueR* are located at some distance from their regulatory targets. ZntR was originally described in *Escherichia coli* and regulates *zntA*, encoding a zinc/cadmium/lead efflux ATPase (Brocklehurst et al. 1999). CueR likewise controls expression of the copper-exporting P-type ATPase CopA (Outten et al. 2000). Based on sequence similarity, many other putative metal-responsive MerR regulators can be identified in other organisms, which may extend the range of metals sensed by this family.

Several other MerR family members have been identified that are not regulated by metal ions. The *E. coli* SoxR regulator responds to oxidative stress by activating transcription of the *soxS* gene, which in turn activates expression of ~12 genes

Fig. 1. Structure of the MerR operator/promoter. The locations of the –10 and –35 regions, involved in recruitment of the RNA polymerase, are shown, respectively, in white and black for their associated *merR* and *merTPAD* operons. The grey box indicates the promoter area involved in MerR binding. Transcription start sites are marked by arrows.

in the SoxS regulon including Fur (Wu and Weiss 1991; Demple 1996) (Zheng et al. 1999). In addition, BltR, BmrR, and Mta regulate drug efflux transporters in *Bacillus subtilus* in response to inducers such as rhodamin and TTP and closely related substances (Ahmed et al. 1994, 1995). It is likely that MerR evolution started from a common DNA binding domain with a dimerization domain, which then acquired ligand-specific regulator domains through fusion with ligand-binding domains from existing proteins (Busenlehner et al. 2003). The metal-responsive MerR regulators are thought to constitute a subfamily that arose from a single ancestral protein. Different metal specificities then arose by mutations of coordinating residues in the metal-binding sites.

Structural and mutagenesis studies have revealed that MerR regulators bind to promoters as dimers and contain a homologous N-terminal DNA binding domain with a helix-turn-helix motif that is also found in many of the other transcription factor families discussed here. The N-terminal domain is connected to a ligand-specific C-terminal binding domain, involved in activation of gene expression (Shewchuk et al. 1989a). Sequence similarity in the C-terminal domain is far lower, reflecting the diversity in signals that individual MerR regulators can respond to.

2.1.1 MerR-dependent transcription initiation and deactivation

Besides sharing sequence similarity in the N-terminal domain, MerR regulators use a common structural mechanism for activation and repression of gene transcription. Initial insight into this mechanism came from the structure of the $P_{merT-PAD}$ promoter. Normal activation of transcription in prokaryotes requires correct spacing of the –35 and –10 promoter elements. It was found that while the typical spacer length between these regions is 17 bp (Lewin 2000), the –35 and –10 regions in the $P_{merTPAD}$ promoter are separated by a sub-optimal distance of 19 bp (Lund and Brown 1989). As a consequence, these regions are misaligned, which prevents a normal, open complex formation by RNA polymerase. Promoters of comparable sub-optimal length have also been found in O/P regions targeted by other members of the MerR family, including SoxR (Amabile-Cuevas and Demple 1991; Nunoshiba et al. 1992), BmrR (Ahmed et al. 1994), and other metal-responsive regulators such as ZntR and CueR (Brocklehurst et al. 1999; Outten et al. 2000; Petersen and Moller 2000; Stoyanov et al. 2001). Similar activation mechanisms have been shown for these regulators (Hidalgo and Demple 1997; Outten et al. 1999; Heldwein and Brennan 2001; Changela et al. 2003).

Increased understanding of the mechanism of MerR-induced gene expression has come from crystallographic studies. The first structure resolved was that of the *Bacillus subtilus* ligand-bound, multidrug resistance regulator BmrR, complexed to the *bmr* promoter region (Heldwein and Brennan 2001). The overall structure of a single BmrR monomer consists of three domains: an N-terminal DNA binding domain (residues 1-75), a linker with an 11-turn α-helix (residues 76-119) and a C-terminal drug binding domain (residues 120-278). The activated regulator-DNA complex points towards an activation mechanism that involves localized base-pair breaking and base sliding. The resulting promoter structure is twisted, compressed in the middle, and bent by ~50º away from the protein, changing the arrangements of the –10 and the –35 regions from opposite sides of the DNA helix to the same side, thus enabling recognition by the RNA polymerase II holoenzyme. A similar structure has been observed for MtaN, which lacks the Mta ligand binding domains and is constitutively active (Goosey et al. 2001). The compressed DNA structure is stabilized by interactions between tyrosine and lysine residues in BmrR and the phosphate backbone. These residues are highly conserved between other MerR family members, which provides strong evidence for similar activated DNA structures in their target promoters. A model for transcriptional regulation by MerR is given in Figure 2.

2.1.2 Determinants of metal specificity in MerR family members

The structures for two metal-responsive MerR family members from *E. coli*, CueR and ZntR, have also been resolved, both in their ligand-bound states (Changela et al. 2003). These regulators share the same topology as BmrR and MtaN, consisting of a dimerization domain flanked by a C-terminal DNA-binding domain and an N-terminal metal-binding domain. CueR activates transcription of the copper-exporting ATPase CopA in response to monovalent metals such as copper, silver, and gold in their reduced state, but not of divalent metals (Outten et al. 2000; Petersen and Moller 2000; Stoyanov et al. 2001; Stoyanov and Brown 2003). In contrast, the zinc resistance regulator ZntR responds to divalent metals such as zinc(II). The difference in specificity could be mediated by accessory factors that control metal loading, or by intrinsic properties of the metal-binding site itself. The distinct structural features of the metal-binding sites in CueR and ZntR suggest the latter (Changela et al. 2003). Copper(I) binds CueR in a linear S-Cu(I)-S center that is located in a solvent-inaccessible metal-binding loop at the dimer interface. Both coordinating ligands are supplied by conserved cysteine residues that form the end points of the 10-residue binding loop. The ZntR metal-binding site shows a similar loop structure, but contains additional cysteine and histidine residues that help to bind two zinc(II) ions. As +2 metal ions typically require higher coordination numbers, the availability of coordinating ligands is an important factor in metal-ion selectivity. The main determinant for mono- or divalent metal specificity that is involved in metal binding is Cys^{79} in ZntR, whereas the equivalent Ser^{77} in CueR does not possess coordination ligands. Additional selection for low-coordination number metals comes from sterical and hydrophobic restrictions at the CueR binding site. Based on these characteristics, it has now been possible

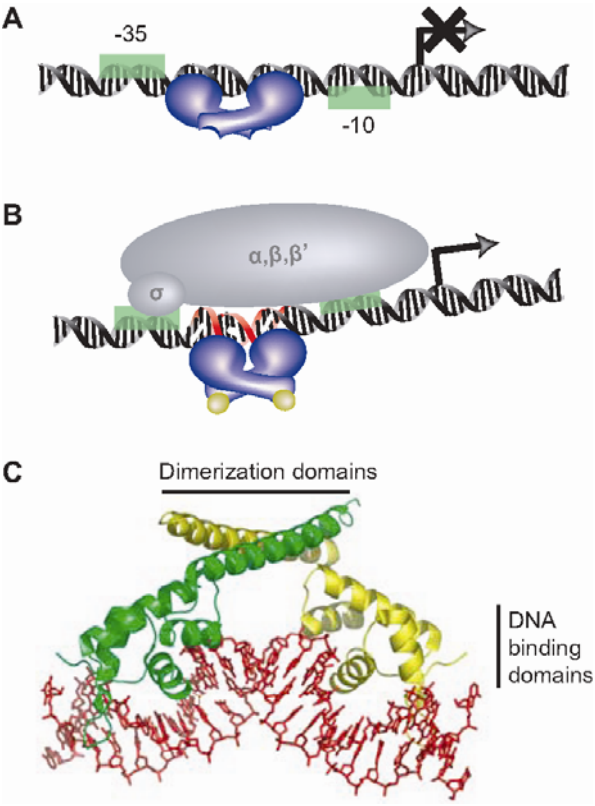


Fig. 2. Model of transcriptional regulation by MerR family members. (A) When the inactive form of MerR (blue) is bound to the promoter, incorrect alignment of the –10 and –35 regions prevents transcription initiation. (B) Upon ligand binding, MerR (blue) undergoes a conformational change, bending, twisting, and compressing the operator DNA to reposition the –10 and –35 regions. This then allows for formation of the open complex with RNA polymerase holoenzyme (grey) and initiation of transcription. (C) Structure of MtaN complexed with its target operator DNA, indicating the compressed and bent DNA helix (red). The individual chains in the dimer are shown in different colors and the DNA-binding domains and dimerization domains are indicated.

to assign putative metal-binding characteristics to novel metal-responsive MerR regulators by structural alignment with known regulators.

Much research has focused on unraveling the mechanism by which MerR family members activate gene expression, however, efficient deactivation is also vital to controlled transcription regulation. Based on the high affinity of MerR for Hg(II) (Ralston and O'Halloran 1990; Ansari et al. 1995), it is unlikely that release of Hg(II) plays an important role in returning DNA-bound MerR to a repressive state. A more likely mechanism involves the displacement of metal-bound MerR by unbound MerR, as it has been shown that the latter form has a higher affinity for DNA (Parkhill et al. 1993). Displacement of metal-bound MerR may also in-

volve additional factors such as MerD, which has been shown to be associated with the *mer* operator, and can repress transcription of the *mer* resistance operon (Nucifora et al. 1989; Mukhopadhyay et al. 1991). In a model proposed by (Champier et al. 2004), MerD could be involved in displacing Hg-bound MerR from the operator, allowing apo-MerR to repress transcription of the mercury resistance operon. The high metal affinity of other metal-responsive MerR family members such as ZntR and CueR (Outten and O'Halloran 2001; Changela et al. 2003) suggests that similar displacement mechanisms play a role in termination of their transcriptional signal, though no MerD-like genes have been reported that could mediate this process. An alternate mechanism is used by the oxidative stress sensor SoxR, which is inactivated by rapid reduction of its Fe-S cluster to the $[2Fe\text{-}2S]^{1+}$ state after termination of the oxidative stress (Ding and Demple 1997). A mutant screen in *E. coli* revealed two loci, the *rsxABCDE* operon and the *rseC* gene, that are thought to constitute the reducing system responsible for this inactivation (Koo et al. 2003).

2.2 Fur family

The founding member of the Fur family was discovered in *fur* mutants of *Salmonella typhimurium* and *Escherichia coli,* which showed constitutive expression of siderophore iron uptake systems and their outer membrane receptor proteins (Ernst et al. 1978; Hantke 1981) (Table 2). Siderophores are specific chelators for iron that are excreted by many bacteria to scavenge iron from the environment (Winkelmann 2002). Subsequent studies led to the identification of the responsible gene (Bagg and Neilands 1985; Schaffer et al. 1985) and its characterization as a transcriptional regulator (Hantke 1981, 1984; Escolar et al. 1997). In the presence of iron, Fe(II)-bound Fur binds a palindromic repeat in the promoter regions of iron-regulated genes and represses transcription (Bagg and Neilands 1987; de Lorenzo et al. 1987). In contrast, apo-Fur has low affinity for DNA and is displaced from the promoter in the absence of Fe(II), allowing access by RNA polymerase and initiation of transcription (Bagg and Neilands 1987).

In the past few years, the number of genes known to be regulated by Fur has increased to more than 90 in different *E. coli* strains (Hantke 2001) with similar numbers in other prokaryotes (Stojiljkovic et al. 1994; Baichoo et al. 2002; Wan et al. 2004), while new targets are still being discovered (Osorio et al. 2004). The majority of these genes encode proteins that are directly related to iron metabolism, but other target genes are involved in diverse cellular processes such as metabolic pathways (Hantke 1987), responses against oxidative stress (Niederhoffer et al. 1990) and acid shock (Hall and Foster 1996), and chemotaxis (Karjalainen et al. 1991). Fur also autoregulates its own expression (De Lorenzo et al. 1988). Despite its general function as repressor, several genes such as *sodB* (Niederhoffer et al. 1990) and aconitase A (Abdul-Tehrani et al. 1999) show increased expression in the presence of Fur. In the absence of evidence for direct interactions between Fur and promoter regions of these genes, it is difficult to say whether the majority of these effects are direct or indirect. However, it is now

clear that Fur regulates the iron-dependent superoxide dismutase SodB by positively affecting its mRNA half-life (Dubrac and Touati 2000). This Fur-dependent stabilization is thought to be controlled by a large palindromic sequence and an AU-rich region in the 5' untranslated part of the SodB mRNA.

The importance of iron to the bacterial cell is further emphasized by regulatory connections of metabolic pathways and the oxidative stress response to Fur (Fig. 3). The promoter region of *fur* contains a binding site for the cAMP-catabolite repressor protein, Crp, providing a link between carbon and iron metabolism (De Lorenzo et al. 1988). The oxidative stress regulator OxyR can also bind the *fur* promoter and activate gene expression in response to hydrogen peroxide (Zheng et al. 1999). A second connection to the oxidative stress response is provided by the SoxR and SoxS transcriptional regulators. In the presence of superoxide and nitric oxide, SoxR activates transcription of SoxS. In turn, SoxS binds the *fldA* O/P region, leading to induction of *fldA*, encoding a flavodoxin, and the downstream *fur* gene (Zheng et al. 1999). The link between oxidative stress and iron metabolism is understandable since deregulation of iron metabolism in Δ*fur* mutants results in oxidative stress and DNA damage due to iron overload (Touati et al. 1995).

2.2.1 Additional Fur family members

A second iron-dependent regulator, Irr, was found to regulate heme biosynthesis in *Bradyrhizobium japonicum* in response to iron availability (Hamza et al. 1998) (Table 2). Irr is closely related to Fur and shares 29% sequence identity with its nearest neighbor in *Pseudomonas aeruginosa* (Hamza et al. 1998). In contrast to Fur, Irr functions as a repressor when iron is scarce and targets *hemB,* encoding the protein that catalyzes the second step of heme biosynthesis. The levels of Irr itself are also regulated and when iron is sufficient, expression of *irr* is repressed at the transcriptional level by Fur (Hamza et al. 2000) (Fig. 3). Additional down-regulation in high-iron conditions occurs through increased protein degradation, mediated by iron-dependent heme binding to a regulatory motif in the Irr protein itself (Qi et al. 1999; Yang et al. 2004).

Other Fur family members have been identified that respond to different types of signaling (Table 2). The zinc uptake repressor Zur has been found in several bacteria, including *E. coli* (Patzer and Hantke 1998), *Bacillus subtilus* (Gaballa and Helmann 1998), *Listeria monocytogenes* (Dalet et al. 1999) and *Staphylococcus aureus* (Lindsay and Foster 2001), and shares significant sequence similarity with Fur (27% and 24% sequence identity in *E. coli* and *B. subtilus,* respectively). In *E. coli*, Zur regulates the divergently transcribed *znuA* and *znuBC* genes, which encode an ATP-binding cassette (ABC) zinc permease involved in zinc uptake (Patzer and Hantke 1998) (Fig. 3). In the presence of zinc, Zur binds the combined operator/promoter (O/P) region and represses transcription (Patzer and Hantke 2000). Similar ABC permease operons have been identified as Zur targets in other prokaryotes, together with an additional *yciC* locus in *B. subtilus*, which may be part of a low-affinity Zn(II) uptake system (Gaballa and Helmann 1998).

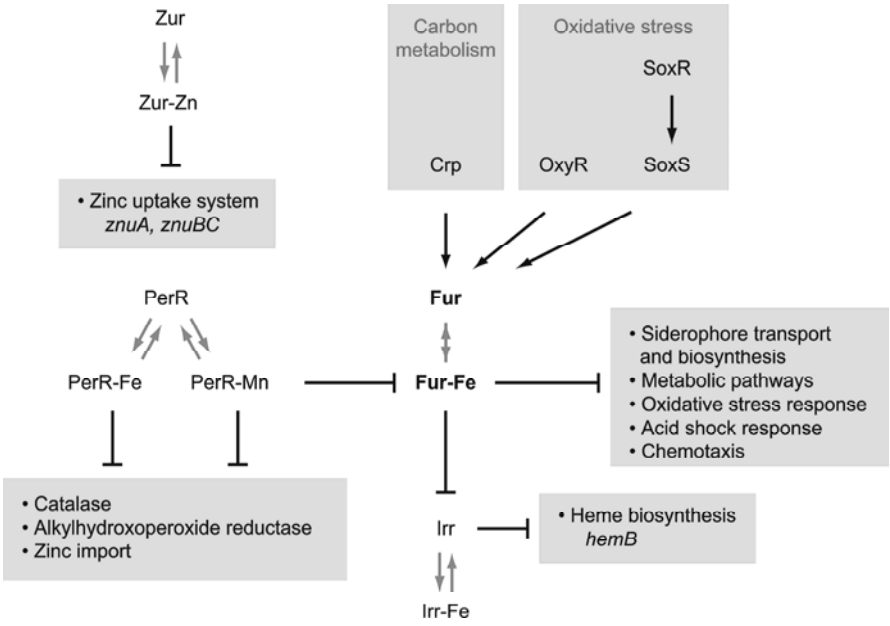


Fig. 3. Regulatory pathways involving Fur family members. Regulatory connections between individual Fur family members, as well as connections with cellular metabolism and oxidative stress, are indicated. Details of the figure are given in the text.

The Fur-like regulator, PerR, was initially found in *B. subtilus* where it regulates the response to hydrogen peroxide (Bsat et al. 1998). The PerR regulon includes *katA* (catalase), *hemAXCDBL* (heme biosynthesis), *ahpCF* (alkylhydroperoxide reductase), *mrgA* (DNA-binding protein), *zosA* (import of zinc), as well as the *fur* regulator and *perR* itself. Although it plays a role in the oxidative stress response, transcriptional repression by PerR requires bound metal ions such as Fe(II) or Mn(II). When hydrogen peroxide is added, it reacts with the metal-bound PerR to cause derepression (Fig. 3). The iron-bound form of PerR is more sensitive to hydrogen peroxide exposure than Mn(II)-PerR, which is largely resistant (Fuangthong et al. 2002). It has also been shown that the bound metal ion influences which genes are targeted, since repression of *perR* and *fur* is only mediated by manganese-bound PerR (Fuangthong et al. 2002) (Fig. 3). The rationale for *fur* regulation by Mn(II)-PerR is unclear, but could be linked to displacement of Mn(II) by Fe(II) in high-iron conditions, resulting in derepression of *fur*. Additional PerR homologs have been described in *Campylobacter jejuni* (van Vliet et al. 1999), *Streptococcus pyogenes* (King et al. 2000), and *Staphylococcus aureus* (Horsburgh et al. 2001).

The broader ligand specificity of the Fur family is also found in other metal-responsive regulators such as the MerR and ArsR/SmtB families. However, despite their differences in ligands, all members of the Fur family are thought to share the same mechanism of transcriptional regulation that has been long estab-

lished for Fur-mediated repression. Homologs of Fur have been found in many other prokaryotes, but not eukaryotes. Sequence analysis of the Fur family shows that there are many members that have been classified as Fur homologs whereas they actually share more similarity with other family members such as Zur and Irr, indicating that they are more likely to be functionally similar to these regulators (Hamza et al. 1998; Patzer and Hantke 1998).

2.2.2 Structural characterization and identification of Fur metal-binding sites

Initial studies have indicated that Fur binds target promoters as a dimer and consists of distinct N-terminal and C-terminal domains (Coy and Neilands 1991; Stojiljkovic and Hantke 1995; Michaud-Soret et al. 1997). The amino terminal domain is responsible for DNA binding and contains a helix-turn-helix motif that is found in many other transcription factors, whereas the C-terminus is involved in dimerization. This basic structure of Fur has now been confirmed in the first crystal structures of Zn(II)-Fur in *Pseudomonas aeruginosa* and a low-resolution apo-Fur in *Rhizobium leguminosarum* (Kolade et al. 2002; Pohl et al. 2003). The availability of the ligand-bound Fur has provided important information on the structure of the metal-binding sites. Two distinct coordination sites with different functions were identified in *P. aeruginosa* Zn(II)-Fur. One of the sites binds zinc with very high affinity and connects the DNA-binding and the dimerization domains. Iron was unable to substitute for bound zinc in this site and it is therefore thought to play an important role in maintaining the structural integrity of the protein. In contrast, the zinc ion at the second metal-binding site could be exchanged with iron by dialysis against a Fe(II) solution, indicating that this site may constitute the regulatory position (Pohl et al. 2003). In a proposed model for Fur activation, metal binding to the regulatory site induces a small local configuration change, moving the DNA-binding domains relative to the dimerization domains and resulting in increased DNA affinity of the Fur dimer.

The structure of *P. aeruginosa* Fur also provides additional insight into the interaction between the regulator and its target inverted repeat at the operator site. According to the structural model, binding of a single Fur dimer would only protect approximately 20 bp in a DNase I assay (Pohl et al. 2003), while previous studies have consistently shown regions of 27-30 bp (Ochsner and Vasil 1996; Escolar et al. 2000). This extended region of DNase protection could be explained by the binding of two dimers on opposite sides of the DNA, consistent with a model proposed by Baichoo et al. (Baichoo and Helmann 2002) (Fig. 4). While this model has not yet been validated, a similar structure has been found in crystallographic studies of another metal-responsive regulator DtxR, complexed to its *tox* operator (Fig. 5).

Spectroscopic studies of zinc-regulated Zur have allowed for the comparison of the metal-binding sites of the Zur and Fur (Outten et al. 2001). Zur monomers bind one zinc through cysteine residues that correspond to the proposed structural zinc binding site in *P. aeruginosa* Fur and are conserved between Fur family members. Similar to Fur, this zinc is thought to form a structural component since

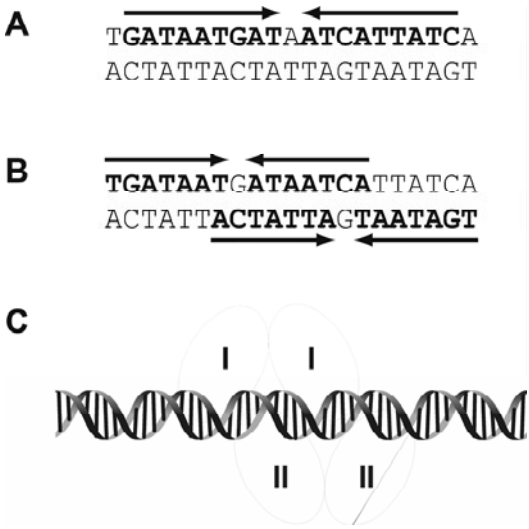


Fig. 4. Model for Fur operator binding. (A) Classical view of the *B. subtilus* Fur consensus binding site, consisting of a single inverted repeat, indicated by two arrows. (B) Model according to (Baichoo and Helmann 2002) that distinguishes two smaller overlapping inverted repeats, which each bind a single dimer. (C) Proposed model for binding of Fur dimers on opposite sides of the DNA. The figure is modified with permission from Baichoo and Helmann 2002.

it cannot be removed from the intact protein, even by extensive chelation (Jacquamet et al. 1998; Althaus et al. 1999; Outten et al. 2001). Metal specificity is therefore thought to reside in the second site that binds Zn(II) in Zur and Fe(II) in Fur. The metal coordination environment of these sites differ between Zur and Fur, such that Zn(II) binding would be thermodynamically favored in the Zur protein, while Fe(II) binding is optimal in the Fur protein (Outten et al. 2001). Considering that only femtomolar concentrations (2.0×10^{-16} M) of Zn(II) are needed to activate Zur (Outten and O'Halloran 2001), quantitative analysis of the metal-binding affinity of both Zn(II) binding sites in Zur is still needed to confirm which site constitutes the actual zinc-sensing site *in vivo*.

2.3 Diphtheria toxin regulator family

The diphtheria toxin regulator family (DtxR) was named after the first member of this family, which was characterized in *Corynebacterium diphtheria* as an iron-dependent repressor of the diphtheria toxin (*tox*) gene (Boyd et al. 1990). Subsequent studies indicated that DtxR also regulates genes involved in iron uptake, similar to those targeted by the Fur protein in other bacteria (Schmitt and Holmes 1994; Lee et al. 1997). However, in contrast to Fur-like regulators, which are mainly confined to Gram-negative bacteria and Gram-positive bacteria with a low guanine and cytosine (GC) content, orthologs of DtxR are found in Gram-positive

bacteria with a high GC content such as streptomycetes, rhodococcus, and mycobacteria, where they are named IdeR (Schmitt et al. 1995; Dussurget et al. 1996; Boland and Meijer 2000) (Table 2). Although the latter group of bacteria also expresses members of the Fur family, these homologs are involved in regulation of oxidative stress responses rather than iron uptake (Zou et al. 1999). Both DtxR and Fur function as dimers that respond to divalent metal ions by binding palindromic repeats in their target genes, resulting in transcriptional repression (Tao et al. 1995). However, despite their comparable function, DtxR and Fur share no sequence similarity, nor do they recognize the same consensus DNA-binding sequences.

Several additional DtxR family members have now been identified that respond to metal ions such as manganese and zinc, rather than iron. These regulators operate similarly to DtxR and IdeR and repress transcription of their target genes upon binding of their metal ligands. The TroR protein regulates transcription of an operon that contains *troR* itself in addition to *troABCD* and *gpm* (Posey et al. 1999). The *troABCD* genes respectively encode a solute binding protein (SBP), an ATPase and two membrane permeases, which together assemble into an ABC transporter. After some initial confusion over the presumed divergent metal specificities of the TroR regulator (manganese) (Posey et al. 1999) and the TroA SBP (zinc) (Lee et al. 1999), it has now been determined that Zn(II) is the most likely metal substrate for both the regulator as well as the ABC uptake system (Hazlett et al. 2003). The last gene in the operon, *gpm*, encodes the glycolytic enzyme phosphoglycerate mutase. Interestingly, *gpm* expression continues in the presence of high zinc when the TroR repressor is active, indicating the presence of a second autonomous *gpr*-specific promoter (Hazlett et al. 2003).

MntR is a manganese-dependent DtxR family member that regulates expression of the manganese uptake systems *mntABCD* and *mntH* in *Bacillus subtilus* and *Salmonella enterica* (Kehres et al. 2000; Que and Helmann 2000; Kehres et al. 2002). The *mntABCD* operon encodes an ABC metal transporter that is very similar to TroABCD. MntH is a member of the family of natural-resistance-associated macrophage proteins (NRAMP), responsible for the transport of Fe(II) ions in mammals. Unlike their eukaryotic counterparts, the MntH transporters in *B. subtilus* and *S. enterica* are highly specific to Mn(II) and play no physiological role in iron uptake (Kehres et al. 2000). A third MntH ortholog which takes up Fe(II) as well as Mn(II) has been characterized in *E. coli*, where it is regulated by both MntR and Fur in response to Mn(II) and Fe(II), respectively (Patzer and Hantke 2001). It is unlikely that an ABC transporter for Mg(II) uptake is present in *E. coli* since it has been found that Mn(II) uptake is solely dependent on the membrane potential rather than on ATP (Silver et al. 1970).

2.3.1 DtxR gene repression and metal specificity

A large number of studies have resulted in crystal structures of *C. diphtheria* DtxR in its apo and metal-bound state (Qiu et al. 1995; Schiering et al. 1995; Ding et al. 1996; Pohl et al. 1998, 2001), as well as in complex with DNA (White et al. 1998; Pohl et al. 1999a). The structures of the closely related IdeR regulator from

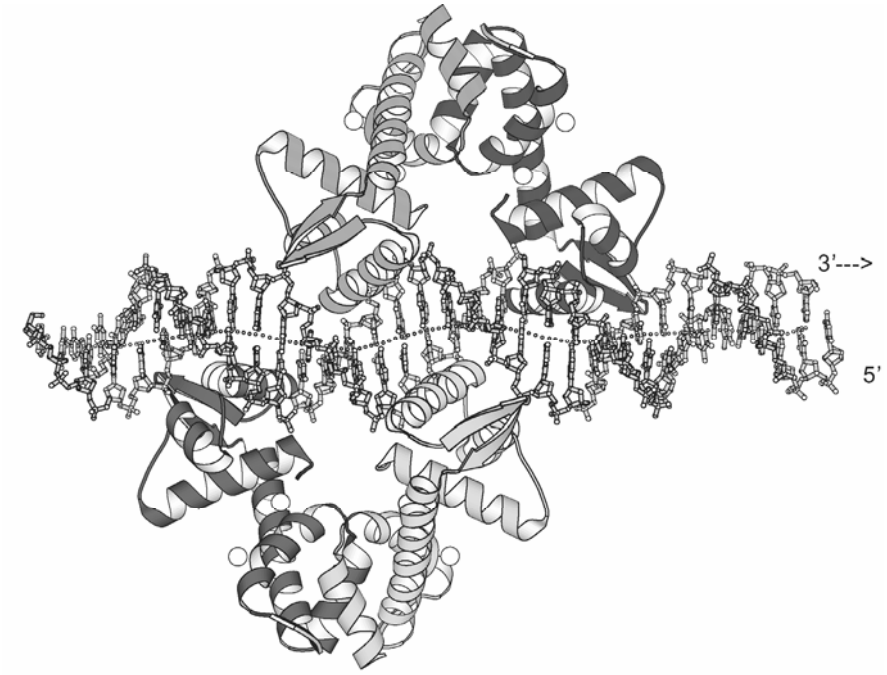


Fig. 5. Structure of DtxR complexed with the tox operator. The DtxR/*tox* complex was resolved with bound nickel instead of iron, and positions of individual nickel ions are indicated with shades of grey. Individual monomers are shown in different colors. The figure is reproduced with permission from White et al. 1998.

M. tuberculosis and *B. subtilus* MntR have also been determined (Pohl et al. 1999b; Glasfeld et al. 2003).

The DtxR protein is composed of two main structural domains that are linked by a flexible 23-residue linker containing a proline-rich region (Fig. 5). The N-terminal region contains the classical helix-turn-helix motif that is involved in DNA recognition, the two metal-binding sites and a hydrophobic surface that is responsible for dimerization. The C-terminal domain adopts an SH3 domain fold that is thought to regulate repressor activity (Pohl et al. 1999a; Wang et al. 1999) and also contributes two ligands to one of the metal-binding sites (Love et al. 2003). Interestingly, the C-terminal SH3 domain is absent in some of the DtxR family members, including the Zn(II)-dependent TroR (Posey et al. 1999) and Mn(II)-dependent MntR (Glasfeld et al. 2003). Aside from the absence of the SH3 domain, the structure of MntR closely resembles those of DtxR and IdeR.

The apo form of DtxR exists as an inactive monomer that is only in weak equilibrium with the dimeric form (Tao et al. 1995). Upon metal binding, the dimeric state is stabilized and the activated dimers specifically bind an interrupted palindromic repeat in the promoter region of their target genes (Tao et al. 1995; White et al. 1998). These palindromic repeats are located close to the –10 and –35 regions and binding of the repressor inhibits transcription by preventing recruitment

of the RNA polymerase holoenzyme to the promoter. The structure of the *tox* operator complexed with DtxR revealed that it binds two DtxR dimers on opposite sides of the DNA (Fig. 5). Each of the two dimers interacts with 19 bp regions that partially overlap and the complete area covered by the two dimers encompasses the full 27 bp of the palindromic repeat (White et al. 1998). While no crystal structures of the MntR and IdeR repressors in complex with their target DNA sequences are currently available, both repressors also function as dimers and bind similar palindromic repeats, making it likely that the observed model of promoter binding for DtxR also applies to these regulators.

Two metal-binding sites have been identified in DtxR monomers: site 1 (ancillary) and site 2 (regulatory) (Qiu et al. 1995; Schiering et al. 1995). Mutations in the ancillary site only result in a partial loss of repressor activity while changes in any of the residues involved in metal coordination at the regulatory site lead to complete inactivation (Ding et al. 1996; Love et al. 2003). Recent studies have suggested that activation of DxtR by metal binding is a multistep process (Spiering et al. 2003; Love et al. 2004). Before metal binding can occur, the regulator needs to be in a closed state where the SH3 domain that is linked by the flexible tether is in close proximity to the N-terminal domain. Binding of the first metal ion to the ancillary site then leads to conformational changes that are needed for the formation of the second, regulatory, binding site. Subsequent binding of the second metal ion results in the formation of the final tertiary structure that is required for dimerization and DNA binding. Similar to what has been found for DtxR, metal binding to IdeR in *Mycobacterium tuberculosis* also occurs in a cooperative manner, with metal-binding site 1 showing a higher affinity than binding site 2 (Chou et al. 2004).

Additional insights into the determinants of metal specificity have come from the structural comparison of Fe(II)-bound DtxR with Mn(II)-bound MntR and mutation analysis of the metal-binding sites in these proteins (Guedon and Helmann 2003). These studies suggest that the metal specificity of the individual DtxR family members is mainly determined by the geometry and composition of the metal-binding sites. When the composition of the MntR metal-binding sites was changed to resemble DxtR by incorporating putative DtxR metal coordinating residues, the resulting protein became responsive to both manganese and iron (Guedon and Helmann 2003).

2.4 ArsR/SmtB family

The ArsR/SmtB family was named after ArsR and SmtB, which were found to regulate arsenic and zinc resistance in *Synechococcus sp*. and *E. coli,* respectively (San Francisco et al. 1990; Huckle et al. 1993) (Table 2). Arsenic resistance is mediated by the *ars* operon in many microrganisms and contains the *arsDABC* genes in addition to the *arsR* regulator itself (Wu and Rosen 1991; Ji and Silver 1992b). In the absence of arsenic, ArsR is associated with the promoter and represses transcription of the *ars* operon. Upon addition of As(III), ArsR loses its affinity for DNA resulting in a rapid dissociation from the promoter and initiation of

transcription (Wu and Rosen 1993b; Rosenstein et al. 1994). ArsC is an arsenate reductase that is needed to convert arsenate (As(V)) to arsenite (As(III)) (Ji and Silver 1992a), which can then be exported by the efflux system encoded by *arsAB*. ArsB is a uniporter that uses the membrane potential to drive the export of arsenite (Wu et al. 1992). When the ArsA ATPase is co-expressed, an ArsAB complex is formed that couples transport to ATP hydrolysis (Rosen et al. 1992; Dey et al. 1994) and is much more efficient as an arsenite exporter (Dey and Rosen 1995). ArsD is thought to encode an additional *ars* repressor that binds the same promoter element as ArsR with twofold lower affinity, but they share no sequence similarity (Wu and Rosen 1993a; Chen and Rosen 1997). The two regulators are thought to form an integrated regulatory system to keep the expression level of the *ars* operon within a defined range, with ArsR controlling the basal expression, and ArsD controlling the maximum expression (Chen and Rosen 1997).

The other founding member, SmtB, represses its own expression and that of *smtA* by binding a shared operator region between the two divergently transcribed genes. SmtA encodes a class II metallothionein that acts as a buffer protein by binding excess zinc (Morby et al. 1993). Similar to ArsR, the presence of zinc(II) severely reduces the affinity of the regulator for the promoter, resulting in transcription initiation (Erbe et al. 1995; Cook et al. 1998; VanZile et al. 2002a).

ArsR and SmtB homologs have been found in many organisms and constitute a family with a diverse metal selectivity to toxic metals such as cadmium, lead, and bismuth (CadC) (Dell et al. 1994; Endo and Silver 1995) and to essential metals, including nickel and cobalt (NmtR, CzrA) (Kuroda et al. 1999; Busenlehner et al. 2002; Cavet et al. 2003) (Table 2). The strong reduction in promoter affinity upon metal binding is shared by all ArsR/SmtB family members and none of the regulators appear to play an active role in transcription initiation (Rosenstein et al. 1994). Target genes and operons generally encode proteins involved in metal resistance and can be located on the chromosome and/or incorporated as part of resistance plasmids (Silver 1992; Diorio et al. 1995). Besides metallothioneins and diffusion transporters, the major class of resistance proteins encoded by these operons consist of P-type ATPase metal exporters such as the zinc exporter ZiaA regulated by ZiaR (Thelwell et al. 1998), the cadmium efflux protein CadA controlled by CadC (Yoon et al. 1991) and the nickel exporter NmtA regulated by NmtR (Cavet et al. 2002).

2.4.1 Structural features of ArsR/SmtB metal-binding sites

The crystal structures of *Synechococcus* PCC7942 SmtB and *Staphylococcus aureus* CzrA have been resolved in metal-bound and -unbound states, revealing an α+β topology that strongly resembles that of the diphtheria toxin repressor proteins (Cook et al. 1998; Eicken et al. 2003). The structure of apo-SmtB is displayed in Figure 6 as an example. Both SmtB and CzrA form elongated homodimers with a twofold axis of symmetry. The subunit interface is formed between the two helices of the N-terminal α1 and C-terminal α5. DNA binding occurs through the classical helix-turn-helix motif that has been found in many other transcriptional regulators described here. The sequence of the DNA recognition

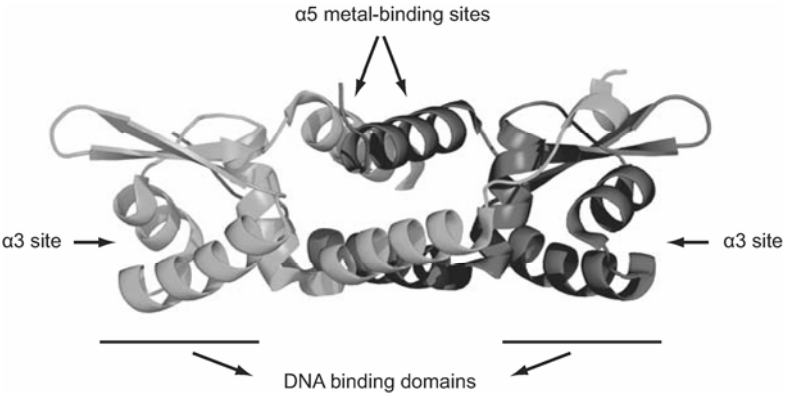


Fig. 6. Metal-binding sites in SmtB. The crystal structure of dimeric apo-SmtB, showing the two monomers in different shades of grey. The positions of the α3 and α5 sites, detected by incubation of apo-SmtB crystals with mercuric acetate are indicated (Cook et al. 1998). The two helix-loop-helix domains responsible for recognition of the DNA-binding motif are also indicated. The figure is modified with permission from Busenlehner et al. 2003.

helix is strongly conserved between individual members and constitutes one of the distinctive features for inclusion in the ArsR/SmtB family.

In order to locate the SmtB metal-binding sites, apo-SmtB crystals were exposed to mercury acetate, revealing two pairs of symmetrical Hg(II) binding sites that were likely to correspond to the native Zn(II) coordination sites (Cook et al. 1998). One of these sites was designated α3N, since metal coordination is mediated by residues from the third α-helix and part of the N-terminus, while a second α5 metal-binding site uniquely consists of residues from the fifth α-helices of the two monomers (Fig. 6). The presence of the α5 site was confirmed in a Zn(II)-bound structure of an α5-SmtB mutant (Eicken et al. 2003). The α3N site corresponds to the highly conserved ELCV(C|G)D metal-binding motif that was initially identified in a multiple alignment of ArsR/SmtB family members (Shi et al. 1994). Originally this conserved ELCV(C|G)D motif was thought to be essential for metal-responsive regulation since mutations of one or more cysteins in the motif inhibited the ability of arsenic to displace ArsR from the *ars* operator (Shi et al. 1996). However, mutation of a similar key residue Cys61 in the SmtB motif was found not to have any effect on *in vivo* Zn(II) sensing (Shi et al. 1996; Turner et al. 1996) and the α5-SmtB mutant retains strong negative regulation of promoter binding in the presence of Zn(II) (VanZile et al. 2002b). In addition, despite the overall structural similarities and conservation of the α5 metal-binding site, CzrA lacks the N-terminal extension and key residues in the α3 helix that are thought to form the α3N binding site in SmtB, providing further evidence that the presence of an α3N binding site is not strictly necessary for metal-responsive regulation in ArsR/SmtB family members.

Based on studies of metal-binding sites in other ArsR/SmtB family members, a complex picture emerges in which some family members possess either only an

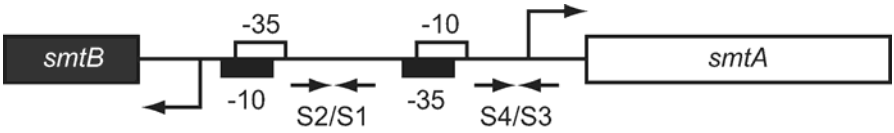


Fig. 7. SmtB operator/promoter structure. The structure of the *smt* locus with the divergently transcribed *smtB* and *smtA* genes is shown, together with the S2/S1 and S4/S3 inverted repeats involved in SmtB binding.

α3/α3N (e.g. ArsR) or α5/α5C metal-binding site (e.g. CzrA, NmtR), or a combination of both (e.g. SmtB, ZiaR). The functional significance of having both metal-binding sites is unclear and, thus far, only one of the sites appear to be important for *in vivo* metal sensing in the regulators involved. The finding that Co(II) appears to bind the *Synechococcus* SmtB at a ratio of two metal ions per homodimer, with one ion bound in the α5 and the other in the α3 site suggests that the latter site could be involved in responses to other metals (VanZile et al. 2002b). The presence of an additional α3 site could also play a facilitating role in zinc-dependent regulation as the affinity of SmtB for Zn(II) is 20-fold higher in the presence of a functional α3 site than without it (VanZile et al. 2002b). Recently, the picture has become more complex with the discovery of a novel cadmium-lead-sensing ArsR/SmtB repressor, CmtR, in *Mycobacterium tuberculosis* (Cavet et al. 2003). Contrary to the norm, mutation of potential metal-binding residues in or near the α3/α3N and α5/α5C sites did not abolish repression and inducer recognition. Mutation of other putative metal-binding residues revealed three cysteine residues derived from the putative α2 and αR (DNA-recognition) helices and a predicted α6 helix that were essential for Cd(II) regulation, defining a completely new sensory site (Cavet et al. 2003).

2.4.2 ArsR/SmtB operator/promoter binding

ArsR/SmtB regulators bind to a palindromic repeat close to the –10 and –35 regions, inhibiting RNA polymerase binding. Most promoters identified to date contain a single repeat, with the exception of SmtB that contains two similar imperfect repeats that are named 'S2/S1' and 'S4/S3' (Fig. 7). Each repeat consists of two units of 12 nucleotides, separated by a two-nucleotide spacer. The S2/S1 repeat is needed for Zn(II) regulation of *smtA* expression *in vivo*, while the S4/S3 repeat is of little to no importance (Erbe et al. 1995; Turner et al. 1996). Other imperfect repeats have also been found in the O/P regions of the *ars*, *czr*, *zia*, *nmt*, and *cad* operons (Endo and Silver 1995; Xu et al. 1996; Xiong and Jayaswal 1998; Singh et al. 1999; Cavet et al. 2002; Pennella et al. 2003).

Analysis of the association of an oligonucleotides probe containing a single inverted repeat from the SmtB promoter (S1/S2) revealed tight binding with two SmtB dimers (Kar et al. 2001; VanZile et al. 2002a). Two additional dimers could be bound at a respectively 10- and 30-fold lower affinity. Two models have been proposed for SmtB binding the imperfect repeats. The first model assumes that a single SmtB homodimer binds the repeat, with a second dimer interacting only di-

rectly with the first homodimer (Kar et al. 2001; VanZile et al. 2002a). This model is supported by the observation that SmtB can form weak tetramers in solution (Kar et al. 1997). The second model states that two dimers bind on opposite sides of the DNA, each one centered on a conserved TGAA sequence in one of the repeat arms (VanZile et al. 2002a). The fact that DNase I footprints of the homologous CadC bound to the *cad* O/P are slightly offset from the midpoint of the repeat and centered on the conserved sequences (Endo and Silver 1995), lends support to this model. It also explains why adding base pairs on both sides of the repeat increases the overall affinity of the DNA-regulator complex (VanZile et al. 2002a). Contrary to expectations, it was found that the complete SmtB O/P with two palindromic repeats binds two dimers, rather than four (Kar et al. 2001). As a result of this, it has been proposed that the full *smt* promoter forms a condensed structure where the DNA is looped around two dimers that interact back-to-back (Kar et al. 2001). Evidence for the formation of multimeric promoter complexes has also been found for other regulators, including CzrA, ZiaR, and CadC (Thelwell et al. 1998; Busenlehner et al. 2002).

2.5 Additional prokaryotic metal-responsive transcriptional systems

A number of smaller prokaryotic transcription factor families involved in regulation of metal metabolism have been described (Table 2). These factors are generally specific for a single metal, such as the well-known CopY regulator of *Enterococcus hirae*. CopY mediates copper resistance and binds the O/P region of the *copYZAB* operon in response to elevated copper levels. A full review on the *E. hirae* copper homeostatic system can be found in (Solioz and Stoyanov 2003). In contrast to other transcription factor families, homologs of CopY are only found in other Gram-positive bacteria that are closely related to *E. hirae*. Additional families are discussed briefly below.

2.5.1 ModE

ModE was initially described in *E. coli* as a repressor of the molybdate uptake operon *modABCD*, which encodes the subunits for a high-affinity ABC uptake transporter (Walkenhorst et al. 1995; Grunden et al. 1996; McNicholas et al. 1997). Molybdate (MoO_4^{2-}) is the biological form of molybdenum that is taken up by virtually all species, including plants and animals, to be incorporated into molybdopterin. Molybdopterin is used as an essential cofactor in a group of oxidoreductases, including dimethyl sulfoxide (DMSO) reductase and biotin sulfoxide reductase (Table 2). In addition to its role as transcriptional repressor, ModE can also activate expression of the *moaABCDE* (Anderson et al. 2000) and *dmsABC* (McNicholas et al. 1998) operons, which respectively encode enzymes responsible for molybdenum cofactor biosynthesis and the molybdenum-dependent DMSO reductase. Finally, ModE was identified as a secondary activator of the *nap*, *nar*, and *hyc* operons, all encoding molybdoenzymes (Self et al. 1999; McNicholas and Gunsalus 2002).

Crystal structures of *E. coli* ModE in its apo and metal-bound form have been resolved (Hall et al. 1999; Gourley et al. 2001; Schuttelkopf et al. 2003) and reveal four distinct domains in the polypeptide. The N-terminal domain is involved in DNA binding, followed by a linker region and two mop domains (mop1 and mop2). These two latter domains are responsible for molybdate binding and coordinate a single molybdate anion. As in the other families described thus far, molybdate-bound ModE forms a dimer (Hall et al. 1999) that binds to an inverted repeat overlapping the –10 region the promoter of target genes (Grunden et al. 1996). This inverted repeat may bind a total of two ModE dimers on opposite sites of the DNA helix, similar to the proposed structures of the DtxR, Fur and SmtB regulator-operator complexes. It is generally assumed that this binding sterically inhibits attachment of RNA polymerase and, therefore, initiation of transcription.

The availability of both the apo and metal-bound form of ModE has provided some insight into how metal binding in the mop domains influences the affinity of the clearly distinct DNA-binding domains (Gourley et al. 2001). The differences between apo-ModE and molybdate-ModE indicate that the protein undergoes a large conformation change where the DNA-binding domains move relative to the mop domain and to each other. The resulting structure is more symmetrical and shows a decreased distance between the recognition helices of the DNA binding domains (32.5 Å relative to 34.5 Å for the metal-bound form). It is likely that this altered position of the DNA-binding domains increases ModE affinity for binding to its target operator sites.

ModE homologs have been described in a wide range of bacteria including *Azotobacter vinelandii* (Mouncey et al. 1996) and *Rhodobacter capsulatus* (Kutsche et al. 1996). In these organisms, ModE is responsible for the regulation of molybdate uptake operons similar to *modABCD* in *E. coli*, as well as indirect control of molybdate-independent nitrogenases through repression of their respective transcriptional activators (Kutsche et al. 1996; Premakumar et al. 1998).

2.5.2 NikR

NikR, originally identified in *E. coli*, is a metal-responsive repressor that senses and controls nickel uptake when the nickel level in cells is sufficient. The operon regulated by *E. coli* NikR, consists of six genes (Navarro et al. 1993).The first five genes, *nikABCDE*, encode a nickel-specific ATP-binding cassette (ABC) transporter (Navarro et al. 1993) while the last gene encodes NikR itself (De Pina et al. 1999). Beside regulation by NikR, transcription of the *nik* operon is activated by the fumarate nitrate regulatory protein (Fnr), an oxygen-sensitive transcription factor (Wu and Mandrand-Berthelot 1986). Fnr activates transcription of the nickel-dependent *hypA* gene (*hypA-E* operon, involved in the maturation of NiFe hydrogenases) under anaerobic growth conditions only (Messenger and Green 2003).

NikR is the only known metal-responsive member of the ribbon-helix-helix family of transcription factors (Chivers and Sauer 1999) and consists of two parts: an N-terminal domain, which forms dimers and binds to the operator (Chivers and Sauer 1999), and a C-terminal high affinity Ni(II)-binding domain that is involved in tetramerization (Chivers and Sauer 2000). In the absence of nickel, the NikR

DNA-binding domain forms dimers that only weakly associate with DNA. When nickel is available, a complete NikR tetramer binds to an operator sequence within the *nikABCDE* promoter, which spans the -10 position of the *nikABCDE* promoter and contains two dyad-symmetric half-sites 5'-GTATGA-3' separated by 16 base pairs (Chivers and Sauer 2000), turning off expression of the uptake operon and decreasing nickel import. The *E. coli nikR* gene, can be expressed either from its own promoter, located 51 bp upstream of *nikR* transcription site, or from the FNR-dependent promoter of the *nik* operon, located upstream of *nikA* (De Pina et al. 1999), which governs expression of genes involved in the transition from aerobic to anaerobic respiration (Wu et al. 1989). Crystal structures of the apo and metal-bound C-terminal forms in *E. coli* have been resolved and reveal that the tetrameric nickel-binding domain is wedged between the two DNA-binding dimers, thus inserting the large spacing of 16 bp between the two contact regions in the operator (Schreiter et al. 2003).

Metal-binding analysis has revealed that NikR can bind a variety of divalent transition metals other than nickel, including Cu(II), Zn(II), Co(II), and Cd(II). However, only nickel induces a distinct conformation change in NikR and exerts the selective allosteric effect that stabilizes the protein-DNA complex and leads to transcription activation (Kim et al. 2004). The enhanced DNA-binding affinity of the NikR-metal complex is also selective for nickel (Wang et al. 2004a).

NikR orthologs are found in Gram-negative bacteria and archaea (Table 2) (Chivers and Sauer 1999), suggesting a common mechanism of nickel regulation across diverse microrganisms. NikR is also distantly related to the Fur family, although sequence similarity is very low (Eitinger and Mandrand-Berthelot 2000). In the human pathogens *Helicobacter pylori* (Contreras et al. 2003) and *Brucella suis* (Jubier-Maurin et al. 2001), NikR induces the expression of urease, a nickel-dependent enzyme and important virulence factor that protects these bacteria against their acidic environment (van Vliet et al. 2002). It also activates the expression of genes associated with nickel metabolism, nickel transport (*nixA, copA2*) and nickel storage (*hpn*) (Contreras et al. 2003). On the other hand, like its *E.coli* homologue, *H. pylori* NikR also acts as repressor to the *nikR-nadD* operon and the divergent operon that encodes the ExbB/ExbD/TonB proteins which provide energy for the various ferric iron uptake and storage systems. This connection may explain the indirect repression that is observed for iron metabolism genes that belong to the Fur regulatory system (Contreras et al. 2003). Finally, NikR also inhibits the expression of genes involved in stress responses, motility and encoding outer membrane proteins (Contreras et al. 2003).

2.5.3 Two-component systems

Two-component regulatory systems are found in many bacteria and constitute a superfamily of conserved proteins (For review see: Foussard et al. 2001). They consist of two partner proteins; a membrane-bound sensor histidine kinase and a cytoplasmic response regulator. Upon binding of a ligand, the histidine kinase is activated and phosphorylates the response regulator, which then orchestrates a cellular response. The sensor protein has a modular organization and contains an N-

terminal periplasmic domain involved in ligand binding as well as a core C-terminal domain that is responsible for transmission of the signal to the cytoplasm. A similar organization is found in the response regulators that contain a phosphorylatable receiver domain and an effector domain. The latter domain is responsible for carrying the signal to the rest of the cell and can have an enzymatic function, e.g. an ATPase, or mediate binding to other proteins and DNA (Foussard et al. 2001).

Several bacterial two-component systems that initiate a transcriptional response upon metal binding of the sensor have now been identified (Table 2). The PcoRS regulators in *E. coli* induce expression of the plasmid-borne *pcoABCDE* resistance system in response to copper (Tetaz and Luke 1983; Brown et al. 1992). PcoA and PcoC respectively encode a multicopper oxidase and a putative Cu(I) chaperone that are thought to operate together in the periplasm to convert Cu(I) to the less toxic Cu(II) form (Huffman et al. 2002). The function of the other proteins encoded by the *pco* operon is less clear, but also seems to be related to periplasmic copper handling. PcoE localizes to the periplasmic space while PcoB appears to be an outer membrane protein that may prevent copper uptake (Lee et al. 2002). The finding that mutations in PcoR and PcoS could not completely abolish expression from P_{pcoA} and P_{pcoE} (Rouch and Brown 1997), in combination with the identification of chromosomal homologs of the related plasmid-borne *cop* system in *Pseudomonas syringae* (Lim and Cooksey 1993; Mills et al. 1993), has lead to the discovery of further chromosomal *cueRS* genes in *E. coli* (Munson et al. 2000). CueRS are required for the expression of *pcoE*, as well as the chromosomal *cusCFBA* operon that is thought to encode an efflux transporter that directly transports Cu(I) across the periplasmic space (Munson et al. 2000; Franke et al. 2003). Additional two-component regulatory systems involved in heavy metal resistance have been identified in *Synechocystis sp*. PCC 6803 (nickel, manganese), *Pseudomonas aeruginosa* (zinc, cadmium), *Ralstonia eutropha* (cobalt, zinc, cadmium), and *Burkholderia pseudomallei* (zinc, cadmium) (Jones et al. 1997; van der Lelie et al. 1997; Hassan et al. 1999; Lopez-Maury et al. 2002; Ogawa et al. 2002).

The metal-responsive sensory kinases and response regulators that were identified in these prokaryotes share considerable sequence similarity. In the sensory kinases, this similarity is, however, mainly confined to the transmission domain and there is little resemblance between the periplasmic domains of sensors with different metal specificities.

3 Eukaryotic metal-responsive transcription regulation

In contrast to the large number of prokaryotic transcription factor families involved in regulation of metal metabolism, only a few metal-responsive regulators have been described in eukaryotes. These regulators control the expression of a large number of genes encoding copper, iron, and zinc uptake and detoxification systems (Table 3). The majority of eukaryotic transcription factors have been de-

scribed in the yeast *Saccharomyces cerevisiae* (baker's yeast) and to a lesser extent in other fungi. Only one transcription factor, the zinc resistance regulator MTF-1, has currently been found in metazoa (multicellular animals), including fish, insects, and mammals (Westin and Schaffner 1988; Auf der Maur et al. 1999). The identification of the putative copper-responsive regulator Crr1 in *Chlamydomonas reinhardtii* indicates that additional factors may also exist in plants (Eriksson et al. 2004). The combined set of genes identified as targets of the eukaryotic metal-responsive regulators has grown considerably over the past few years, in part because of the availability of microarrays that have enabled genome-wide screens for expression changes. As a complete overview of these genes is beyond the scope of this review, readers are referred to other reviews in this volume for more details on their roles in metal homeostatic pathways.

The small number of eukaryotic metal-responsive regulators that have been identified to date makes it difficult to group them into families. Moreover, some factors are composed of domains that are derived from different classes of transcriptional regulators (Beaudoin et al. 2003). Therefore, the eukaryotic transcription factors will be discussed here according to the type of metal they respond to and their mechanism of transcription activation (activator/repressor) (Table 3).

3.1 Copper-responsive transcription factors

Transcription regulation plays an important role in maintaining fungal copper levels within an optimal range. When copper is low, these organisms react by inducing expression of genes encoding copper uptake systems (Chapters 2, 5). This response is mediated by a group of transcriptional regulators that are currently known to include Mac1 (*Saccharomyces cerevisiae*), Cuf1 (*Schizosaccharomyces pombe*), and GRISEA (*Podospora anserina*) (Jungmann et al. 1993; Borghouts and Osiewacz 1998; Labbe et al. 1999) (Table 3). As soon as homeostatic balance is restored, these regulators are inhibited in a copper-dependent manner. In contrast, exposure to toxic copper levels results in activation of genes involved in the oxidative stress response and copper sequestration (Chapters 2,5) by another set of regulators that is presently comprised of Ace1 (*Saccharomyces cerevisiae*), Crf1 (*Yarrowia lipolytica*), and Amt1 (*Candida glabrata*) (Thiele 1988; Zhou and Thiele 1991; Garcia et al. 2002) (Table 3). Gene expression responses related to copper metabolism have also been extensively characterized in microarray experiments (Gross et al. 2000; De Freitas et al. 2004; van Bakel et al. 2005).

Insight into the molecular mechanism responsible for copper-dependent inhibition of the uptake regulators has mainly come from mutational analysis of conserved domains in these proteins. Mac1 and GRISEA both consist of an N-terminal domain with zinc finger motifs that are involved in DNA binding, followed by a C-terminal transactivation domain. The transactivation domains contain two conserved cysteine-rich repeats, which have been named REPI and REPII (Graden and Winge 1997; Keller et al. 2000). Both of these motifs have been shown to form multicopper clusters that coordinate four Cu(I) ions each, and are

believed to play an important role in copper-dependent regulation (Brown et al. 2002).

The function of the conserved domains has been most extensively characterized in Mac1. Mac1 can form homodimers that bind copper-responsive elements (CuREs) located in the promoter regions of its target genes (Jamison McDaniels et al. 1999; Joshi et al. 1999). Dimerization occurs independent of copper binding, mediated by a predicted α-helix that is situated C-terminal of the REP motifs (Serpe et al. 1999). REPI is essential for Mac1 regulation and is thought to mediate repression through copper-dependent interactions with the DNA-binding domain, resulting in loss of both DNA affinity and transactivation potential (Graden and Winge 1997; Jensen and Winge 1998). As expected, mutations in any of the cysteins in REPI lead to a loss of copper-dependent regulation. REPII has been shown to function as an effective transactivator in fusion proteins with Gal4 DNA-binding domains, but was also found to exert a negative effect on Mac1 DNA affinity (Voutsina et al. 2001). Recent studies indicate that additional mechanisms may play a role in regulation of activity. Mac1 needs to be phosphorylated before it can bind DNA, and it was suggested that this phosphorylation affects interactions between the DNA-binding domain and the transactivation domain (Heredia et al. 2001). Finally, C-terminally tagged Mac1 has been shown to undergo rapid degradation in the presence of copper (Zhu et al. 1998), although a similar effect is absent when Mac1 is overexpressed (Jensen and Winge 1998). A putative model, reflecting current knowledge on the copper-dependent regulation of Mac1 is shown in Figure 8a/b. In contrast to Mac1, copper-responsive repression of GRISEA is thought to be mediated by an interaction between the DNA-binding domain and REPII, rather than REPI (Borghouts and Osiewacz 1998).

The structure of Cuf1 is different from Mac1 and GRISEA, and contains only one cysteine-rich motif in its transactivation domain (Labbe et al. 1999). In addition, the N-terminal domain shows more similarity to the copper detoxification regulator Ace1 than to its functional neighbors, and includes some of the elements that confer copper-dependent regulation to Ace1 (Beaudoin et al. 2003). Interestingly, the copper uptake regulator Cuf1 recognizes CuSE motifs that are very similar to those recognized by the Ace1, and an $Ace1_{1\text{-}63}$-Cuf1 fusion protein is able to complement the growth defect of Δ*cuf1* mutants on low copper medium (Beaudoin and Labbe 2001; Beaudoin et al. 2003). The mechanism of Cuf1 repression could therefore be an intermediate between two different classes of copper-dependent regulators. In addition to activation of genes involved in copper transport, Cuf1 also functions as a repressor of the copper-dependent high-affinity transport system in *S. pombe* (Labbe et al. 1999).

The copper resistance regulators Ace1 and Amt1 have been shown to activate genes in response to copper by binding conserved motifs in the promoter region (Thiele and Hamer 1986; Huibregtse et al. 1989). In contrast to Mac1, both factors bind these motifs as monomers (Zhou et al. 1992) (Fig. 8c/d). Amt1 also autoregulates its own expression, which requires the additional function of the Swi/Snf nucleosome remodeling complex and the histone acyltransferase Gcn5 (Koch et al. 2001). Copper-dependent activation of the Ace1, Amt1, and Crf1 regulators involves different domains than those identified in the uptake factors. The three

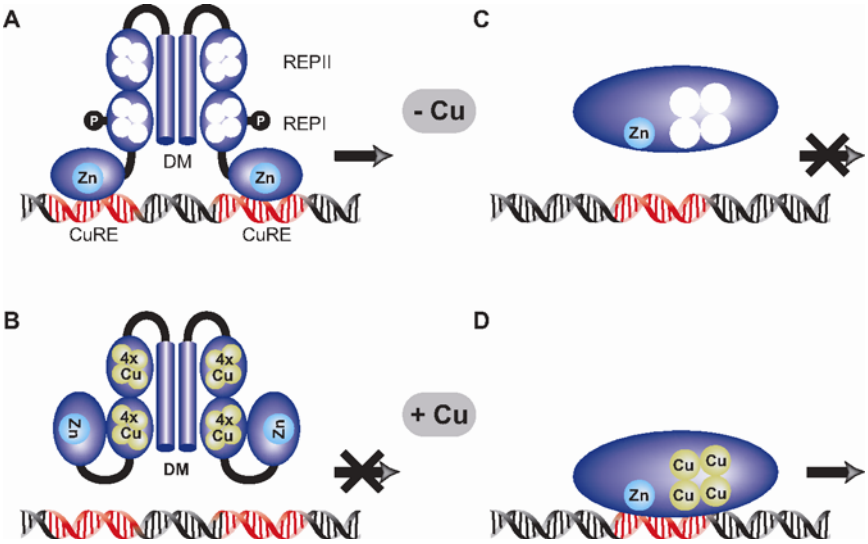


Fig. 8. Putative model for Mac1 and Ace1 regulation. (A) In the absence of copper, Mac1 is in the active state with putatively phosphorylated activation domains (Heredia et al. 2001) that show no interaction with the DNA-binding domains. The positions of the two multicopper-binding domains (REPI, REPII) are indicated, together with the α-helix that is involved in Mac1 dimerization. (B) Addition of copper leads to the formation of the two multicopper centers, resulting in an interaction between the REPI and the DNA-binding domain. The resulting Mac1 conformation has reduced DNA-binding affinity. Another mechanism for Mac1 inactivation is not indicated in the figure but involves targeted protein degradation. (C) In the absence of copper, Ace1 is not able to bind to DNA. The location of the N-terminal zinc finger motif is indicated. (D) Copper binding to four N-terminal cystein motifs leads to stabilization of the DNA-binding domain. Activated Ace1 then binds promoter regions as monomers, where the N-terminal zinc finger motifs stabilize the DNA-protein interaction.

factors contain N-terminal zinc finger motifs (Farrell et al. 1996) that are followed by a conserved (R/K)GRP sequence and eight cystein residues, which are arranged in four Cys-$X_{1\text{-}2}$-Cys motifs (Dameron et al. 1991; Thorvaldsen et al. 1994; Graden et al. 1996; Turner et al. 1998). Copper binding to the latter cystein residues is thought to lead to a conformational change that results in activation and DNA binding (Fig. 8d). Interactions between the zinc finger motifs and the conserved (R/K)GRP motif with the minor groove of the DNA are thought to provide extra stability (Koch and Thiele 1996). An additional regulation mechanism has been identified for Crf1, which shows copper-dependent relocalization to the nucleus, (Garcia et al. 2002).

There is evidence for the existence of additional copper-responsive transcription factors in plants. Normal operation of the *Chlamydomonas reinhardtii* photosynthetic system requires the function of several cuproenzymes including plastocyanin. However, when copper levels are low, the putative regulator Crr1 initiates

a transcriptional remodeling that results in the expression of alternate, heme-dependent, electron carriers, and the redistribution of existing copper by controlled degradation of plastocyanin (Quinn and Merchant 1995; Quinn et al. 1999; Eriksson et al. 2004). The inbred maize line A351 shows much reduced levels of Cu/Zn SOD and additional cytosolic and chloroplastic enzymes (Ruzsa and Scandalios 2003). Subsequent examination of the promoter sequences of Cu/Zn SOD has revealed the presence of motifs that are highly homologous to those recognized by the Ace1 and Amt1 transcription factors (Ruzsa and Scandalios 2003).

3.2 Iron-mediated gene expression

Iron metabolism in *Saccharomyces cerevisiae* is regulated by two transcription factors, Aft1 and Aft2, which activate expression of iron uptake systems and a set of additional genes in response to iron deprivation (Table 3). The Aft1/Aft2 regulon has also been extensively studied by microarray analysis (Rutherford et al. 2003; Shakouly_elizeh et al. 2004; Puig et al. 2005). Both factors are highly similar and may have originated from an ancient gene duplication event. Despite their similarity, yeast cells have different requirements for Aft1 and Aft2. Deletion of Aft1 results in cells that grow poorly in iron-starved conditions (Yamaguchi-Iwai et al. 1996), while no phenotype is observed in a similar Aft2 knockout strain. A double knockout of Aft1 and Aft2 does however result in a stronger phenotype than a knockout of Aft1 alone (Blaiseau et al. 2001; Rutherford et al. 2001). Aft1 and Aft2 bind the same iron-regulatory promoter elements (FeRE) to regulate expression of their target genes (Yamaguchi-Iwai et al. 1996; Rutherford et al. 2003). Both Aft1 and Aft2 consist of an N-terminal DNA-binding domain and a C-terminal regulatory domain; however, no crystal structures are available thus far.

The mechanism by which Aft1 and Aft2 are either activated or inhibited in response to the cellular iron status is not fully understood, although the subcellular location of these transcription factors appears to be an important factor in their regulation. A nuclear export signal (NES) sequence has been identified in Aft1, and it is known that Aft1 translocates from the cytoplasm when sufficient iron is available, to the nucleus in iron-starved cells (Yamaguchi-Iwai et al. 2002). In addition, mutations in the NES sequence that trap Aft1 in the nucleus result in constitutive transcriptional activation of the Aft1 regulon (Yamaguchi-Iwai et al. 2002). Both Aft1 and Aft2 also contain a conserved Cys-Xaa-Cys motif that may be involved in direct iron sensing. Mutations in this motif result in similar constitutively active transcription factors (Yamaguchi-Iwai et al. 1995) that are retained in the nucleus (Yamaguchi-Iwai et al. 2002), indicating that it is important for iron-mediated relocalization.

Recent data suggest a role for Fe-S cluster biosynthesis in the activation of the Aft transcription factors. Defects in *S. cerevisiae* mitochondrial Fe-S biogenesis lead to iron accumulation in the mitochondrion and constitutive expression of iron uptake systems (Kispal et al. 1999), while cytoplasmic iron levels remain normal (Rutherford et al. 2005). Further studies have now suggested that Aft1 and Aft2

respond to an inhibitory signal from mitochondrial Fe-S biosynthesis, but it is unlikely that this signal is mediated by direct binding of Fe-S clusters to these regulators (Rutherford et al. 2005). The inner membrane transporter Amt1 has been implicated as a key component to connect the mitochondrial Fe-S biosynthesis pathway to the iron regulon (Rutherford et al. 2005).

In addition to iron, Aft1-mediated gene regulation is linked to general cellular processes. The glucose metabolism influences Aft1 gene regulation through the global regulators Snf1/Snf4 (Haurie et al. 2003) and the cyclic AMP-dependent protein kinase A (Robertson et al. 2000). This interdependency between cell metabolism and iron uptake can be explained by the increased requirement for iron during respiratory growth. The phosphorylation state of Aft1 is also related to the cell cycle. When cells undergo cell cycle arrest, Aft1 is phosphorylated by a mechanism that is independent of Snf1 or cyclic AMP levels (Haurie et al. 2003). The functional significance of this regulation is currently unknown.

While the Aft1 and Aft2 transcription factors are unique to *S. cerevisiae*, a different family of regulators control iron homeostasis in other fungi, including *Schizosaccharomyces pombe* (Fep1), *Neurospora crassa* (SRE), *Aspergillus nidulans* (SREA), *Penicillium chrysogenum* (SreP), and *Ustilago maydis* (Urbs1) (Voisard et al. 1993; Haas et al. 1997, 1999; Zhou et al. 1998; Pelletier et al. 2002). In contrast to the ones identified in *S. cerevisiae*, these regulators function as repressors that inhibit transcription in the presence of iron and belong to the class of GATA-type transcription factors (Table 3). GATA regulators are zinc finger proteins which bind to core 5'-GATA-3' promoter elements, extended with additional residues that confer specificity. The target genes of these iron-responsive regulators are generally involved in siderophore production and siderophore-iron transport, as well as high-affinity iron uptake systems (Table 3).

Activation of the GATA-type repressors is likely to occur through direct iron binding. This model is supported by the observation that SRE from *N. crassa* exhibits a reddish-brown color, indicative of iron-binding proteins (Harrison and Marzluf 2002). Moreover, significant DNA-binding affinity of purified *S. pombe* Fep1 requires that the protein is isolated from cells that were grown in the presence of iron (Pelletier et al. 2002). The zinc finger domains, most notably the C-terminal zinc finger, are thought to play an important role in coordination of the iron ions, and mutations in these domains result in loss of iron-dependent regulation (Harrison and Marzluf 2002; Pelletier et al. 2002).

The repressor function of the GATA-type regulators may also depend on interactions with additional proteins. *S. pombe* mutant strains with a double deletion of the transcriptional co-repressors *tup11* and *tup12* are unresponsive to gene regulation by iron (Pelletier et al. 2003). Deletion of either gene alone has no effect on iron-dependent gene regulation, indicating that the two co-regulators have functionally overlapping roles. Homologs of Tup11 and Tup12 are also present in other fungi and deletion of the related *tup1* gene in *C. albicans* results in a similar iron phenotype (Knight et al. 2002). The direct interaction between Tup11 and the C-terminal domain of Fep1 has recently been confirmed by two-hybrid analysis and immunoprecipitation (Znaidi et al. 2004).

3.3 The zinc uptake regulator Zap1

The Zap1 regulator is unique to *S. cerevisiae* and responsible for transcriptional activation of a number of genes involved in zinc uptake (*ZRT1*, *ZRT2*, and *FET4*) and release of vacuolar zinc stores (*ZRT3*) (Zhao and Eide 1996a, 1996b; Waters and Eide 2002) (Table 3). Surprisingly, Zap1 also simultaneously induces expression of a vacuolar zinc uptake system (MacDiarmid et al. 2002), which may provide cells with a way of preventing zinc overload when environmental zinc levels return to normal (MacDiarmid et al. 2003). Another recent finding is that Zap1 not only functions as an activator of the *ZRT2* gene, but actually represses *ZRT2* expression during zinc starvation (Bird et al. 2004). Activation of *ZRT2* expression occurs by Zap1 binding to a set of two high-affinity zinc-responsive elements (ZRE), while repression is mediated by a third low-affinity ZRE that is located near the TATA box (Bird et al. 2004). Although it seems strange that a zinc uptake transporter is repressed when zinc levels are low, it makes sense when considering that Zrt2 is only a low-affinity transporter that is unable to function during severe zinc deficiency (Bird et al. 2004). A further set of 46 zinc-responsive genes that may represent additional targets for Zap1 were identified in a microarray study of zinc-depleted cells (Lyons et al. 2000).

Structural characterization of Zap1 has revealed that it contains two acidic N-terminal activation domains (AD1 and AD2), the latter of which contains two zinc finger motifs (Zhao and Eide 1997; Bird et al. 2003). These domains are followed by a C-terminal domain that contains an additional five zinc fingers, which are all required for DNA binding (Zhao et al. 1998; Bird et al. 2000a; Evans-Galea et al. 2003). The importance of zinc as an essential factor for normal cellular function is reflected by the existence of an intricate regulatory system that controls Zap1 activity at multiple levels. Zap1 controls its own expression by binding a zinc-responsive element in its promoter. Deactivation upon zinc exposure is mediated by the two activation domains and the DNA-binding domain itself. Repression by AD1 is thought to involve an allosteric switch that brings the domain in contact with the DNA-binding domain, resulting in loss of transactivation function (Bird et al. 2000b). The AD2 domain uses its two zinc fingers to sense zinc levels. Progressive binding of zinc to the first and then the second site induces structural changes that inhibit the transactivation potential of the AD2 domain (Bird et al. 2003). The DNA-binding domain itself also contributes to zinc-dependent control since constructs containing only the Zap1 DNA-binding domain coupled to a heterologous activation domain still display zinc-dependent activation of a reporter gene (Bird et al. 2000b).

3.4 Metal-responsive transcription factor –1 (MTF-1)

MTF-1 was initially characterized in mice (*Mus musculus*) as a zinc-responsive activator of metallothionein expression (*MT-1* and *MT-2*) (Westin and Schaffner 1988; Heuchel et al. 1994). An additional target gene, *ZnT-1*, was identified later and encodes a zinc efflux system (Langmade et al. 2000). Interestingly, knockouts

of MTF-1 in mice are embryonically lethal as a result of liver degeneration on day 14 of gestation (Gunes et al. 1998). Orthologs of MTF-1 have been found in several species, including *Homo sapiens* (Brugnera et al. 1994), *Fugu rubripes* (Auf der Maur et al. 1999), *Drosophila melanogaster* (Egli et al. 2003), in which they regulate similar sets of target genes (Table 3). In addition to zinc, MTF-1 can also respond to other divalent metal ions such as cadmium and copper, as well as hydrogen peroxide in these organisms. For example, *D. melanogaster* MTF-1 gene regulation is most responsive to copper. In contrast to mice, an MTF-1 knockout in *D. melanogaster* is viable and results in flies that are hypersensitive to increased levels of copper, cadmium, and zinc (Egli et al. 2003). In humans, *hZTL1* was identified as a putative novel target gene, encoding a zinc uptake transporter expressed in the apical membrane of enterocytes (Cragg et al. 2002).

MTF-1 induction of target genes is mediated by *cis*-acting metal-responsive elements (MRE) with a core consensus sequence of TGC(A/G)CnC. It has recently been shown that differences in the sequence context of this core MRE may regulate MTF-1 binding affinity at different zinc concentrations (Wang et al. 2004b). Like Zap1, the activity of MTF-1 is coordinated by several other mechanisms. Addition of zinc or cadmium results in translocation of human and mouse MTF-1 from the cytoplasm to the nucleus (Smirnova et al. 2000; Saydam et al. 2001). When zinc levels are returned to normal, redistribution to the cytoplasm depends on the presence of a NES in the C-terminal domain. At another level, MTF-1 activity is controlled by modulation of its DNA-binding affinity. MTF-1 contains six zinc finger domains in combination with three transactivation domains (Brugnera et al. 1994; Radtke et al. 1995). The zinc fingers have been shown to differ in their affinity for zinc and it is believed that some of them (ZF1, ZF5, ZF6) only become occupied when zinc levels are high (Chen et al. 1998, 1999). Since all zinc fingers are required for maximal DNA-binding affinity (Westin and Schaffner 1988; Heuchel et al. 1994), full activation of MTF-1 is only achieved in zinc-replete conditions. Finally, MTF-1 activity can be stimulated up to fourfold by phosphorylation (LaRochelle et al. 2001). This phosphorylation is believed to mainly affect the function of the transactivation domains, as studies with kinase inhibitors have shown little effect on either subcellular localization or DNA binding (LaRochelle et al. 2001).

4 Interplay of transcriptional systems in determining limits to metal ion levels

Members of the different families of transcriptional regulators do not operate as individual units, but form integrated transcriptional metal-homeostasis systems. Transcription factors from different families often play opposing roles depending on whether they activate or repress transcription in their metal-bound states. Combined with different metal specificities and affinities, they can form intricate systems to maintain intracellular concentrations of multiple metals within the optimal

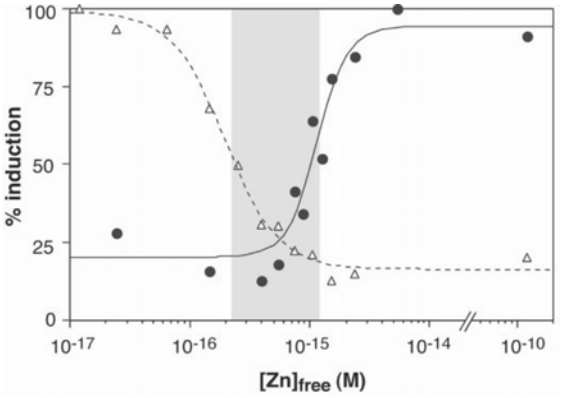


Fig. 9. Interplay between Zur and ZntR balances zinc uptake and efflux. The ability of Zur and ZntR to drive transcription of their respective *znuC* and *zntA* promoters was measured as a function of the free zinc concentration. The dotted line and solid line represent the fit of measured transcript levels driven by Zur (triangles) and ZntR (closed circles), respectively. The grey area is the range of free zinc between the half-maximal induction points of Zur and ZntR. Concentrations of RNA-polymerase, Zur, and Zntr were at 50nM, DNA was at 4nM, and TPEN was at 25 μM. The figure is reproduced with permission from Outten and O'Halloran (2001).

range for cellular processes. As previously discussed, iron-dependent gene regulation in microrganisms involves the joint actions of members from the Fur, DtxR and MerR families (Fig. 3). The latter family is indirectly involved through the oxidative control of Fur by SoxR (Zheng et al. 1999). Similarly, microarray studies of the global transcriptional response of *B. subtilis* to manganese indicate the involvement of the MntR, Fur, TnrA, and sigmaB regulons (Guedon et al. 2003).

The first observation of the degree to which these regulatory mechanisms can control metal levels came from *S. cerevisiae*, where the number of free copper atoms was found to be less than one per cell (Rae et al. 1999). This low concentration of unbound copper is reflected in a subfemtomolar Cu(I) affinity of the copper trafficking proteins (Xiao et al. 2004). An even tighter regulation is found for the copper-responsive CueR transcription factor in *E. coli*, which responds to free copper concentrations in the zeptomolar range (Changela et al. 2003). More evidence for extremely low concentrations of free zinc and mercury in *E. coli* comes from similar observations on the Zur, ZntR, and MerR regulators (Ralston and O'Halloran 1990; Ansari et al. 1995; Outten and O'Halloran 2001). Both Zur and Zntr respond to femtomolar free zinc concentrations, while MerR senses nanomolar concentrations of its metal ligand.

Zinc-dependent gene transcription in *E. coli* is regulated by Zur and ZntR, which are part of the Fur and MerR families, respectively. The interplay between these factors to maintain zinc homeostasis was demonstrated by *in vitro* transcription profiling of gene expression directed by the Zur-P_{znuC} and ZntR-P_{zntA} promoters as a function of the free zinc concentration (Outten and O'Halloran 2001) (Fig. 9). In conditions of zinc starvation, *E.coli* expresses *zurABC,* which encodes a

high-affinity ABC transporter for zinc uptake (Patzer and Hantke 1998). As intracellular zinc levels approach normal levels, the Zur regulator acquires Zn(II) and represses transcription of the uptake system by binding to the bidirectional promoter region of *znuA* and *znuCB* (Patzer and Hantke 1998, 2000). Half-maximal repression by Zur was found to occur at a free zinc concentration of $2x10^{-16}$ M. When zinc levels continue to rise and slightly exceed normal levels, Zn(II)-ZntR induces expression of *zntA,* encoding a zinc efflux pump (Rensing et al. 1997; Singh et al. 1999). ZntR shows half-maximal induction at higher levels than Zur, at a free zinc concentration of $11.5x10^{-16}$ M (Fig. 9).

The concentration boundaries set by these factors delineate a very narrow window for homeostatic regulation of cytoplasmic zinc levels, especially considering the volume of a typical *E. coli* cell. At a maximum cell volume of $1.8x10^{-15}$ liter, a free zinc concentration of 1 atom per cell would correspond to a concentration of $2x10^{-9}$ M, which is six orders of magnitude higher than the concentration needed to saturate both Zur and ZntR. It can therefore be concluded that no free pool of zinc exists in *E. coli*. This finding is consistent with observations in *Synechococcus* that suggest a Zn(II) affinity for the SmtB transcription factor in the 10^{-11} range (VanZile et al. 2000). In contrast to the prokaryotic systems, the sensitivity of the eukaryotic zinc binding factors MTF-1 and Zap1 is in the nanomolar to subnanomolar range (Giedroc et al. 2001; Bird et al. 2003). It therefore remains to be seen if eukaryotic cells also function without free zinc in their cytoplasm.

The data obtained from these studies of copper and zinc homeostasis point to a cellular environment that can maintain a large, high-affinity buffer capacity for these metals, far in excess of the total metal ion content of the cell. This is illustrated by the fact that the total zinc content of an *E. coli* cell is at least $2x10^5$ atoms, corresponding to a intracellular concentration of 0.2 mM, while free zinc concentrations are in the femtomolar range. The high sensitivity of zinc-responsive transcription factors in *E. coli* indicates that they are perfectly adapted to maintain this intracellular metal balance.

4.1 Metal ion acquisition and regulator specificity

The fact that free metal concentrations can be so low has implications for the selectivity of metallo-regulatory proteins. Even though a large number of metal-responsive transcription factors have been shown to bind different metals with varying affinities *in vitro*, the low biological availability of some of these metals in cells means that they may never play a regulatory role *in vivo*. Mechanisms that influence metal availability to transcription factors, such as biological pools of metal chelators, can therefore be important determinants for the specificity of metal homeostatic systems. A good example of the importance of the cellular environment in metal-selectivity of eukaryotic transcriptional regulators comes from *D. melanogaster* MTF-1, which predominantly responds to copper and cadmium (Zhang et al. 2001). However, when expressed in mammalian cells, the same regulator displays a zinc-dependent regulation similar to endogenous MTF-1 (Zhang et al. 2001).

Cells contain several compounds that can be involved in metal buffering, including glutathione and metallothioneins. In the case of *E. coli*, glutathione seems unlikely to be involved in copper buffering, since the affinity for this metal is lower than that of the copper-responsive transcription factors. If copper were available in the levels required for association with glutathione, this would result in constitutive gene expression by the CueR regulator. The CopY regulator of *E. hirae* is known to use the copper chaperone CopZ as a copper donor, thereby overcoming the limited concentrations of free copper (Solioz and Stoyanov 2003). Metal chaperones have also been identified in other organisms, including yeast and humans (Klomp et al. 1997; Lin et al. 1997), but their role in transcription regulation remains unclear.

Metallothioneins have been found to play an important role in the regulation of eukaryotic MTF-1 (Zhang et al. 2003). Activation of MTF-1 dependent gene transcription by copper or cadmium depends on the availability of zinc-saturated metallothioneins. It is presumed that exposure to these metals leads to displacement of zinc from the metallothioneins, which then results in activation of MTF-1.

The overall metal specificity of regulons involved in metal trafficking not only depends on metal selection by transcription factors, but also of their target genes. The relative contribution of the regulators and detoxification system in this selection is still unclear. Experiments on the ArsR/SmtB-regulated zinc and cobalt resistance systems in *Synechocystis* PCC 6803 indicate that swapping control of the zinc and cobalt export ATPases genes *ziaA* or *coaT* to their opposite regulators CoaA and ZntA, is not sufficient to restore resistance to metals sensed by the regulators (Borrelly et al. 2004). This indicates that metal specificity in this system is not exclusively determined at the level of the transcription factors. Replacement of the cytosolic N-terminal domain of CoaA by that of ZiaA does restore ZntR-mediated zinc resistance in a Δ*zia*Δ*coa* strain, indicating that this domain plays an important role in metal selection by metal export ATPases (Borrelly et al. 2004). Therefore, for correct reconstruction of pathways involved in metal regulation and trafficking, it is important to know the relative metal affinity of their individual components. Obtaining metal association/dissociation constants, combined with measurements of transcriptional levels at different metal ion concentrations would greatly facilitate accurate modeling of these homeostatic pathways.

5 Conclusions

Based on the number of transcription factors identified to date, metal-responsive gene regulation appears to play a more important role in prokaryotic metal homeostasis than in eukaryotes. Although this may in part be due to bias in the accessibility of these two different domains for research, it is also likely to reflect the greater complexity of eukaryotic systems. Interestingly, despite the large degree of conservation in prokaryotic transcription factor families, there appears to be a great diversity in the pathways that they regulate. A study of the presence of similar *S. cerevisiae* copper trafficking pathways in prokaryotes indicates that although

individual pathway components are highly conserved, these are used in a wide variety of distinct pathways (van Bakel et al. 2004). Sequence similarity searches with known regulators suggest the presence of many additional metal-regulated transcription factors in prokaryotic genomes. Characterization of these factors in the coming years will almost certainly lead to the identification of novel metal specificities and regulatory mechanisms, as well as novel metal homeostatic pathways.

New eukaryotic metal-responsive transcription systems are also likely to be discovered, including those that are sensitive to metals other than copper, iron, and zinc. For example, it has been observed that gene expression of the *S. cerevisiae* P-type Ca(II)/Mn(II)-ATPase PMR1 is induced in a calcineurin-dependent manner when Mn(II) or Ca(II) are added to the medium (Maeda et al. 2004). The relative contribution of transcriptional versus post-transcriptional in regulation of metal homeostatic systems in eukaryotes remains to be determined. The recent discovery of post-transcriptional regulation mediated by controlled RNA degradation in response to iron deprivation in *S. cerevisiae* (Puig et al. 2005) certainly indicates that the complete picture will be complex and most likely will involve control at many different levels.

Acknowledgements

We would like to thank Jackie Senior for editing the manuscript. This work was supported by the Netherlands Organization for Scientific Research (NWO) grant 901-04-219.

References

Abdul-Tehrani H, Hudson A, Chang Y, Timms A, Hawkins C, Williams J, Harrison P, Guest J, Andrews S (1999) Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and fur mutants are iron deficient. J Bacteriol 181:1415-1428

Ahmed M, Borsch CM, Taylor SS, Vazquez-Laslop N, Neyfakh AA (1994) A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. J Biol Chem 269:28506-28513

Ahmed M, Lyass L, Markham P, Taylor S, Vazquez-Laslop N, Neyfakh A (1995) Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. J Bacteriol 177:3904-3910

Althaus EW, Outten CE, Olson KE, Cao H, O'Halloran TV (1999) The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. Biochemistry 38:6559-6569

Amabile-Cuevas CF, Demple B (1991) Molecular characterization of the soxRS genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res 19:4479-4484

Anderson L, McNairn E, Lubke T, Pau R, Boxer D (2000) ModE-dependent molybdate regulation of the molybdenum cofactor operon moa in *Escherichia coli*. J Bacteriol 182:7035-7043

Ansari AZ, Bradner JE, O'Halloran TV (1995) DNA-bend modulation in a repressor-to-activator switching mechanism. Nature 374:371-375

Auf der Maur A, Belser T, Elgar G, Georgiev O, Schaffner W (1999) Characterization of the transcription factor MTF-1 from the Japanese pufferfish (*Fugu rubripes*) reveals evolutionary conservation of heavy metal stress response. Biol Chem 380:175-185

Bagg A, Neilands J (1985) Mapping of a mutation affecting regulation of iron uptake systems in *Escherichia coli* K-12. J Bacteriol 161:450-453

Bagg A, Neilands J (1987) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry 26:5471-5477

Baichoo N, Helmann J (2002) Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J Bacteriol 184:5826-5832

Baichoo N, Wang T, Ye R, Helmann JD (2002) Global analysis of the *Bacillus subtilis* Fur regulon and the iron starvation stimulon. Mol Microbiol 45:1613-1629

Bearden SW, Perry RD (1999) The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. Mol Microbiol 32:403-414

Beaudoin J, Labbe S (2001) The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. J Biol Chem 276:15472-15480

Beaudoin J, Mercier A, Langlois R, Labbe S (2003) The *Schizosaccharomyces pombe* Cuf1 is composed of functional modules from two distinct classes of copper metalloregulatory transcription factors. J Biol Chem 278:14565-14577

Bender CL, Cooksey DA (1987) Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. tomato. J Bacteriol 169:470-474

Bird A, Blankman E, Stillman D, Eide D, Winge D (2004) The Zap1 transcriptional activator also acts as a repressor by binding downstream of the TATA box in ZRT2. Embo J 23:1123-1132

Bird A, Evans-Galea M, Blankman E, Zhao H, Luo H, Winge D, Eide D (2000a) Mapping the DNA binding domain of the Zap1 zinc-responsive transcriptional activator. J Biol Chem 275:16160-16166

Bird A, Zhao H, Luo H, Jensen L, Srinivasan C, Evans-Galea M, Winge D, Eide D (2000b) A dual role for zinc fingers in both DNA binding and zinc sensing by the Zap1 transcriptional activator. Embo J 19:3704-3713

Bird AJ, McCall K, Kramer M, Blankman E, Winge DR, Eide DJ (2003) Zinc fingers can act as Zn2+ sensors to regulate transcriptional activation domain function. Embo J 22:5137-5146

Blaiseau P, Lesuisse E, Camadro J (2001) Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. J Biol Chem 276:34221-34226

Boland C, Meijer W (2000) The iron dependent regulatory protein IdeR (DtxR) of *Rhodococcus equi*. FEMS Microbiol Lett 191:1-5

Borghouts C, Osiewacz H (1998) GRISEA, a copper-modulated transcription factor from *Podospora anserina* involved in senescence and morphogenesis, is an ortholog of MAC1 in *Saccharomyces cerevisiae*. Mol Gen Genet 260:492-502

Borrelly G, Rondet S, Tottey S, Robinson N (2004) Chimeras of P-type ATPases and their transcriptional regulators: contributions of a cytosolic amino-terminal domain to metal specificity. Mol Microbiol 53:217-227

Borremans B, Hobman J, Provoost A, Brown N, van Der Lelie D (2001) Cloning and functional analysis of the pbr lead resistance determinant of *Ralstonia metallidurans* CH34. J Bacteriol 183:5651-5658

Boyd J, Oza M, Murphy J (1990) Molecular cloning and DNA sequence analysis of a diphtheria tox iron-dependent regulatory element (dtxR) from *Corynebacterium diphtheriae*. Proc Natl Acad Sci USA 87:5968-5972

Braun V (2003) Iron uptake by *Escherichia coli*. Front Biosci 8:s1409-1421

Brocklehurst KR, Hobman JL, Lawley B, Blank L, Marshall SJ, Brown NL, Morby AP (1999) ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of zntA in *Escherichia coli*. Mol Microbiol 31:893-902

Brown K, Keller G, Pickering I, Harris H, George G, Winge D (2002) Structures of the cuprous-thiolate clusters of the Mac1 and Ace1 transcriptional activators. Biochemistry 41:6469-6476

Brown N, Rouch D, Lee B (1992) Copper resistance determinants in bacteria. Plasmid 27:41-51

Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland G, Schaffner W (1994) Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. Nucleic Acids Res 22:3167-3173

Bsat N, Herbig A, Casillas-Martinez L, Setlow P, Helmann J (1998) *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol Microbiol 29:189-198

Busenlehner LS, Pennella MA, Giedroc DP (2003) The SmtB/ArsR family of metalloregulatory transcriptional repressors: Structural insights into prokaryotic metal resistance. FEMS Microbiol Rev 27:131-143

Busenlehner LS, Weng TC, Penner-Hahn JE, Giedroc DP (2002) Elucidation of primary (alpha(3)N) and vestigial (alpha(5)) heavy metal-binding sites in *Staphylococcus aureus* pI258 CadC: evolutionary implications for metal ion selectivity of ArsR/SmtB metal sensor proteins. J Mol Biol 319:685-701

Cavet J, Meng W, Pennella M, Appelhoff R, Giedroc D, Robinson N (2002) A nickel-cobalt-sensing ArsR-SmtB family repressor. Contributions of cytosol and effector binding sites to metal selectivity. J Biol Chem 277:38441-38448

Cavet JS, Graham AI, Meng W, Robinson NJ (2003) A cadmium-lead-sensing ArsR-SmtB repressor with novel sensory sites. Complementary metal discrimination by NmtR AND CmtR in a common cytosol. J Biol Chem 278:44560-44566

Champier L, Duarte V, Michaud-Soret I, Coves J (2004) Characterization of the MerD protein from *Ralstonia metallidurans* CH34: a possible role in bacterial mercury resistance by switching off the induction of the mer operon. Mol Microbiol 52:1475-1485

Changela A, Chen K, Xue Y, Holschen J, Outten CE, O'Halloran TV, Mondragon A (2003) Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science 301:1383-1387

Chen X, Agarwal A, Giedroc D (1998) Structural and functional heterogeneity among the zinc fingers of human MRE-binding transcription factor-1. Biochemistry 37:11152-11161

Chen X, Chu M, Giedroc D (1999) MRE-Binding transcription factor-1: weak zinc-binding finger domains 5 and 6 modulate the structure, affinity, and specificity of the metal-response element complex. Biochemistry 38:12915-12925

Chen Y, Rosen B (1997) Metalloregulatory properties of the ArsD repressor. J Biol Chem 272:14257-14262

Chivers PT, Sauer RT (1999) NikR is a ribbon-helix-helix DNA-binding protein. Protein Sci 8:2494-2500

Chivers PT, Sauer RT (2000) Regulation of high affinity nickel uptake in bacteria. Ni2+-Dependent interaction of NikR with wild-type and mutant operator sites. J Biol Chem 275:19735-19741

Chou C, Wisedchaisri G, Monfeli R, Oram D, Holmes R, Hol W, Beeson C (2004) Functional studies of the iron-dependent regulator. J Biol Chem 279:53554-53561

Contreras M, Thiberge JM, Mandrand-Berthelot MA, Labigne A (2003) Characterization of the roles of NikR, a nickel-responsive pleiotropic autoregulator of *Helicobacter pylori*. Mol Microbiol 49:947-963

Cook W, Kar S, Taylor K, Hall L (1998) Crystal structure of the cyanobacterial metallothionein repressor SmtB: a model for metalloregulatory proteins. J Mol Biol 275:337-346

Coy M, Neilands J (1991) Structural dynamics and functional domains of the fur protein. Biochemistry 30:8201-8210

Cragg R, Christie G, Phillips S, Russi R, Kury S, Mathers J, Taylor P, Ford D (2002) A novel zinc-regulated human zinc transporter, hZTL1, is localized to the enterocyte apical membrane. J Biol Chem 277:22789-22797

Dalet K, Gouin E, Cenatiempo Y, Cossart P, Hechard Y (1999) Characterisation of a new operon encoding a Zur-like protein and an associated ABC zinc permease in *Listeria monocytogenes*. FEMS Microbiol Lett 174:111-116

Dameron C, Winge D, George G, Sansone M, Hu S, Hamer D (1991) A copper-thiolate polynuclear cluster in the ACE1 transcription factor. Proc Natl Acad Sci USA 88:6127-6131

De Freitas JM, Kim JH, Poynton H, Su T, Wintz H, Fox T, Holman P, Loguinov A, Keles S, van der Laan M, Vulpe C (2004) Exploratory and confirmatory gene expression profiling of mac1Delta. J Biol Chem 279:4450-4458

De Lorenzo V, Herrero M, Giovannini F, Neilands JB (1988) Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of fur gene in *Escherichia coli*. Eur J Biochem 173:537-546

de Lorenzo V, Wee S, Herrero M, Neilands J (1987) Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor. J Bacteriol 169:2624-2630

De Pina K, Desjardin V, Mandrand-Berthelot MA, Giordano G, Wu LF (1999) Isolation and characterization of the nikR gene encoding a nickel-responsive regulator in *Escherichia coli*. J Bacteriol 181:670-674

Dell CL, Neely MN, Olson ER (1994) Altered pH and lysine signalling mutants of cadC, a gene encoding a membrane-bound transcriptional activator of the *Escherichia coli* cadBA operon. Mol Microbiol 14:7-16

Demple B (1996) Redox signaling and gene control in the *Escherichia coli* soxRS oxidative stress regulon--a review. Gene 179:53-57

Dey S, Dou D, Tisa L, Rosen B (1994) Interaction of the catalytic and the membrane subunits of an oxyanion-translocating ATPase. Arch Biochem Biophys 311:418-424

Dey S, Rosen B (1995) Dual mode of energy coupling by the oxyanion-translocating ArsB protein. J Bacteriol 177:385-389

Ding H, Demple B (1997) *In vivo* kinetics of a redox-regulated transcriptional switch. Proc Natl Acad Sci USA 94:8445-8449

Ding X, Zeng H, Schiering N, Ringe D, Murphy J (1996) Identification of the primary metal ion-activation sites of the diphtheria tox repressor by X-ray crystallography and site-directed mutational analysis. Nat Struct Biol 3:382-387

Diorio C, Cai J, Marmor J, Shinder R, DuBow M (1995) An *Escherichia coli* chromosomal ars operon homolog is functional in arsenic detoxification and is conserved in gram-negative bacteria. J Bacteriol 177:2050-2056

Dubrac S, Touati D (2000) Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the sodB promoter. J Bacteriol 182:3802-3808

Dussurget O, Rodriguez M, Smith I (1996) An ideR mutant of *Mycobacterium smegmatis* has derepressed siderophore production and an altered oxidative-stress response. Mol Microbiol 22:535-544

Dussurget O, Timm J, Gomez M, Gold B, Yu S, Sabol SZ, Holmes RK, Jacobs WR Jr, Smith I (1999) Transcriptional control of the iron-responsive fxbA gene by the mycobacterial regulator IdeR. J Bacteriol 181:3402-3408

Egli D, Selvaraj A, Yepiskoposyan H, Zhang B, Hafen E, Georgiev O, Schaffner W (2003) Knockout of 'metal-responsive transcription factor' MTF-1 in *Drosophila* by homologous recombination reveals its central role in heavy metal homeostasis. Embo J 22:100-108

Eicken C, Pennella M, Chen X, Koshlap K, VanZile M, Sacchettini J, Giedroc D (2003) A metal-ligand-mediated intersubunit allosteric switch in related SmtB/ArsR zinc sensor proteins. J Mol Biol 333:683-695

Eitinger T, Mandrand-Berthelot MA (2000) Nickel transport systems in microorganisms. Arch Microbiol 173:1-9

Endo G, Silver S (1995) CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. J Bacteriol 177:4437-4441

Erbe J, Taylor K, Hall L (1995) Metalloregulation of the cyanobacterial smt locus: identification of SmtB binding sites and direct interaction with metals. Nucleic Acids Res 23:2472-2478

Eriksson M, Moseley J, Tottey S, Del Campo J, Quinn J, Kim Y, Merchant S (2004) Genetic dissection of nutritional copper signaling in chlamydomonas distinguishes regulatory and target genes. Genetics 168:795-807

Ernst J, Bennett R, Rothfield L (1978) Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium*. J Bacteriol 135:928-934

Escolar L, de Lorenzo V, Perez-Martin J (1997) Metalloregulation *in vitro* of the aerobactin promoter of *Escherichia coli* by the Fur (ferric uptake regulation) protein. Mol Microbiol 26:799-808

Escolar L, Perez-Martin J, de Lorenzo V (2000) Evidence of an unusually long operator for the fur repressor in the aerobactin promoter of *Escherichia coli*. J Biol Chem 275:24709-24714

Evans-Galea M, Blankman E, Myszka D, Bird A, Eide D, Winge D (2003) Two of the five zinc fingers in the Zap1 transcription factor DNA binding domain dominate site-specific DNA binding. Biochemistry 42:1053-1061

Farrell R, Thorvaldsen J, Winge D (1996) Identification of the Zn(II) site in the copper-responsive yeast transcription factor, AMT1: a conserved Zn module. Biochemistry 35:1571-1580

Foussard M, Cabantous S, Pedelacq J, Guillet V, Tranier S, Mourey L, Birck C, Samama J (2001) The molecular puzzle of two-component signaling cascades. Microbes Infect 3:417-424

Franke S, Grass G, Rensing C, Nies D (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J Bacteriol 185:3804-3812

Fuangthong M, Herbig A, Bsat N, Helmann J (2002) Regulation of the *Bacillus subtilis* fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. J Bacteriol 184:3276-3286

Gaballa A, Helmann J (1998) Identification of a zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. J Bacteriol 180:5815-5821

Garcia S, Prado M, Degano R, Dominguez A (2002) A copper-responsive transcription factor, CRF1, mediates copper and cadmium resistance in *Yarrowia lipolytica*. J Biol Chem 277:37359-37368

Garcia-Dominguez M, Lopez-Maury L, Florencio F, Reyes J (2000) A gene cluster involved in metal homeostasis in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 182:1507-1514

Giedroc DP, Chen X, Pennella MA, LiWang AC (2001) Conformational heterogeneity in the C-terminal zinc fingers of human MTF-1: an NMR and zinc-binding study. J Biol Chem 276:42322-42332

Glasfeld A, Guedon E, Helmann J, Brennan R (2003) Structure of the manganese-bound manganese transport regulator of *Bacillus subtilis*. Nat Struct Biol 10:652-657

Gourley D, Schuttelkopf A, Anderson L, Price N, Boxer D, Hunter W (2001) Oxyanion binding alters conformation and quaternary structure of the c-terminal domain of the transcriptional regulator mode. Implications for molybdate-dependent regulation, signaling, storage, and transport. J Biol Chem 276:20641-20647

Graden J, Posewitz M, Simon J, George G, Pickering I, Winge D (1996) Presence of a copper(I)-thiolate regulatory domain in the copper-activated transcription factor Amt1. Biochemistry 35:14583-14589

Graden J, Winge D (1997) Copper-mediated repression of the activation domain in the yeast Mac1p transcription factor. Proc Natl Acad Sci USA 94:5550-5555

Gross C, Kelleher M, Iyer VR, Brown PO, Winge DR (2000) Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. J Biol Chem 275:32310-32316

Grunden A, Ray R, Rosentel J, Healy F, Shanmugam K (1996) Repression of the *Escherichia coli* modABCD (molybdate transport) operon by ModE. J Bacteriol 178:735-744

Guedon E, Helmann J (2003) Origins of metal ion selectivity in the DtxR/MntR family of metalloregulators. Mol Microbiol 48:495-506

Guedon E, Moore C, Que Q, Wang T, Ye R, Helmann J (2003) The global transcriptional response of *Bacillus subtilis* to manganese involves the MntR, Fur, TnrA and sigmaB regulons. Mol Microbiol 49:1477-1491

Gunes C, Heuchel R, Georgiev O, Muller K, Lichtlen P, Bluthmann H, Marino S, Aguzzi A, Schaffner W (1998) Embryonic lethality and liver degeneration in mice lacking the metal-responsive transcriptional activator MTF-1. Embo J 17:2846-2854

Haas H, Angermayr K, Stoffler G (1997) Molecular analysis of a *Penicillium chrysogenum* GATA factor encoding gene (sreP) exhibiting significant homology to the *Ustilago maydis* urbs1 gene. Gene 184:33-37

Haas H, Zadra I, Stoffler G, Angermayr K (1999) The *Aspergillus nidulans* GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake. J Biol Chem 274:4613-4619

Hall D, Gourley D, Leonard G, Duke E, Anderson L, Boxer D, Hunter W (1999) The high-resolution crystal structure of the molybdate-dependent transcriptional regulator (ModE) from *Escherichia coli*: a novel combination of domain folds. Embo J 18:1435-1446

Hall H, Foster J (1996) The role of fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. J Bacteriol 178:5683-5691

Hamza I, Chauhan S, Hassett R, O'Brian M (1998) The bacterial irr protein is required for coordination of heme biosynthesis with iron availability. J Biol Chem 273:21669-21674

Hamza I, Qi Z, King N, O'Brian M (2000) Fur-independent regulation of iron metabolism by Irr in *Bradyrhizobium japonicum*. Microbiology 146 (Pt 3):669-676

Hantke K (1981) Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol Gen Genet 182:288-292

Hantke K (1984) Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K12. Mol Gen Genet 197:337-341

Hantke K (1987) Selection procedure for deregulated iron transport mutants (fur) in *Escherichia coli* K 12: fur not only affects iron metabolism. Mol Gen Genet 210:135-139

Hantke K (2001) Iron and metal regulation in bacteria. Curr Opin Microbiol 4:172-177

Harrison K, Marzluf G (2002) Characterization of DNA binding and the cysteine rich region of SRE, a GATA factor in *Neurospora crassa* involved in siderophore synthesis. Biochemistry 41:15288-15295

Hassan M, van der Lelie D, Springael D, Romling U, Ahmed N, Mergeay M (1999) Identification of a gene cluster, czr, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. Gene 238:417-425

Haurie V, Boucherie H, Sagliocco F (2003) The Snf1 protein kinase controls the induction of genes of the iron uptake pathway at the diauxic shift in *Saccharomyces cerevisiae*. J Biol Chem 278:45391-45396

Hazlett K, Rusnak F, Kehres D, Bearden S, La Vake C, La Vake M, Maguire M, Perry R, Radolf J (2003) The *Treponema pallidum* tro operon encodes a multiple metal transporter, a zinc-dependent transcriptional repressor, and a semi-autonomously expressed phosphoglycerate mutase. J Biol Chem 278:20687-20694

Heldwein EE, Brennan RG (2001) Crystal structure of the transcription activator BmrR bound to DNA and a drug. Nature 409:378-382

Hentze MW, Muckenthaler MU, Andrews NC (2004) Balancing acts: molecular control of mammalian iron metabolism. Cell 117:285-297

Heredia J, Crooks M, Zhu Z (2001) Phosphorylation and Cu+ coordination-dependent DNA binding of the transcription factor Mac1p in the regulation of copper transport. J Biol Chem 276:8793-8797

Heuchel R, Radtke F, Georgiev O, Stark G, Aguet M, Schaffner W (1994) The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. Embo J 13:2870-2875

Hidalgo E, Demple B (1997) Spacing of promoter elements regulates the basal expression of the soxS gene and converts SoxR from a transcriptional activator into a repressor. Embo J 16:1056-1065

Horsburgh M, Clements M, Crossley H, Ingham E, Foster S (2001) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infect Immun 69:3744-3754

Huckle JW, Morby AP, Turner JS, Robinson NJ (1993) Isolation of a prokaryotic metallothionein locus and analysis of transcriptional control by trace metal ions. Mol Microbiol 7:177-187

Huffman D, Huyett J, Outten F, Doan P, Finney L, Hoffman B, O'Halloran T (2002) Spectroscopy of Cu(II)-PcoC and the multicopper oxidase function of PcoA, two essential components of *Escherichia coli* pco copper resistance operon. Biochemistry 41:10046-10055

Huibregtse J, Engelke D, Thiele D (1989) Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences. Proc Natl Acad Sci USA 86:65-69

Jacquamet L, Aberdam D, Adrait A, Hazemann J, Latour J, Michaud-Soret I (1998) X-ray absorption spectroscopy of a new zinc site in the fur protein from *Escherichia coli*. Biochemistry 37:2564-2571

Jamison McDaniels C, Jensen L, Srinivasan C, Winge D, Tullius T (1999) The yeast transcription factor Mac1 binds to DNA in a modular fashion. J Biol Chem 274:26962-26967

Jensen L, Winge D (1998) Identification of a copper-induced intramolecular interaction in the transcription factor Mac1 from *Saccharomyces cerevisiae*. Embo J 17:5400-5408

Ji G, Silver S (1992a) Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of *Staphylococcus aureus* plasmid pI258. Proc Natl Acad Sci USA 89:9474-9478

Ji G, Silver S (1992b) Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid pI258. J Bacteriol 174:3684-3694

Jones A, DeShazer D, Woods D (1997) Identification and characterization of a two-component regulatory system involved in invasion of eukaryotic cells and heavy-metal resistance in *Burkholderia pseudomallei*. Infect Immun 65:4972-4977

Joshi A, Serpe M, Kosman D (1999) Evidence for (Mac1p)2.DNA ternary complex formation in Mac1p-dependent transactivation at the CTR1 promoter. J Biol Chem 274:218-226

Jubier-Maurin V, Rodrigue A, Ouahrani-Bettache S, Layssac M, Mandrand-Berthelot MA, Kohler S, Liautard JP (2001) Identification of the nik gene cluster of *Brucella suis*: regulation and contribution to urease activity. J Bacteriol 183:426-434

Jungmann J, Reins H, Lee J, Romeo A, Hassett R, Kosman D, Jentsch S (1993) MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. Embo J 12:5051-5056

Kar S, Adams A, Lebowitz J, Taylor K, Hall L (1997) The cyanobacterial repressor SmtB is predominantly a dimer and binds two Zn2+ ions per subunit. Biochemistry 36:15343-15348

Kar S, Lebowitz J, Blume S, Taylor K, Hall L (2001) SmtB-DNA and protein-protein interactions in the formation of the cyanobacterial metallothionein repression complex: Zn2+ does not dissociate the protein-DNA complex *in vitro*. Biochemistry 40:13378-13389

Karjalainen T, Evans D, Evans D Jr, Graham D, Lee C (1991) Iron represses the expression of CFA/I fimbriae of enterotoxigenic *E. coli*. Microb Pathog 11:317-323

Kehres D, Janakiraman A, Slauch J, Maguire M (2002) SitABCD is the alkaline Mn(2+) transporter of *Salmonella enterica* serovar *Typhimurium*. J Bacteriol 184:3159-3166

Kehres DG, Zaharik ML, Finlay BB, Maguire ME (2000) The NRAMP proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. Mol Microbiol 36:1085-1100

Keller G, Gross C, Kelleher M, Winge D (2000) Functional independence of the two cysteine-rich activation domains in the yeast Mac1 transcription factor. J Biol Chem 275:29193-29199

Kim BE, Wang F, Dufner-Beattie J, Andrews GK, Eide DJ, Petris MJ (2004) Zn2+-stimulated endocytosis of the mZIP4 zinc transporter regulates its location at the plasma membrane. J Biol Chem 279:4523-4530

King KY, Horenstein JA, Caparon MG (2000) Aerotolerance and peroxide resistance in peroxidase and PerR mutants of *Streptococcus pyogenes*. J Bacteriol 182:5290-5299

Kispal G, Csere P, Prohl C, Lill R (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. Embo J 18:3981-3989

Klomp LW, Lin SJ, Yuan DS, Klausner RD, Culotta VC, Gitlin JD (1997) Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis. J Biol Chem 272:9221-9226

Knight S, Lesuisse E, Stearman R, Klausner R, Dancis A (2002) Reductive iron uptake by Candida albicans: role of copper, iron and the TUP1 regulator. Microbiology 148:29-40

Koch K, Allard S, Santoro N, Cote J, Thiele D (2001) The *Candida glabrata* Amt1 copper-sensing transcription factor requires Swi/Snf and Gcn5 at a critical step in copper detoxification. Mol Microbiol 40:1165-1174

Koch K, Thiele D (1996) Autoactivation by a *Candida glabrata* copper metalloregulatory transcription factor requires critical minor groove interactions. Mol Cell Biol 16:724-734

Kolade O, Bellini P, Wexler M, Johnston A, Grossmann J, Hemmings A (2002) Structural studies of the Fur protein from *Rhizobium leguminosarum*. Biochem Soc Trans 30:771-774

Koo MS, Lee JH, Rah SY, Yeo WS, Lee JW, Lee KL, Koh YS, Kang SO, Roe JH (2003) A reducing system of the superoxide sensor SoxR in *Escherichia coli*. Embo J 22:2614-2622

Kuroda M, Hayashi H, Ohta T (1999) Chromosome-determined zinc-responsible operon czr in *Staphylococcus aureus* strain 912. Microbiol Immunol 43:115-125

Kutsche M, Leimkuhler S, Angermuller S, Klipp W (1996) Promoters controlling expression of the alternative nitrogenase and the molybdenum uptake system in *Rhodobacter capsulatus* are activated by NtrC, independent of sigma54, and repressed by molybdenum. J Bacteriol 178:2010-2017

Labbe S, Pena M, Fernandes A, Thiele D (1999) A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. J Biol Chem 274:36252-36260

Langmade S, Ravindra R, Daniels P, Andrews G (2000) The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J Biol Chem 275:34803-34809

LaRochelle O, Gagne V, Charron J, Soh J, Seguin C (2001) Phosphorylation is involved in the activation of metal-regulatory transcription factor 1 in response to metal ions. J Biol Chem 276:41879-41888

Lee JH, Wang T, Ault K, Liu J, Schmitt MP, Holmes RK (1997) Identification and characterization of three new promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. Infect Immun 65:4273-4280

Lee S, Grass G, Rensing C, Barrett S, Yates C, Stoyanov J, Brown N (2002) The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*. Biochem Biophys Res Commun 295:616-620

Lee Y, Deka R, Norgard M, Radolf J, Hasemann C (1999) *Treponema pallidum* TroA is a periplasmic zinc-binding protein with a helical backbone. Nat Struct Biol 6:628-633

Lewin B (2000) Promotor recognition depends on consensus sequences. In: Lewin B (ed) Genes VII. Oxford University Press Inc., New York, pp 244-246

Lim C, Cooksey D (1993) Characterization of chromosomal homologs of the plasmid-borne copper resistance operon of *Pseudomonas syringae*. J Bacteriol 175:4492-4498

Lin SJ, Pufahl RA, Dancis A, O'Halloran TV, Culotta VC (1997) A role for the *Saccharomyces cerevisiae* ATX1 gene in copper trafficking and iron transport. J Biol Chem 272:9215-9220

Lindsay JA, Foster SJ (2001) zur: a Zn(2+)-responsive regulatory element of *Staphylococcus aureus*. Microbiology 147:1259-1266

Lopez-Maury L, Garcia-Dominguez M, Florencio F, Reyes J (2002) A two-component signal transduction system involved in nickel sensing in the cyanobacterium *Synechocystis* sp. PCC 6803. Mol Microbiol 43:247-256

Love J, vanderSpek J, Marin V, Guerrero L, Logan T, Murphy J (2004) Genetic and biophysical studies of diphtheria toxin repressor (DtxR) and the hyperactive mutant DtxR(E175K) support a multistep model of activation. Proc Natl Acad Sci USA 101:2506-2511

Love J, VanderSpek J, Murphy J (2003) The src homology 3-like domain of the diphtheria toxin repressor (DtxR) modulates repressor activation through interaction with the ancillary metal ion-binding site. J Bacteriol 185:2251-2258

Lund PA, Brown NL (1989) Regulation of transcription in *Escherichia coli* from the mer and merR promoters in the transposon Tn501. J Mol Biol 205:343-353

Lund PA, Ford SJ, Brown NL (1986) Transcriptional regulation of the mercury-resistance genes of transposon Tn501. J Gen Microbiol 132 (Pt 2):465-480

Lyons T, Gasch A, Gaither L, Botstein D, Brown P, Eide D (2000) Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast. Proc Natl Acad Sci USA 97:7957-7962

MacDiarmid C, Milanick M, Eide D (2002) Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. J Biol Chem 277:39187-39194

MacDiarmid C, Milanick M, Eide D (2003) Induction of the ZRC1 metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. J Biol Chem 278:15065-15072

Maeda T, Sugiura R, Kita A, Saito M, Deng L, He Y, Yabin L, Fujita Y, Takegawa K, Shuntoh H, Kuno T (2004) Pmr1, a P-type ATPase, and Pdt1, an Nramp homologue, cooperatively regulate cell morphogenesis in fission yeast: the importance of Mn2+ homeostasis. Genes Cells 9:71-82

Masepohl B, Klipp W (1996) Organization and regulation of genes encoding the molybdenum nitrogenase and the alternative nitrogenase in *Rhodobacter capsulatus*. Arch Microbiol 165:80-90

McNicholas P, Chiang R, Gunsalus R (1998) Anaerobic regulation of the *Escherichia coli* dmsABC operon requires the molybdate-responsive regulator ModE. Mol Microbiol 27:197-208

McNicholas P, Gunsalus R (2002) The molybdate-responsive *Escherichia coli* ModE transcriptional regulator coordinates periplasmic nitrate reductase (napFDAGHBC) operon expression with nitrate and molybdate availability. J Bacteriol 184:3253-3259

McNicholas P, Rech S, Gunsalus R (1997) Characterization of the ModE DNA-binding sites in the control regions of modABCD and moaABCDE of *Escherichia coli*. Mol Microbiol 23:515-524

Messenger SL, Green J (2003) FNR-mediated regulation of hyp expression in *Escherichia coli*. FEMS Microbiol Lett 228:81-86

Michaud-Soret I, Adrait A, Jaquinod M, Forest E, Touati D, Latour J (1997) Electrospray ionization mass spectrometry analysis of the apo- and metal-substituted forms of the Fur protein. FEBS Lett 413:473-476

Mills S, Jasalavich C, Cooksey D (1993) A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. J Bacteriol 175:1656-1664

Morby AP, Turner JS, Huckle JW, Robinson NJ (1993) SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene smtA: identification of a Zn inhibited DNA-protein complex. Nucleic Acids Res 21:921-925

Mouncey N, Mitchenall L, Pau R (1996) The modE gene product mediates molybdenum-dependent expression of genes for the high-affinity molybdate transporter and modG in *Azotobacter vinelandii*. Microbiology 142 (Pt 8):1997-2004

Mukhopadhyay D, Yu HR, Nucifora G, Misra TK (1991) Purification and functional characterization of MerD. A coregulator of the mercury resistance operon in gram-negative bacteria. J Biol Chem 266:18538-18542

Munson G, Lam D, Outten F, O'Halloran T (2000) Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J Bacteriol 182:5864-5871

Navarro C, Wu LF, Mandrand-Berthelot MA (1993) The nik operon of *Escherichia coli* encodes a periplasmic binding-protein-dependent transport system for nickel. Mol Microbiol 9:1181-1191

Niederhoffer EC, Naranjo CM, Bradley KL, Fee JA (1990) Control of *Escherichia coli* superoxide dismutase (sodA and sodB) genes by the ferric uptake regulation (fur) locus. J Bacteriol 172:1930-1938

Noll M, Petrukhin K, Lutsenko S (1998) Identification of a novel transcription regulator from *Proteus mirabilis*, PMTR, revealed a possible role of YJAI protein in balancing zinc in *Escherichia coli*. J Biol Chem 273:21393-21401

Nucifora G, Silver S, Misra TK (1989) Down regulation of the mercury resistance operon by the most promoter-distal gene merD. Mol Gen Genet 220:69-72

Nunoshiba T, Hidalgo E, Amabile Cuevas CF, Demple B (1992) Two-stage control of an oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the soxS regulatory gene. J Bacteriol 174:6054-6060

Ochsner UA, Vasil ML (1996) Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc Natl Acad Sci USA 93:4409-4414

Ogawa T, Bao D, Katoh H, Shibata M, Pakrasi H, Bhattacharyya-Pakrasi M (2002) A two-component signal transduction pathway regulates manganese homeostasis in *Synechocystis* 6803, a photosynthetic organism. J Biol Chem 277:28981-28986

O'Halloran TV, Walsh CT (1986) Positive and negative control of prokaryotic gene-expression by a metalloprotein - purification and characterization of the MerR regulatory protein. J Cell Biochem Suppl 10D

Osorio C, Lemos M, Braun V (2004) Identification of Fur regulated genes in the bacterial fish pathogen *Photobacterium damselae* ssp. piscicida using the Fur titration assay. Biometals 17:725-733

Outten C, Tobin D, Penner-Hahn J, O'Halloran T (2001) Characterization of the metal receptor sites in *Escherichia coli* Zur, an ultrasensitive zinc(II) metalloregulatory protein. Biochemistry 40:10417-10423

Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488-2492

Outten CE, Outten FW, O'Halloran TV (1999) DNA distortion mechanism for transcriptional activation by ZntR, a Zn(II)-responsive MerR homologue in *Escherichia coli*. J Biol Chem 274:37517-37524

Outten FW, Outten CE, Hale J, O'Halloran TV (2000) Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, cueR. J Biol Chem 275:31024-31029

Parkhill J, Ansari AZ, Wright JG, Brown NL, O'Halloran TV (1993) Construction and characterization of a mercury-independent MerR activator (MerRAC): transcriptional activation in the absence of Hg(II) is accompanied by DNA distortion. Embo J 12:413-421

Patzer S, Hantke K (2001) Dual repression by Fe(2+)-Fur and Mn(2+)-MntR of the mntH gene, encoding an NRAMP-like Mn(2+) transporter in *Escherichia coli*. J Bacteriol 183:4806-4813

Patzer SI, Hantke K (1998) The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol Microbiol 28:1199-1210

Patzer SI, Hantke K (2000) The zinc-responsive regulator Zur and its control of the znu gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. J Biol Chem 275:24321-24332

Pelletier B, Beaudoin J, Mukai Y, Labbe S (2002) Fep1, an iron sensor regulating iron transporter gene expression in *Schizosaccharomyces pombe*. J Biol Chem 277:22950-22958

Pelletier B, Beaudoin J, Philpott C, Labbe S (2003) Fep1 represses expression of the fission yeast *Schizosaccharomyces pombe* siderophore-iron transport system. Nucleic Acids Res 31:4332-4344

Pennella M, Shokes J, Cosper N, Scott R, Giedroc D (2003) Structural elements of metal selectivity in metal sensor proteins. Proc Natl Acad Sci USA 100:3713-3718

Petersen C, Moller LB (2000) Control of copper homeostasis in *Escherichia coli* by a P-type ATPase, CopA, and a MerR-like transcriptional activator, CopR. Gene 261:289-298

Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J (1996) Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi

apparatus to the plasma membrane: a novel mechanism of regulated trafficking. Embo J 15:6084-6095

Petris MJ, Smith K, Lee J, Thiele DJ (2003) Copper-stimulated endocytosis and degradation of the human copper transporter, hCtr1. J Biol Chem 278:9639-9646

Pohl E, Goranson-Siekierke J, Choi M, Roosild T, Holmes R, Hol W (2001) Structures of three diphtheria toxin repressor (DtxR) variants with decreased repressor activity. Acta Crystallogr D Biol Crystallogr 57:619-627

Pohl E, Haller J, Mijovilovich A, Meyer-Klaucke W, Garman E, Vasil M (2003) Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. Mol Microbiol 47:903-915

Pohl E, Holmes R, Hol W (1998) Motion of the DNA-binding domain with respect to the core of the diphtheria toxin repressor (DtxR) revealed in the crystal structures of apo- and holo-DtxR. J Biol Chem 273:22420-22427

Pohl E, Holmes R, Hol W (1999a) Crystal structure of a cobalt-activated diphtheria toxin repressor-DNA complex reveals a metal-binding SH3-like domain. J Mol Biol 292:653-667

Pohl E, Holmes R, Hol W (1999b) Crystal structure of the iron-dependent regulator (IdeR) from *Mycobacterium tuberculosis* shows both metal binding sites fully occupied. J Mol Biol 285:1145-1156

Posey J, Hardham J, Norris S, Gherardini F (1999) Characterization of a manganese-dependent regulatory protein, TroR, from *Treponema pallidum*. Proc Natl Acad Sci U S A 96:10887-10892

Premakumar R, Pau R, Mitchenall L, Easo M, Bishop P (1998) Regulation of the transcriptional activators AnfA and VnfA by metals and ammonium in *Azotobacter vinelandii*. FEMS Microbiol Lett 164:63-68

Puig S, Askeland E, Thiele DJ (2005) Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. Cell 120:99-110

Qi Z, Hamza I, O'Brian M (1999) Heme is an effector molecule for iron-dependent degradation of the bacterial iron response regulator (Irr) protein. Proc Natl Acad Sci USA 96:13056-13061

Qiu X, Verlinde C, Zhang S, Schmitt M, Holmes R, Hol W (1995) Three-dimensional structure of the diphtheria toxin repressor in complex with divalent cation co-repressors. Structure 3:87-100

Que Q, Helmann J (2000) Manganese homeostasis in *Bacillus subtilis* is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol Microbiol 35:1454-1468

Quinn J, Merchant S (1995) Two copper-responsive elements associated with the Chlamydomonas Cyc6 gene function as targets for transcriptional activators. Plant Cell 7:623-628

Quinn J, Nakamoto S, Merchant S (1999) Induction of coproporphyrinogen oxidase in Chlamydomonas chloroplasts occurs via transcriptional regulation of Cpx1 mediated by copper response elements and increased translation from a copper deficiency-specific form of the transcript. J Biol Chem 274:14444-14454

Radtke F, Georgiev O, Muller H, Brugnera E, Schaffner W (1995) Functional domains of the heavy metal-responsive transcription regulator MTF-1. Nucleic Acids Res 23:2277-2286

Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 284:805-808

Ralston DM, O'Halloran TV (1990) Ultrasensitivity and heavy-metal selectivity of the allosterically modulated MerR transcription complex. Proc Natl Acad Sci USA 87:3846-3850

Rensing C, Mitra B, Rosen BP (1997) The zntA gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. Proc Natl Acad Sci USA 94:14326-14331

Rivers SL, McNairn E, Blasco F, Giordano G, Boxer DH (1993) Molecular genetic analysis of the moa operon of *Escherichia coli* K-12 required for molybdenum cofactor biosynthesis. Mol Microbiol 8:1071-1081

Robertson L, Causton H, Young R, Fink G (2000) The yeast A kinases differentially regulate iron uptake and respiratory function. Proc Natl Acad Sci USA 97:5984-5988

Rosen B, Dey S, Dou D, Ji G, Kaur P, Ksenzenko M, Silver S, Wu J (1992) Evolution of an ion-translocating ATPase. Ann N Y Acad Sci 671:257-272

Rosenstein R, Nikoleit K, Gotz F (1994) Binding of ArsR, the repressor of the *Staphylococcus xylosus* (pSX267) arsenic resistance operon to a sequence with dyad symmetry within the ars promoter. Mol Gen Genet 242:566-572

Rouch D, Brown N (1997) Copper-inducible transcriptional regulation at two promoters in the *Escherichia coli* copper resistance determinant pco. Microbiology 143 (Pt 4):1191-1202

Rutherford J, Cavet J, Robinson N (1999) Cobalt-dependent transcriptional switching by a dual-effector MerR-like protein regulates a cobalt-exporting variant CPx-type ATPase. J Biol Chem 274:25827-25832

Rutherford J, Jaron S, Ray E, Brown P, Winge D (2001) A second iron-regulatory system in yeast independent of Aft1p. Proc Natl Acad Sci USA 98:14322-14327

Rutherford J, Jaron S, Winge D (2003) Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. J Biol Chem 278:27636-27643

Rutherford J, Ojeda L, Balk J, Muhlenhoff U, Lill R, Winge D (2005) Activation of the iron regulon by the yeast Aft1/Aft2 transcription factors depends on mitochondrial, but not cytosolic iron-sulfur protein biogenesis. J Biol Chem 280:10135-10140

Ruzsa S, Scandalios J (2003) Altered Cu metabolism and differential transcription of Cu/ZnSod genes in a Cu/ZnSOD-deficient mutant of maize: evidence for a Cu-responsive transcription factor. Biochemistry 42:1508-1516

San Francisco MJ, Hope CL, Owolabi JB, Tisa LS, Rosen BP (1990) Identification of the metalloregulatory element of the plasmid-encoded arsenical resistance operon. Nucleic Acids Res 18:619-624

Saydam N, Georgiev O, Nakano M, Greber U, Schaffner W (2001) Nucleo-cytoplasmic trafficking of metal-regulatory transcription factor 1 is regulated by diverse stress signals. J Biol Chem 276:25487-25495

Schaffer S, Hantke K, Braun V (1985) Nucleotide sequence of the iron regulatory gene fur. Mol Gen Genet 200:110-113

Schiering N, Kabsch W, Moore MJ, Distefano MD, Walsh CT, Pai EF (1991) Structure of the detoxification catalyst mercuric ion reductase from *Bacillus* sp. strain RC607. Nature 352:168-172

Schiering N, Tao X, Zeng H, Murphy J, Petsko G, Ringe D (1995) Structures of the apo- and the metal ion-activated forms of the diphtheria tox repressor from *Corynebacterium diphtheriae*. Proc Natl Acad Sci USA 92:9843-9850

Schmitt M, Predich M, Doukhan L, Smith I, Holmes R (1995) Characterization of an iron-dependent regulatory protein (IdeR) of *Mycobacterium tuberculosis* as a functional homolog of the diphtheria toxin repressor (DtxR) from *Corynebacterium diphtheriae*. Infect Immun 63:4284-4289

Schmitt MP (1997a) Transcription of the *Corynebacterium diphtheriae* hmuO gene is regulated by iron and heme. Infect Immun 65:4634-4641

Schmitt MP (1997b) Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. J Bacteriol 179:838-845

Schmitt MP, Holmes RK (1994) Cloning, sequence, and footprint analysis of two promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. J Bacteriol 176:1141-1149

Schneider R, Hantke K (1993) Iron-hydroxamate uptake systems in *Bacillus subtilis*: identification of a lipoprotein as part of a binding protein-dependent transport system. Mol Microbiol 8:111-121

Schreiter ER, Sintchak MD, Guo Y, Chivers PT, Sauer RT, Drennan CL (2003) Crystal structure of the nickel-responsive transcription factor NikR. Nat Struct Biol 10:794-799

Schuttelkopf A, Boxer D, Hunter W (2003) Crystal structure of activated ModE reveals conformational changes involving both oxyanion and DNA-binding domains. J Mol Biol 326:761-767

Self W, Grunden A, Hasona A, Shanmugam K (1999) Transcriptional regulation of molybdoenzyme synthesis in *Escherichia coli* in response to molybdenum: ModE-molybdate, a repressor of the modABCD (molybdate transport) operon is a secondary transcriptional activator for the hyc and nar operons. Microbiology 145 (Pt 1):41-55

Serpe M, Joshi A, Kosman D (1999) Structure-function analysis of the protein-binding domains of Mac1p, a copper-dependent transcriptional activator of copper uptake in *Saccharomyces cerevisiae*. J Biol Chem 274:29211-29219

Shewchuk LM, Helmann JD, Ross W, Park SJ, Summers AO, Walsh CT (1989a) Transcriptional switching by the MerR protein: activation and repression mutants implicate distinct DNA and mercury(II) binding domains. Biochemistry 28:2340-2344

Shewchuk LM, Verdine GL, Walsh CT (1989b) Transcriptional switching by the metalloregulatory MerR protein: initial characterization of DNA and mercury (II) binding activities. Biochemistry 28:2331-2339

Shi W, Dong J, Scott RA, Ksenzenko MY, Rosen BP (1996) The role of arsenic-thiol interactions in metalloregulation of the ars operon. J Biol Chem 271:9291-9297

Shi W, Wu J, Rosen B (1994) Identification of a putative metal binding site in a new family of metalloregulatory proteins. J Biol Chem 269:19826-19829

Silver S (1992) Plasmid-determined metal resistance mechanisms: range and overview. Plasmid 27:1-3

Silver S, Johnseine P, King K (1970) Manganese active transport in *Eschericia coli*. J. Bacteriol 104:1299-1306

Singh VK, Xiong A, Usgaard TR, Chakrabarti S, Deora R, Misra TK, Jayaswal RK (1999) ZntR is an autoregulatory protein and negatively regulates the chromosomal zinc resistance operon znt of *Staphylococcus aureus*. Mol Microbiol 33:200-207

Smirnova I, Bittel D, Ravindra R, Jiang H, Andrews G (2000) Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. J Biol Chem 275:9377-9384

Solioz M, Stoyanov J (2003) Copper homeostasis in *Enterococcus hirae*. FEMS Microbiol Rev 27:183-195

Spiering M, Ringe D, Murphy J, Marletta M (2003) Metal stoichiometry and functional studies of the diphtheria toxin repressor. Proc Natl Acad Sci USA 100:3808-3813

Stojiljkovic I, Baumler AJ, Hantke K (1994) Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a fur titration assay. J Mol Biol 236:531-545

Stojiljkovic I, Hantke K (1995) Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). Mol Gen Genet 247:199-205

Stoyanov JV, Brown NL (2003) The *Escherichia coli* copper-responsive copA promoter is activated by gold. J Biol Chem 278:1407-1410

Stoyanov JV, Hobman JL, Brown NL (2001) CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. Mol Microbiol 39:502-511

Strausak D, Solioz M (1997) CopY is a copper-inducible repressor of the *Enterococcus hirae* copper ATPases. J Biol Chem 272:8932-8936

Tang Y, Guest JR (1999) Direct evidence for mRNA binding and post-transcriptional regulation by *Escherichia coli* aconitases. Microbiology 145 (Pt 11):3069-3079

Tao X, Zeng H, Murphy J (1995) Transition metal ion activation of DNA binding by the diphtheria tox repressor requires the formation of stable homodimers. Proc Natl Acad Sci USA 92:6803-6807

Tetaz T, Luke R (1983) Plasmid-controlled resistance to copper in *Escherichia coli*. J Bacteriol 154:1263-1268

Thelwell C, Robinson N, Turner-Cavet J (1998) An SmtB-like repressor from *Synechocystis* PCC 6803 regulates a zinc exporter. Proc Natl Acad Sci USA 95:10728-10733

Thiele D (1988) ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. Mol Cell Biol 8:2745-2752

Thiele D, Hamer D (1986) Tandemly duplicated upstream control sequences mediate copper-induced transcription of the *Saccharomyces cerevisiae* copper-metallothionein gene. Mol Cell Biol 6:1158-1163

Thorvaldsen J, Sewell A, Tanner A, Peltier J, Pickering I, George G, Winge D (1994) Mixed Cu+ and Zn2+ coordination in the DNA-binding domain of the AMT1 transcription factor from *Candida glabrata*. Biochemistry 33:9566-9577

Touati D, Jacques M, Tardat B, Bouchard L, Despied S (1995) Lethal oxidative damage and mutagenesis are generated by iron in delta fur mutants of *Escherichia coli*: protective role of superoxide dismutase. J Bacteriol 177:2305-2314

Turner J, Glands P, Samson A, Robinson N (1996) Zn2+-sensing by the cyanobacterial metallothionein repressor SmtB: different motifs mediate metal-induced protein-DNA dissociation. Nucleic Acids Res 24:3714-3721

Turner R, Smith D, Zawrotny M, Summers M, Posewitz M, Winge D (1998) Solution structure of a zinc domain conserved in yeast copper-regulated transcription factors. Nat Struct Biol 5:551-555

van Bakel H, Huynen M, Wijmenga C (2004) Prokaryotic diversity of the *Saccharomyces cerevisiae* Atx1p-mediated copper pathway. Bioinformatics 20:2644-2655

van der Lelie D, Schwuchow T, Schwidetzky U, Wuertz S, Baeyens W, Mergeay M, Nies D (1997) Two-component regulatory system involved in transcriptional control of heavy-metal homoeostasis in *Alcaligenes eutrophus*. Mol Microbiol 23:493-503

van Vliet A, Baillon M, Penn C, Ketley J (1999) *Campylobacter jejuni* contains two fur homologs: characterization of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. J Bacteriol 181:6371-6376

van Vliet AH, Poppelaars SW, Davies BJ, Stoof J, Bereswill S, Kist M, Penn CW, Kuipers EJ, Kusters JG (2002) NikR mediates nickel-responsive transcriptional induction of urease expression in *Helicobacter pylori*. Infect Immun 70:2846-2852

VanZile M, Chen X, Giedroc D (2002a) Allosteric negative regulation of smt O/P binding of the zinc sensor, SmtB, by metal ions: a coupled equilibrium analysis. Biochemistry 41:9776-9786

VanZile M, Chen X, Giedroc D (2002b) Structural characterization of distinct alpha3N and alpha5 metal sites in the cyanobacterial zinc sensor SmtB. Biochemistry 41:9765-9775

VanZile ML, Cosper NJ, Scott RA, Giedroc DP (2000) The zinc metalloregulatory protein *Synechococcus* PCC7942 SmtB binds a single zinc ion per monomer with high affinity in a tetrahedral coordination geometry. Biochemistry 39:11818-11829

Voisard C, Wang J, McEvoy J, Xu P, Leong S (1993) urbs1, a gene regulating siderophore biosynthesis in *Ustilago maydis*, encodes a protein similar to the erythroid transcription factor GATA-1. Mol Cell Biol 13:7091-7100

Voutsina A, Fragiadakis G, Boutla A, Alexandraki D (2001) The second cysteine-rich domain of Mac1p is a potent transactivator that modulates DNA binding efficiency and functionality of the protein. FEBS Lett 494:38-43

Walkenhorst H, Hemschemeier S, Eichenlaub R (1995) Molecular analysis of the molybdate uptake operon, modABCD, of *Escherichia coli* and modR, a regulatory gene. Microbiol Res 150:347-361

Wan X, Verberkmoes N, McCue L, Stanek D, Connelly H, Hauser L, Wu L, Liu X, Yan T, Leaphart A, Hettich R, Zhou J, Thompson D (2004) Transcriptomic and proteomic characterization of the Fur modulon in the metal-reducing bacterium *Shewanella oneidensis*. J Bacteriol 186:8385-8400

Wang G, Wylie G, Twigg P, Caspar D, Murphy J, Logan T (1999) Solution structure and peptide binding studies of the C-terminal src homology 3-like domain of the diphtheria toxin repressor protein. Proc Natl Acad Sci USA 96:6119-6124

Wang SC, Dias AV, Bloom SL, Zamble DB (2004a) Selectivity of metal binding and metal-induced stability of *Escherichia coli* NikR. Biochemistry 43:10018-10028

Wang Y, Lorenzi I, Georgiev O, Schaffner W (2004b) Metal-responsive transcription factor-1 (MTF-1) selects different types of metal response elements at low vs. high zinc concentration. Biol Chem 385:623-632

Waters B, Eide D (2002) Combinatorial control of yeast FET4 gene expression by iron, zinc, and oxygen. J Biol Chem 277:33749-33757

Westin G, Schaffner W (1988) A zinc-responsive factor interacts with a metal-regulated enhancer element (MRE) of the mouse metallothionein-I gene. Embo J 7:3763-3770

White A, Ding X, vanderSpek J, Murphy J, Ringe D (1998) Structure of the metal-ion-activated diphtheria toxin repressor/tox operator complex. Nature 394:502-506

Winkelmann G (2002) Microbial siderophore-mediated transport. Biochem Soc Trans 30:691-696

Wu J, Rosen B (1991) The ArsR protein is a trans-acting regulatory protein. Mol Microbiol 5:1331-1336

Wu J, Rosen B (1993a) The arsD gene encodes a second trans-acting regulatory protein of the plasmid-encoded arsenical resistance operon. Mol Microbiol 8:615-623

Wu J, Rosen BP (1993b) Metalloregulated expression of the ars operon. J Biol Chem 268:52-58

Wu J, Tisa L, Rosen B (1992) Membrane topology of the ArsB protein, the membrane subunit of an anion-translocating ATPase. J Biol Chem 267:12570-12576

Wu J, Weiss B (1991) Two divergently transcribed genes, soxR and soxS, control a superoxide response regulon of *Escherichia coli*. J Bacteriol 173:2864-2871

Wu LF, Mandrand-Berthelot MA (1986) Genetic and physiological characterization of new *Escherichia coli* mutants impaired in hydrogenase activity. Biochimie 68:167-179

Wu LF, Mandrand-Berthelot MA, Waugh R, Edmonds CJ, Holt SE, Boxer DH (1989) Nickel deficiency gives rise to the defective hydrogenase phenotype of hydC and fnr mutants in *Escherichia coli*. Mol Microbiol 3:1709-1718

Xiao Z, Loughlin F, George GN, Howlett GJ, Wedd AG (2004) C-terminal domain of the membrane copper transporter Ctr1 from *Saccharomyces cerevisiae* binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster: sub-femtomolar Cu(I) affinity of three proteins involved in copper trafficking. J Am Chem Soc 126:3081-3090

Xiong A, Jayaswal R (1998) Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. J Bacteriol 180:4024-4029

Xu C, Shi W, Rosen B (1996) The chromosomal arsR gene of *Escherichia coli* encodes a trans-acting metalloregulatory protein. J Biol Chem 271:2427-2432

Yamaguchi-Iwai Y, Dancis A, Klausner R (1995) AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. Embo J 14:1231-1239

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner R (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. Embo J 15:3377-3384

Yamaguchi-Iwai Y, Ueta R, Fukunaka A, Sasaki R (2002) Subcellular localization of Aft1 transcription factor responds to iron status in *Saccharomyces cerevisiae*. J Biol Chem 277:18914-18918

Yang J, Ishimori K, O'Brian M (2004) Two heme binding sites are involved in the regulated degradation of the bacterial iron response regulator (Irr) protein. J Biol Chem 280:7671-7676

Yoon K, Misra T, Silver S (1991) Regulation of the cadA cadmium resistance determinant of *Staphylococcus aureus* plasmid pI258. J Bacteriol 173:7643-7649

Zhang B, Egli D, Georgiev O, Schaffner W (2001) The *Drosophila* homolog of mammalian zinc finger factor MTF-1 activates transcription in response to heavy metals. Mol Cell Biol 21:4505-4514

Zhang B, Georgiev O, Hagmann M, Gunes C, Cramer M, Faller P, Vasak M, Schaffner W (2003) Activity of metal-responsive transcription factor 1 by toxic heavy metals and H2O2 *in vitro* is modulated by metallothionein. Mol Cell Biol 23:8471-8485

Zhao H, Butler E, Rodgers J, Spizzo T, Duesterhoeft S, Eide D (1998) Regulation of zinc homeostasis in yeast by binding of the ZAP1 transcriptional activator to zinc-responsive promoter elements. J Biol Chem 273:28713-28720

Zhao H, Eide D (1996a) The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc Natl Acad Sci USA 93:2454-2458

Zhao H, Eide D (1996b) The ZRT2 gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*. J Biol Chem 271:23203-23210

Zhao H, Eide D (1997) Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*. Mol Cell Biol 17:5044-5052

Zheng M, Doan B, Schneider T, Storz G (1999) OxyR and SoxRS regulation of fur. J Bacteriol 181:4639-4643

Zhou L, Haas H, Marzluf G (1998) Isolation and characterization of a new gene, sre, which encodes a GATA-type regulatory protein that controls iron transport in *Neurospora crassa*. Mol Gen Genet 259:532-540

Zhou P, Szczypka M, Sosinowski T, Thiele D (1992) Expression of a yeast metallothionein gene family is activated by a single metalloregulatory transcription factor. Mol Cell Biol 12:3766-3775

Zhou P, Thiele D (1991) Isolation of a metal-activated transcription factor gene from *Candida glabrata* by complementation in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 88:6112-6116

Zhu Z, Labbe S, Pena M, Thiele D (1998) Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. J Biol Chem 273:1277-1280

Znaidi S, Pelletier B, Mukai Y, Labbe S (2004) The Schizosaccharomyces pombe corepressor Tup11 interacts with the iron-responsive transcription factor Fep1. J Biol Chem 279:9462-9474

Zou P, Borovok I, Ortiz de Orue Lucana D, Muller D, Schrempf H (1999) The mycelium-associated *Streptomyces reticuli* catalase-peroxidase, its gene and regulation by FurS. Microbiology 145 (Pt 3):549-559

Abbreviations

ABC: ATP-binding cassette
Dtx: diphtheria toxin
SBP: solute binding protein
NRAMP: natural resistance-associated macrophage proteins
DMSO: Dimethyl sulfoxide
FeRE: Iron-regulatory element
CuRE: Copper-responsive element
MRE: metal-responsive element
NES: nuclear export signal
O/P: Operator/Promoter

van Bakel, Harm
Complex Genetics Group, Dept. of Biomedical Genetics, Stratenum 2.117, University Medical Center Utrecht, PO Box 85060, 3508 AB Utrecht, The Netherlands
H.H.M.J.vanBakel@med.uu.nl

Wijmenga, Cisca
Complex Genetics Group, Dept. of Biomedical Genetics, Stratenum 2.117, University Medical Center Utrecht, PO Box 85060, 3508 AB Utrecht, The Netherlands

Mechanisms of toxic metal tolerance in yeast

Markus J. Tamás, Jean Labarre, Michel B. Toledano, and Robert Wysocki

Abstract

Toxic metals are an integral part of our environment and all organisms possess systems to evade toxicity and acquire tolerance. Studies in yeast have revealed a number of important tolerance systems encompassing metal uptake and export pathways, metal binding and sequestration systems as well as the regulatory mechanisms that the cell utilizes to control these systems. The study of the physiological, molecular, and genetic details of the function of these systems has significantly contributed to our understanding of toxic metal tolerance acquisition. This review will focus on tolerance mechanisms to toxic metals including cadmium, arsenic, antimony, mercury, and selenium in the model eukaryote *Saccharomyces cerevisiae* (bakers' yeast) and other fungi.

1 Introduction

All living organisms are exposed to metals through natural geological as well as anthropogenic sources. Many metals serve as essential nutrients, while others are either toxic or harmful in excessive quantities. Deposition of nonessential toxic metals in the environment has dramatically increased during the last century. Cadmium, arsenic, mercury, and lead are extensively distributed in nature and can reach relatively high concentrations in some locations. These metals are highly toxic and pose a considerable threat to the environment and to human health. Metal intoxication often occurs through occupational exposure or through ingestion of contaminated food and water. In fact, organisms have been exposed to toxic metals since the origin of life and have therefore developed various tolerance mechanisms early during evolution. Currently, metal pollution leads to the spread of plasmids containing resistance genes among prokaryota.

Metal tolerance mechanisms in bacteria are relatively well-described where plasmids containing specific operons account for this phenomenon (Silver 1998, 2003; Nies 1999; Rosen 2002). Similarly, there has been a tremendous advance in the understanding of nutrient metal homeostasis and detoxification in many organisms. These mechanisms are extensively reviewed in other chapters in this volume and will not be considered here. However, the mechanisms of tolerance to various nonessential metals in eukaryotic organisms have remained poorly explored. The increasing use of toxic metals in medical therapy, *e.g.*, the use of arsenic for the treatment of certain forms of cancer and of diseases caused by protozoan parasites,

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_105 / Published online: 15 September 2005

as well as the need to develop systems for phytoremediation of contaminated sites, has spurred research in this field and led to a significant progress in understanding metal responses and tolerance acquisition mechanisms in eukaryotic organisms (Tamás and Wysocki 2001; Alkorta et al. 2004; Desoize 2004; Macek et al. 2004). In particular, the use of the yeast *Saccharomyces cerevisiae* (bakers' yeast) as a eukaryotic model organism has proved very useful to unravel the molecular mechanism of many cellular metal tolerance systems. Here, we review the current knowledge about tolerance mechanisms to cadmium, arsenic, antimony, mercury, and selenium in *S. cerevisiae*.

1.1 Metal abundance, distribution, and usage

Arsenic is a semimetal or metalloid and as such, it has intermediate properties between those of metals and nonmetals. Arsenic compounds, in the form of sulphides and oxides as well as in the form of calcium, sodium, and potassium salts, are naturally occurring and ubiquitous in the environment. Arsenic contamination of drinking water is a serious problem worldwide: Bangladesh, West Bengal, Vietnam, and Taiwan are the most affected areas where global epidemic of arsenic poisoning is observed (Frisbie et al. 2002; Nordstrom 2002). The sources of arsenic in underground water supplies in these areas are geologically deposited sediments. Elevated concentrations of arsenic in soil and surface water are also associated with the use of arsenic compounds as pesticides, fungicides, insecticides, and wood preservatives (Mukhopadhyay and Rosen 2002). Nonferrous ore smelting, semiconductor, and glass manufacturing as well as power generation by the burning of arsenic-contaminated coal further contributes to arsenic pollution (Hei and Filipic 2004).

Arsenic has a long and well-documented history of usage in medicine since ancient times (Waxman and Anderson 2001; Ravandi 2004). In the 18th century, potassium arsenite in the form of Fowler's solution was used to treat a number of ailments. The use of arsenic in treating leukaemia was first described in the 19th century and its efficacy was confirmed in the 1930s (Evens et al. 2004). At the beginning of the 20th century, Paul Ehrlich and his co-worker Sahachiro Hata developed the arsenic-containing 'compound 606' (Salvarsan) and introduced this drug for the treatment of syphilis and trypanosomiasis (Sorgel 2004). This was the first example of modern chemotherapy; in fact, Paul Ehrlich coined the term 'chemotherapy'. However, arsenic was often overdosed producing severe side effects. With the introduction of penicillin and other less toxic drugs, arsenic was no longer in use. Recently, arsenic trioxide was re-introduced in the treatment of acute promyelocytic leukaemia (APL) and multiple myeloma (Zhu et al. 2002; Ravandi 2004). Arsenic trioxide exerts its effect by inducing differentiation of leukaemia cells (by promoting degradation of the leukemogenic PML-RARα fusion protein) and/or by inducing apoptosis (Hu et al. 2005).

The metalloid antimony is related to arsenic. Antimony is not abundant but is found in more than 100 mineral species. Antimony, in the form of sulphide called stibnite, has been known since Biblical times as a medicine and as a cos-

metic. Today, antimony compounds are used for instance in the making of flame-proofing formulations and glass paints. In addition, pentavalent antimony is an active component of Pentostam and Glucantime, the first line drugs in the treatment of leishmaniasis (Sundar and Rai 2002; Berman 2003).

Cadmium is a highly toxic metal at very low concentrations and is found in increasing abundance in the environment due to industrial activities. Cadmium occurs naturally in zinc, lead, copper, and other ores, which can serve as sources to ground and surface waters, especially when in contact with soft, acidic waters. Nowadays, cadmium is released in air, water, and soil, mainly due to mining, smelting, battery, and paint manufacturing and car exhaust (Goyer and Clarkson 2001).

Although, mercury is a very rare element in the earth's crust, it was used already in ancient Egypt from 3500 BC. Mercury is primarily obtained from the mineral cinnabar (HgS). Mercury easily forms alloys with other metals called amalgams, which are used in gold extraction from ores, dental fillings, and batteries. Due to its high toxicity, mercury chloride ($HgCl_2$) was once used as pesticide, antiseptic, and wood preservative (Goyer and Clarkson 2001).

In contrast to the metals described above, selenium [Se(0)] is an essential trace element in all organisms and non-toxic at low levels. In nature, selenium is found in a few rare minerals such as eucairite (CuAgSe), crooksite (CuThSe), and clausthalite (PbSe) as well as in many sulphide ores. In living organisms, it is a component of the amino acid selenocysteine, which is present in several enzymes with oxidoreductase activity (Stadtman 1996). On the other hand, selenate [Se(VI)], selenite [Se(IV)], and selenide [Se(II)] are highly reactive and may cause increased production of reactive oxygen species (Turner et al. 1998).

2 Effects of nonessential metals on biological systems

The toxicity of a compound depends on the dose as well as on the contamination pathway, *i.e.*, whether it is absorbed via skin, ingested via the intestinal tract, or inhaled via the lungs. In the case of metals, other parameters influencing toxicity include the oxidation state and the speciation of the metal. Most metals affect various organ systems but at the lowest dose where effects occur, each metal tends to affect first a specific organ or tissue. A common property of nearly all toxic metals, including Hg(II), As(III), Cd(II), and Pb(II) is their high reactivity with sulphhydryl groups. Other toxic metals such as Cr(VI), Se(III), and Se(VI) are less sulphhydryl reactive though they are generally reduced in the cell by glutathione. Many metals have been shown to cause oxidative stress, lipid peroxidation, DNA strand breaks, and alteration of the cellular glutathione pool. In addition, the toxic and carcinogenic effects of metals may be induced by targeting cellular regulatory proteins or signalling proteins involved in proliferation, cell cycle regulation, apoptosis, DNA repair and differentiation (reviewed in: Stohs and Bagchi 1995; Ercal et al. 2001; Goyer and Clarkson 2001; Chen and Shi 2002; Harris and Shi 2003).

2.1 Effects on human health

Arsenic has for long been known to be toxic and carcinogenic. Epidemiological studies have indicated that arsenic can cause cancers of the skin, bladder, liver, and lung. In addition, arsenic exposure has been associated with diabetes and hypertension (Smith et al. 1992; Evens et al. 2004; Shi et al. 2004). Arsenic exists in various oxidation states exhibiting different biological properties and degrees of toxicity. Arsenate [As(V)] is a phosphate analogue and it inhibits oxidative phosphorylation, short-circuiting the cell's main energy-generation system (Goyer and Clarkson 2001). Arsenite [As(III)] is more potently toxic and exposure leads to increased production of free radicals in mammals, especially hydroxyl radicals (Liu et al. 2001). Enhanced oxidative stress is proposed to be responsible for arsenic-induced toxicity and carcinogenicity. However, the mechanisms of free radical production and the details of arsenic-induced cytotoxicity remain largely unknown (Ercal et al. 2001; Evens et al. 2004; Hei and Filipic 2004; Shi et al. 2004)

Antimony belongs to the same periodic group as arsenic and has the same oxidation states. In contrast to arsenic, the genotoxic and carcinogenic properties of antimony has received little attention and most information has been obtained from industrial experiences. Chronic exposure to antimony may affect the skin and lungs and also produce alterations in cardiac function. Pentavalent antimony [Sb(V)] inhibits glucose catabolism and ATP-formation while trivalent antimonite [Sb(III)] may impair protein function by interacting with sulphhydryl groups. Whether antimony can induce oxidative stress is unclear (Gebel 1997; Goyer and Clarkson 2001).

Cadmium contaminating food is absorbed through the intestinal tract and transferred to the liver where it is chelated to glutathione and transferred to metallothionein. This complex is reabsorbed into the kidney where it may stay up to 20 years; its accumulation over time and progressive release when chelating capacities are exceeded may cause toxicity. In Japan, the accidental exposure of inhabitants of the Jinzu river basin eating cadmium-contaminated rice provoked a severe disease called Itai Itai disease (Itai! means Ouch!), which is particularly painful to kidneys and bones. Cadmium is also considered genotoxic and carcinogenic for lung, kidney, and prostate (Waalkes 2003).

The major forms of mercury are methylmercury (MeHg) and Hg(0) (vapour). Inside the cell, Hg(0) may be converted to Hg(II), which is thought to produce H_2O_2 leading to oxidative stress. Mercury has also been described to perturb calcium homeostasis and to inhibit oxidative phosphorylation. Finally, mercury accumulates in liver, kidney, and brain and is poorly excreted (Ercal et al. 2001).

Selenium salts are toxic at high concentrations and selenium sulphide is considered carcinogenic. However, selenium is an essential trace element; small amounts of selenium are required to synthesize the amino acid selenocysteine present in glutathione peroxidases and thioredoxin reductases in mammals (Stadtman 1996). Dietary selenium has also been shown to prevent chemicals from inducing tumours in mammals (Rayman 2000). Selenium inhibits the intracellular JNK/SAPK signalling and $p38^{MAPK}$ cascades (Park et al. 2000) as well as some transcription factors (Handel et al. 1995; Kim and Stadtman 1997). Some of these inhibitory ef-

fects occur through a thiol redox mechanism (Park et al. 2000), but it is not known whether this mechanism is responsible for the anti-carcinogenic properties of this element.

2.2 Toxic metals cause oxidative stress

Oxidative stress originates from toxic levels of oxygen-derived reactive species. Reactive oxygen species (ROS) are mainly singlet oxygen ($O^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^{\bullet}$). Hydrogen peroxide is also included as ROS although this molecule is reactive only in some specific conditions (*e.g.* in the presence of reduced iron or copper; see Section 2.3.3). Hydroxyl radical is considered the most toxic ROS since it can attack and damage all macromolecules of the cell leading to protein oxidation, lipid peroxidation and DNA damage (reviewed in: Halliwell and Gutteridge 1984; Toledano et al. 2003).

2.2.1 Genetic data

Cadmium has been described to cause oxidative stress (Brennan and Schiestl 1996), lipid peroxidation (Howlett and Avery 1997), and mutagenesis (Jin et al. 2003). In line with this, yeast genes encoding oxidative stress defence functions (*SOD1*, *SOD2*, *TRR1*, *GLR1*, *TRX1*, *TRX2*, *GPX3*; see also Table 1 for abbreviations of genes/proteins and their function) are necessary for cadmium tolerance (Brennan and Schiestl 1996; Vido et al. 2001; Avery et al. 2004). Yap1, the main transcriptional activator controlling the oxidative stress response is central for cadmium tolerance (Wemmie et al. 1994b) and also for tolerance to Hg(II) (Westwater et al. 2002), Se(III) (Pinson et al. 2000), As(III) (Menezes et al. 2004; Wysocki et al. 2004), and Sb(III) (Wysocki et al. 2004). In contrast to these data, a recent genome-wide analysis found that strains lacking genes encoding oxidative stress defence functions were not particularly As(III) sensitive (Haugen et al. 2004). On the other hand, the same study confirmed the importance of Yap1-mediated induction of oxidative stress defence genes by As(III) (Haugen et al. 2004).

2.2.2 Lipid peroxidation

Yeast cells exposed to Cd(II) have an elevated level of lipid peroxidation and cadmium-induced lipid peroxidation is influenced by the degree of plasma membrane fatty acid unsaturation (Howlett and Avery 1997). A correlation between membrane peroxidation, membrane permeabilisation, and cadmium sensitivity has been reported, suggesting that lipid peroxidation is contributing to cadmium toxicity (Howlett and Avery 1997). Phospholipid hydroperoxidases are considered the principal cellular enzymes capable of repairing membrane lipid peroxides. *S. cerevisiae* expresses at least three different enzymes (Gpx1, Gpx2, and Gpx3) with phospholipid hydroperoxidase activity (Avery and Avery 2001). Among them,

Table 1. List of genes described in this review, the function of the corresponding gene products and their role in metal tolerance.

Gene	Function and role in metal tolerance
Sulphur assimilation and methyl cycle	
SUL1, SUL2	Sulphate transport
MET3	ATP sulphurylase
MET14	APS kinase
MET16	PAPS reductase
MET5, MET10	Sulphite reductase
MET25	O-acetylhomoserine sulphydrylase
MET6	Homocysteine methyl-transferase
SAM1, SAM2	S-adenosylmethionine synthase
SAH1	S-adenosylhomocysteinase
Glutathione biosynthesis	
CYS4	Cystathionine β-synthase
CYS3	Cystathionine γ-lyase
GSH1	γ-glutamyl-cysteine synthase
GSH2	Glutathione synthetase
GTT2	Glutathione S-transferase
Oxidative stress defence and antioxidants	
GLR1	Glutathione reductase
GPX1, GPX2, GPX3	Glutathione peroxidase
GRX1, GRX2, GRX5	Glutaredoxin
TRX1, TRX2	Thioredoxin
TRR1	Thioredoxin reductase
TSA1	Thiol peroxidase
SOD1, SOD2	Superoxide dismutase
CUP1, CRS5	Metallothionein
Signalling proteins and transcriptional regulators	
HOG1	Mitogen-activated protein kinase (MAPK); mediates As(III) and Sb(III) tolerance through multiple mechanisms
YAP1	AP-1-like bZIP transcription factor; controls expression of genes encoding oxidative and metal stress defence functions as well as functions in redox metabolism; activated by oxidants and metals; mutant is sensitive to oxidants and metals
YAP8/ACR1/ARR1	AP-1-like bZIP transcription factor; controls expression of ACR2 and ACR3; activated by As(III); mutant is As(V) and As(III) sensitive
ORP1/GPX3	Required for Yap1 activation by peroxide
YBP1	Required for Yap1 activation by peroxide
SKN7	Activator of the heat shock factor family; involved in numerous cellular processes including oxidative stress; role in metal tolerance unclear
MET4	bZIP transcriptional activator of MET genes; required for Cd(II) and As(III) tolerance
MET28, MET31, MET32, CBF1	DNA-binding factors that are required for tethering Met4 to the promoter DNA of target genes
MSN2, MSN4	Zinc finger family of transcriptional activators; activators of the 'general stress response'
RAD9	DNA damage checkpoint protein

Gene	Function and role in metal tolerance
Metal transporters	
PHO84, PHO87, PHO88	Phosphate transport; entry of As(V)
FPS1	Aquaglyceroporin; entry of As(III) and Sb(III)
ZRT1	High affinity zinc uptake; Cd(II) entry
SMF1	Manganese, copper, and iron uptake; Cd(II) entry
FET4	Low affinity iron uptake; Cd(II) entry
ACR3/ARR3	As(III) and perhaps Sb(III) export
SSU1	Sulphite efflux; Cd(II) efflux
PCA1	P-type ATPase involved in copper and iron homeostasis; Cd(II) efflux
YCF1	ABC transporter; sequesters glutathione conjugated substrates into the vacuole; As(III), Sb(III), Cd(II), Hg(II), Pb(II) detoxification
BPT1	ABC transporter in vacuolar membrane, Cd(II) detoxification
ZRC1, COT1	Vacuolar zinc uptake; Cd(II) detoxification
ATR1, FLR1	Multidrug transport
Other functions	
ACR2/ARR2	Arsenate reductase
PDC1, PDC6	Pyruvate decarboxylase; role in sulphur sparing programme
CRM1/XPO1	Nuclear export receptor (exportin)
PSE1/KAP121	Nuclear import receptor (importin)
RCK2	MAPK-activated protein kinase; mutant is As(III) and Cd(II) sensitive
SIC1	CDK-inhibitor protein; mutant is As(III) sensitive
MET30	F subunit of the SCF^{Met30} ubiquitin ligase
REV3	DNA polymerase (zeta subunit)
URE2	Regulator of nitrogen utilization; shows glutathione peroxidase activity

Gpx3 was shown to play a major role in cadmium resistance through its phospholipid hydroperoxidase activity (Avery et al. 2004). Taken together, these data strongly suggest that an important toxic effect of cadmium is membrane lipid peroxidation. Arsenic has also been shown to induce lipid peroxidation in animal models (Hei and Filipic 2004; Shi et al. 2004). Whether this is also the case in yeast is not known.

2.2.3 Mutagenic effect

Cadmium has strong mutagenic effects even at low concentrations; it induces recombination events (Brennan and Schiestl 1996), base substitution mutations and frame-shift mutations at a high rate (Jin et al. 2003). Cadmium causes hypermutability by inhibiting the mutation avoidance system rather than by direct DNA damage; it strongly inhibits the DNA mismatch repair system (Jin et al. 2003) by blocking the ATPase activity of the MSH2-MSH6 complex (Banerjee and Flores-Rozas 2005). Thus, cadmium strongly increases the number of mutations arising from endogenous processes (Jin et al. 2003). Arsenic exposure can also lead to

various types of DNA damage including chromosomal aberrations and sister chromatid exchange. It has been proposed that arsenic interferes with DNA repair systems (Shi et al. 2004). However, yeast strains lacking genes encoding DNA repair functions were not found particularly sensitive to arsenite (Haugen et al. 2004). For the other toxic metals described here, it is not known whether their potential mutagenic properties are due to similar mechanisms (*i.e.* inhibition of DNA repair systems) or to metal-mediated DNA damage. However, the yeast DNA repair mutants *rad9Δ* and *rev3Δ* are hypersensitive to selenite (Pinson et al. 2000).

2.3 Possible molecular mechanisms leading to oxidative stress

Cd(II), Hg(II), and Pb(II) are redox-inactive metals and cannot undergo simple oxidation reactions. The molecular mechanisms leading to oxidative stress are still largely unknown and are probably indirect. From literature, three possible and non-exclusive scenarios can be proposed: (i) binding and inhibition of specific enzymes, (ii) depletion of free glutathione pools, and (iii) Fenton reactions.

2.3.1 Binding and inhibition of specific enzymes

Nonessential toxic metals are generally thought to bind proteins through thiol groups of cysteine residues (Stohs and Bagchi 1995), which may lead to inhibition of essential enzymes. Methyl mercury (MeHg) strongly inhibits the yeast L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) which catalyses the synthesis of glutamine-6-phosphate (Naganuma et al. 2000). Overexpression of this enzyme allows cells to resist MeHg, suggesting that GFAT is a MeHg target. Whether MeHg inhibits this enzyme by binding to a thiol group is unknown.

Cadmium is also described to bind thiol groups in proteins. An *in vitro* search for Cd(II) binding site on phytochelatin synthase (Maier et al. 2003) and thioredoxin (Rollin-Genetet et al. 2004) identified, as expected, thiol-containing peptides. In addition, the frequent implication of Asp and Glu residues in Cd(II) binding-sites suggests an important participation of carboxylate groups (Maier et al. 2003; Rollin-Genetet et al. 2004). *In vitro* analysis showed that human thiol transferases (glutathione reductase, thioredoxin reductase, thioredoxin) are inhibited by Cd(II) (Chrestensen et al. 2000). It has been proposed that Cd(II) binds the two essential cysteine residues of thiol transferase active sites. As these enzymes are involved in oxidative stress defence, their inhibition would lead to increased oxidative stress in the cell. Although the inhibition of yeast thiol transferases has not been tested experimentally, this hypothesis is consistent with the Cd(II) hypersensitive phenotype of the *trr1Δ*, *glr1Δ* and *trx1Δ trx2Δ* mutants (Vido et al. 2001).

Cd(II) may also displace zinc and calcium ions from metalloproteins (Stohs and Bagchi 1995; Schützendübel and Polle 2002; Faller et al. 2005) and from zinc finger proteins (Hartwig 2001) leading to inhibition of essential proteins. However, although this hypothesis is interesting, there is no experimental evidence indicating that such a mechanism is involved in cadmium toxicity. In particular, it is not known whether the inhibition by cadmium of the ATPase activity of the MMR

system is due to cadmium binding to a specific site or to the replacement of an unidentified zinc site (McMurray and Tainer 2003).

2.3.2 Depletion of free glutathione pools

Cd(II), As(III), Hg(II), and Sb(III) are detoxified, at least in part, through chelation of the metal to glutathione (GSH) and subsequent transport of the complex into the vacuole (Li et al. 1997; Ghosh et al. 1999; Gueldry et al. 2003). This detoxification pathway may thus contribute to reduce or deplete free GSH pools from the cytosol. Cytosolic GSH depletion would reduce the activity of GSH dependent enzymes, such as glutathione peroxidases, glutathione S-transferases, and glutaredoxins that are involved in oxidative stress defence and perform essential functions in the cell. Among these enzymes, the activity of the glutaredoxin Grx5 is particularly important for the cell since it is required for the activity of mitochondrial iron/sulphur enzymes (Rodriguez-Manzaneque et al. 2002). Interestingly, another protein, the yeast prion Ure2, showing a glutathione peroxidase activity (Bai et al. 2004) is essential for cadmium tolerance (Rai et al. 2003) indicating that its glutathione peroxidase activity may play a role in combating cadmium toxicity. However, metal concentrations that are high enough to be toxic may still be too low to significantly deplete glutathione; glutathione is estimated to be in the mM range (Lafaye et al. 2005a) whereas cadmium is toxic in the micromolar range. Furthermore, the cellular glutathione pool has been shown to increase in response to cadmium (Lafaye et al. 2005a). Nevertheless, it cannot be excluded that metal exposure decreases the available pool of glutathione to an extent where the activity of GSH dependent enzymes become affected.

2.3.3 Fenton reactions

Fenton-type reactions are described for the nutrient metals Fe(II) and Cu(I) and are supposed to be a major source of hydroxyl radicals and oxidative stress in the cell (Halliwell and Gutteridge 1984).

$$M(n+1) + O_2^- \text{------>} M(n) + O_2$$
$$M(n) + H_2O_2 \text{------>} M(n+1) + OH^\bullet + OH^-$$

In contrast to iron and copper, Cd(II) and Hg(II) are unable to undergo such reactions. However, it cannot be excluded that Cd(II) may perturb intracellular iron metabolism (Lesuisse and Labbe 1995). An increased level of free iron in the cell would enhance Fenton-type reactions and ROS production.

2.3.4 Arsenite and oxidative stress

Arsenite exposure leads to increased ROS production in mammals (Liu et al. 2001), however, the source of ROS is unknown. It has been proposed that arsenite can damage the mitochondrial membrane, which in turn may result in increased intracellular superoxide ($O_2^{\bullet-}$) levels. Moreover, As(III) may activate NADH oxidase which leads to elevated cellular $O_2^{\bullet-}$ levels (Huang et al. 2004; Shi et al. 2004). Similarly, NADPH oxidase, an enzyme complex formed in response to

immune challenge and other stressors, appears to play an important role in arsenic-induced superoxide formation (Chou et al. 2004). In turn, the formation of $O_2^{\bullet-}$ leads to other ROS such as $OH^{\bullet}$ and H_2O_2. As(III) may also increase H_2O_2 production as a result of As(III) oxidation or formation of hydroxyl radicals during the release of iron from ferritin triggered by arsenicals. Cellular H_2O_2 levels may also increase as a result of As(III) inhibition of glutathione peroxidase (Huang et al. 2004; Shi et al. 2004).

2.4 Selenium and chromium salts

In vitro studies (Turner et al. 1998) have shown that reduction of selenite involves reactions with sulphhydryl groups of thiol-containing molecules such as glutathione, leading to production of the intermediate metabolites selenodiglutathione (GS-Se-SG), glutathioselenol (GS-SeH) and hydrogen selenide (HSe^-) and finally to elemental selenium. Certain reactions of this pathway produce hydrogen peroxide and superoxide anion (Seko and Imura 1997). In a similar way, the reduction of chromate Cr(VI) in the cell is supposed to involve reactions with cellular glutathione (Liu et al. 1997a). The reduction intermediates Cr(V) and Cr(IV) are thought to be responsible for the generation of $OH^{\bullet}$ through a Fenton like mechanism (Shi et al. 1994; Stohs and Bagchi 1995). The final product of the reduction pathway is Cr(III), which is thought to present low toxicity.

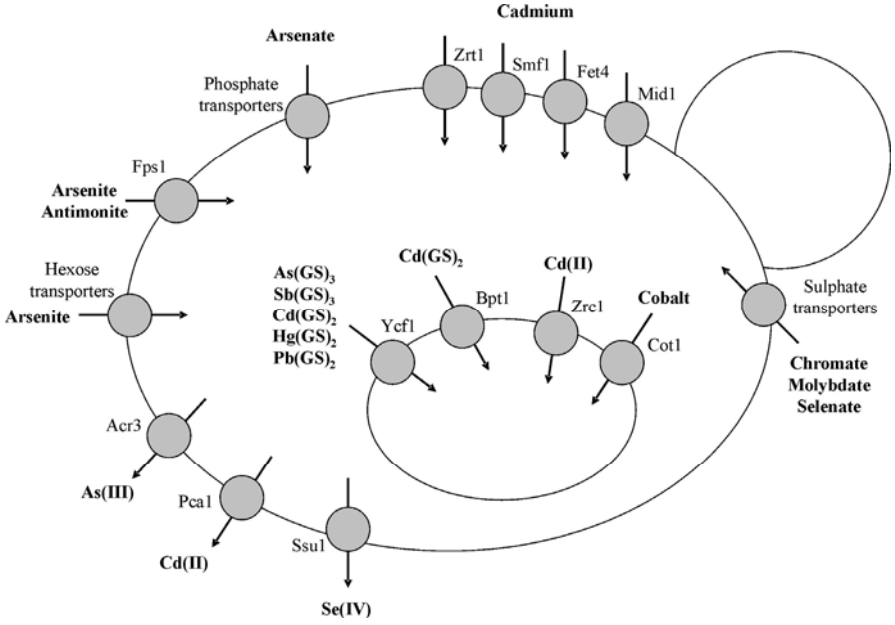


Fig. 1. Pathways of uptake and detoxification of toxic nonessential metals in *Saccharomyces cerevisiae*. See text and Table 1 for explanation of protein abbreviations.

3 Metal uptake pathways

A rapidly increasing number of proteins are being discovered that allow passage of nonessential metals across cell membranes and organelles inside the cell (Fig. 1; see also Chapter 14). While nonessential toxic metals appear to cross cell membranes through transporters responsible for nutrient metal acquisition, specific transporters contribute to their removal from the cytosol involving either metal export and/or sequestration in (an) internal organelle(s). Certain metals are transported in the free ionic form while others may be transported as complexes with various ligands. Inactivation of the uptake pathways in conjunction with enhanced activity of detoxification systems protects cells against metal toxicity.

3.1 Arsenic and antimony

Arsenic exists in two biologically important oxidation states; pentavalent arsenate As(V) and trivalent arsenite As(III). Arsenate is structurally similar to phosphate and competes with phosphate for transport. As(V) uptake in *Escherichia coli* is catalysed by the PhoS-PstABC phosphate translocating ABC-type ATPase complex and bacteria with defective phosphate transport accumulate little As(V) and display enhanced As(V) tolerance (Bennett and Malamy 1970; Willsky and Malamy 1980; Gatti et al. 2000). Similarly, arsenate enters plant roots via phosphate transporters (Meharg and Macnair 1990, 1992; Abedin et al. 2002; Wang et al. 2002) and a common arsenate/phosphate entry pathway may also exist in mammals (Huang and Lee 1996). In *S. cerevisiae*, arsenate uptake is likely to be mediated by the high-affinity phosphate transporter Pho84. Mutations in *PHO84* or in the *PHO87* and *PHO88* genes encoding respectively a low affinity phosphate transporter and a putative phosphate transporter, confer arsenate tolerance (Bun-ya et al. 1996; Yompakdee et al. 1996b). In addition, mutation of the *PHO86* gene also produces cellular arsenate tolerance (Bun-ya et al. 1996; Yompakdee et al. 1996a). Pho86 is an endoplasmic reticulum resident protein that is required for packaging of Pho84 into vesicles for subsequent transport to the plasma membrane (Lau et al. 2000).

The first arsenite influx pathway described was the *S. cerevisiae* aquaglyceroporin Fps1. In addition to arsenite, this protein also mediates antimonite entry into cells (Wysocki et al. 2001). Fps1 is a 74 kDa protein with six transmembrane helices and it belongs to the aquaporin family of channel proteins found in virtually all organisms. Whereas classical aquaporins are thought to be highly specific water channels, aquaglyceroporins may be permeated by a variety of substrates including glycerol and urea (Hohmann et al. 2000; Maurel et al. 2002; King et al. 2004). Fps1 plays a central role in yeast osmoregulation (Tamás and Hohmann 2003). Fps1 is inactive under hyperosmotic stress to permit glycerol accumulation and possibly turgor recovery. Upon hypoosmotic shock, Fps1 is rapidly activated and mediates glycerol export in order to prevent cell bursting and death (Tamás et al. 1999). Regulation of Fps1 activity requires a short domain in the cytoplasmic N-

terminal extension: an Fps1 protein lacking this domain cannot restrict transport and becomes hyperactive (Tamás et al. 1999, 2003).

Inactivation of Fps1, either by increasing external osmolarity or by deleting the *FPS1* gene, confers high level of arsenite and antimonite tolerance to yeast cells. In addition, deletion of *FPS1* reduces the uptake of arsenite into cells. Conversely, cells expressing a hyperactive Fps1 protein accumulate large amounts of arsenite and presumably also antimonite. As a consequence, such cells are highly As(III) and Sb(III) sensitive (Wysocki et al. 2001). Interestingly, this metalloid entry pathway appears to be controlled: expression of the *FPS1* gene is strongly repressed when cells are exposed to As(III) or Sb(III) (Wysocki et al. 2001). While the identity of the protein/signal transduction pathway that controls *FPS1* expression in the presence of these metalloids remains elusive, it was recently shown that the mitogen-activated protein kinase (MAPK) Hog1 modulates Fps1-mediated As(III) and probably also Sb(III) influx into cells (see further). However, repression of *FPS1* in the presence of As(III) occurs independently of Hog1 (Thorsen, Tängemo, Wagner, Wysocki, Boman, and Tamás: in preparation). Importantly, the capacity to transport arsenite and antimonite is not restricted to Fps1 but encompasses other aquaglyceroporins; the GlpF protein mediates As(III) and Sb(III) uptake in *E. coli* (Meng et al. 2004); mammalian AQP9 is permeated by both As(III) and Sb(III) whereas mammalian AQP7 transports As(III) (Liu et al. 2002, 2004b); LmAQP1 mediates uptake of both As(III) and Sb(III) into various *Leishmania* species and disruption of an *LmAQP1* allele in *L. major* results in increased Sb(III) resistance (Gourbal et al. 2004).

The predominant form of arsenite in solution at neutral pH appears to be the polyhydroxylated form $As(OH)_3$ (Ramirez-Solis et al. 2004). Similarly, the chemical form of Sb(III) recognised by aquaglyceroporins is thought to be $Sb(OH)_3$ (Baes and Mesmer 1976). Interestingly, polymerisation of three $As(OH)_3$ molecules is predicted to form a six-membered ring structure that may be similar to the six-membered ring structures of arsenious oxide (As_4O_6) and of hexose sugars (Liu et al. 2004a). In line with this notion, a recent study provided evidence that glucose carriers catalyse the uptake of As(III) in yeast: a strain lacking all glucose carriers exhibited low level of As(III) uptake (Liu et al. 2004a) and improved tolerance (Tängemo and Tamás: unpublished data).

Although Fps1 and hexose transporters are mediating the majority of As(III) influx into cells, genetic evidence (Wysocki et al. 2001) and transport data (Liu et al. 2004a) suggest that additional uptake routes exist. The molecular identity of the protein(s) catalysing residual arsenite uptake remains to be unveiled.

Arsenic and antimony-containing drugs are currently employed in the treatment of a variety of diseases. Arsenic trioxide (Trisenox) is used as a treatment for acute promyelocytic leukaemia and it might also be employed for other haematological and solid cancers (Evens et al. 2004; Ravandi 2004). Drugs containing arsenic or antimony are also employed to treat diseases caused by the protozoan parasites *Trypanosoma* and *Leishmania* (Murray 2001; Barrett et al. 2003). Knowledge of how arsenite and antimonite enters cells is imperative for the understanding of metalloid toxicity as well as of their ability to serve as chemotherapeutic agents. Indeed, recent studies have demonstrated that aquaglyceroporins

can modulate drug sensitivity in leukaemia (Bhattacharjee et al. 2004) as well as in *Leishmania* (Gourbal et al. 2004).

3.2 Cadmium

The molecular identity of the pathways mediating cadmium uptake has for long been elusive, although it was assumed that cadmium enters cells through transport systems for other essential elements such as calcium and iron. For instance, dietary iron and calcium deficiency was shown to promote increased accumulation of cadmium via the intestinal tract (reviewed in: Himeno et al. 2002). Recently, it has become clear that cadmium enters eukaryotic cells through a number of divalent cation transport systems. Cadmium uptake in yeast is mediated by various transport proteins involved in uptake of essential divalent cations including Zrt1 (zinc), Smf1 (manganese, copper, iron), and Fet4 (iron).

Zinc uptake in *S. cerevisiae* is primarily mediated by the high-affinity Zrt1 and low-affinity Zrt2 zinc transporters (Zhao and Eide 1996a, 1996b; see also Chapter 3). Zrt1 and Zrt2 are members of the ZIP (ZRT, IRT-like Protein) family of metal transporters present in bacteria, yeast, plants, and mammals. The ZIP proteins are capable of transporting a variety of cations including cadmium, iron, manganese, and zinc (Guerinot 2000; Gaither and Eide 2001). In addition to zinc, cadmium also appears to be a substrate for Zrt1; cells deleted for *ZRT1* accumulate less cadmium than wild type cells (Gomes et al. 2002) and Zrt1-mediated zinc uptake is strongly inhibited in the presence of cadmium (Gitan et al. 2003). Zrt1 is controlled at multiple levels. Firstly, transcription of the *ZRT1* gene is strongly induced under zinc-limiting conditions (Zhao and Eide 1996a, 1996b). Secondly, Zrt1 is inactivated by ubiquitination and subsequent endocytosis in zinc-replete cells thereby preventing further zinc uptake (Gitan et al. 1998; Gitan and Eide 2000). Interestingly, Zrt1 is also ubiquitinated and removed from the cell surface in the presence of cadmium. This inactivation may be an important mechanism to protect zinc-limited cells from cadmium toxicity (Gitan et al. 2003).

A second pathway of cadmium uptake into cells is through the Smf1 protein, which is a yeast member of the Nramp (neutral resistance-associated macrophage protein) family of metal transporters found in bacteria, fungi, plants, and mammals. As with the proteins of the ZIP family, the Nramp transporters can potentially recognize a broad range of substrates including copper, cadmium, manganese, and iron (Cellier et al. 1995; Thomine et al. 2000; Forbes and Gros 2001). *S. cerevisiae* expresses three functionally distinct members of the Nramp family encoded by the *SMF1*, *SMF2*, and *SMF3* genes (Portnoy et al. 2000). Smf1 was originally described as a high-affinity manganese transport system (Supek et al. 1996) and was later demonstrated to contribute to cellular accumulation of cadmium and copper (Liu et al. 1997b). Smf1 also stimulates iron uptake into *Xenopus* oocytes (Chen et al. 1999). The transport activity of Smf1 is regulated in response to metals; Smf1 is targeted to the vacuole when cells are replete with manganese whereas Smf1 fails to enter the vacuole under manganese starvation

and is instead inserted into the plasma membrane where it mediates metal uptake (Liu and Culotta 1999).

The function of Smf1 in cadmium uptake was revealed by analysis of the *BSD2* gene product. *BSD2* was first isolated as a gene, when inactivated by mutation, suppressed oxidative damage in yeast cells devoid of the copper/zinc superoxide dismutase (Liu and Culotta 1994). Cells lacking *BSD2* exhibited an increased level of Smf1-dependent manganese uptake, accumulated high levels of cadmium and cobalt, and displayed cadmium and cobalt hypersensitivity (Liu and Culotta 1994; Liu et al. 1997b). Interestingly, inactivation of *SMF1* in *bsd2Δ* cells eliminated the cadmium sensitivity of the *bsd2Δ* mutant but not its cobalt sensitivity. Instead, cobalt accumulation in *bsd2Δ* was reduced by deleting another Nramp encoding gene *SMF2* (Liu et al. 1997b). Smf2 is regulated by manganese and Bsd2 in a similar way as Smf1. However, while Smf1 moves to the plasma membrane in response to manganese starvation, Smf2 redistributes to intracellular vesicles. Moreover, Smf2 does not show any cell surface localisation (Portnoy et al. 2000; Luk and Culotta 2001). Hence, it is unclear how Smf2 contributes to cobalt influx into cells. Bsd2 is localised to the membrane of the endoplasmic reticulum. In the presence of manganese, Bsd2 is required to direct Smf1 and Smf2 to the vacuole (Liu et al. 1997b; Liu and Culotta 1999). In fact, Bsd2 is involved in Rsp5-mediated ubiquitination and sorting of various transmembrane proteins (Hettema et al. 2004). Hence, vacuolar targeting of Smf1 might also involve Rsp5.

The *FET4* gene encodes a low affinity iron transporter localised to the plasma membrane (Dix et al. 1994, 1997). In addition to its role in iron uptake, Fet4 has also been shown to be a physiologically relevant copper transporter (Hassett et al. 2000; Portnoy et al. 2001) and may be capable of transporting zinc, cadmium, and cobalt as well (Dix et al. 1994; Li and Kaplan 1998; Waters and Eide 2002). Transcription of the *FET4* gene is tightly regulated by environmental factors; the Atf1 iron responsive transcription factor induces *FET4* expression under iron-limiting conditions, the Zap1 transcription factor controls *FET4* expression in response to zinc and the Rox1 repressor controls *FET4* expression in response to oxygen (Jensen and Culotta 2002; Waters and Eide 2002). Repression of *FET4* in the presence of oxygen may protect cells against cadmium toxicity.

Finally, cadmium may also enter cells through calcium uptake systems such as the wheat LCT1-encoded calcium transporter. Interestingly, wheat LCT1 can restore calcium influx in a yeast strain deleted for the *MID1* gene encoding a stretch-activated calcium-permeable channel (Clemens et al. 1998). However, whether Mid1 indeed mediates influx of cadmium has not been tested.

Also plant members of the ZIP and Nramp protein families catalyse nonessential metal transport in addition to essential metals. For instance, the *Arabidopsis* IRT1 transporter (Korshunova et al. 1999; Rogers et al. 2000) as well as AtNramp1, AtNramp3, and AtNramp4 metal transporters are permeated by cadmium ions (Thomine et al. 2000). Similarly, the mammalian divalent metal transporter 1 DCT1 (also called DMT1), a member of the Nramp family, exhibits broad substrate specificity towards a variety of divalent cations including cadmium and lead (Gunshin et al. 1997).

3.3 Mercury

Mercury is a highly toxic, nonessential metal and the methylated form, *i.e.* methylmercury, is the most important form of mercury in terms of toxicity. Gram-negative bacteria protect themselves against the toxic effects of mercury by transporting Hg(II) into the cell via a specific uptake system. The periplasmic Hg(II)-binding protein MerP binds mercury and delivers it to the mercury transporter MerT for transport into the cell (Hobman and Brown 1996; Qian et al. 1998). An additional route of mercury uptake may involve the MerC protein (Sahlman et al. 1997). Once inside the cell, Hg(II) is reduced using NADPH to Hg(0) by the MerA reductase. Hg(0) is believed to leave the cell by passive diffusion (reviewed in: Nies 1999; Brown et al. 2002).

Mercury has a high affinity for reduced sulphhydryl groups, including those of cysteine and glutathione. It is in form of a methylmercury-cysteine complex that methylmercury enters mammalian cells through amino acid carriers. Methylmercury-L-cysteine is structurally similar to methionine and this complex is a substrate for transport systems that catalyse methionine uptake across cell membranes (reviewed in: Ballatori 2002). How mercury enters eukaryotic microorganisms such as yeast and fungi is not known.

3.4 Other metals

Other micronutrients with a potential for toxicity include selenate, molybdate and chromate. These metals cross cell membranes though sulphate transporters in mammalian cells (Ballatori 2002) whereas cobalt may be transported into yeast cells through the phosphate transporter Pho84 (Jensen et al. 2003). Phosphate and sulphate transporters are likely to carry the metals across the plasma membrane in form of oxyanions.

4 Metal transport and detoxification systems

Cells evade metal toxicity through metal export from the cell, sequestration within internal organelles, chelation by metal-binding proteins and peptides and reduction of uptake. Export of toxic metals and compartmentalisation in specific organelles such as the vacuole are the most efficient detoxification mechanisms found in microbes. Toxic metals may also bind to glutathione, metallothionein, and phytochelatin compounds and the resulting complexes are often substrates for transport systems. In this way, cells reduce the cytosolic concentration of reactive metal ions to sub-toxic levels, which leads to a better ability to survive and to proliferate in a polluted environment. The molecular identity of such detoxification systems may differ between metals and also between organisms.

4.1 Efflux-mediated tolerance systems

Metal export is a common detoxification strategy in prokaryota and a large number of transport proteins catalysing metal export have been characterised (Silver 1998; Rosen 1999b; Nies 2003). In contrast, there are very few such systems known in lower eukaryota. The most well-characterised metal exporter in *S. cerevisiae* is the plasma membrane protein Acr3 (Wysocki et al. 1997; Ghosh et al. 1999).

The *ACR3* gene (also called *ARR3*) was isolated in a screen for genes that confer high-level arsenic resistance to cells when overexpressed (Bobrowicz et al. 1997). *ACR3* encodes a 46 kDa protein with 10 potential membrane-spanning helices. Deletion of *ACR3* sensitises cells to arsenite and arsenate but not to the related metalloid antimony or any other metal tested including cadmium (Wysocki et al. 1997). Based on the fact that Acr3 is not able to contribute to arsenate tolerance without the activity of the arsenate reductase Acr2 (also called Arr2; see Section 4.4), it was suggested that Acr3 is a specific arsenite export protein (Bobrowicz et al. 1997). Indeed, cells expressing multiple copies of the *ACR3* gene accumulated little arsenite (Wysocki et al. 1997), whereas the *acr3Δ* mutant was deficient in arsenite export (Ghosh et al. 1999). The mechanism by which Acr3 transports As(III) is not understood but the lack of an ATP-binding cassette in the Acr3 sequence may indicate that As(III) export is coupled to the membrane potential (Rosen 1999).

The activity of Acr3 is controlled at the level of transcription; *ACR3* expression is strongly induced by As(III), As(V) and to a lesser extent by Sb(III). Metalloid-stimulated expression of *ACR3* requires the AP-1-like transcription factor Yap8 and possibly also Yap1 (Bobrowicz and Ulaszewski 1998; Bouganim et al. 2001; Haugen et al. 2004; Menezes et al. 2004; Wysocki et al. 2004).

The fact that *ACR3* expression is stimulated by antimonite may suggest that arsenite is not the sole substrate for Acr3. However, yeast cells lacking *ACR3* are not Sb(III) sensitive (Wysocki et al. 1997). In addition, an Acr3 homologue from the *Bacillus subtilis ars* operon confers resistance only to arsenicals (Sato and Kobayashi 1998). On the other hand, antimonite sensitivity of double *acr3*Δ *ycf1*Δ mutant (*YCF1* encodes a vacuolar ABC-transporter involved in metal detoxification: see Section 4.2) was only slightly improved by deletion of *FPS1*, while the *ycf1*Δ *fps1*Δ double mutant showed much higher increase in Sb(III) tolerance (Wysocki et al. 2001). This observation suggests that Acr3 might contribute to antimony tolerance under certain conditions.

The *ACR3* gene is located in a cluster of arsenical resistance genes that in terms of function resembles the prokaryotic *ars* operon (Bobrowicz et al. 1997). However, Acr3 shows no structural or sequence similarities to the well-studied arsenite/antimonite transporter ArsB from the *E. coli* plasmid R773 or the *Staphylococcus aureus* plasmid pI258. As a result of sequencing of numerous prokaryotic and fungal genomes, a novel and distinct family of Acr3-like transporters has emerged (Wysocki et al. 2003). Interestingly, *ACR3* homologues are widely distributed in both *Bacteria* and *Archea*, but are not found in eukaryotes except for a few fungal species, including some *Saccharomyces* yeasts, *Kluyveromyces lactis*,

Candida albicans, and *Neurospora crassa*. Phylogenetic analysis of the Acr3-like family suggests that horizontal gene transfer has played a major role in the evolution of the *ACR3* gene and that fungi might have acquired the *ACR3* homologue directly from a prokaryotic organism relatively late in evolution (Wysocki et al. 2003).

The *ars* operon of the *E. coli* plasmid R773 encodes an ATP-driven metalloid pump ArsAB consisting of two proteins; the arsenite- and antimonite-stimulated ATPase ArsA bound to the inner-membrane protein ArsB (Rosen 1999). Interestingly, in the absence of ArsA, ArsB uses the membrane potential for arsenite and antimonite export. No homologues of *E. coli* ArsB transporter have been found in eukaryotic genomes. In contrast, ArsA homologues are ubiquitous but the physiological function of eukaryotic ArsA remains elusive (Kurdi-Haidar et al. 1998; Zuniga et al. 1999; Bhattacharjee et al. 2001; Shen et al. 2003). For instance, the *S. cerevisiae* ArsA homologue encoded by the *ARR4* (*YDL100c*) gene is not associated with the plasma membrane and the ATPase activity of Arr4 is not stimulated by metalloids (Shen et al. 2003). Deletion of *ARR4* results in a slight sensitivity to several metals like copper, zinc, cobalt, chromium, vanadate, and arsenic (Zuniga et al. 1999; Shen et al. 2003) and this sensitivity is enhanced at 37°C suggesting a general role of Arr4 in stress response that is not related to a specific metal detoxification pathway.

The plasma membrane protein Ssu1 belongs to the major facilitator superfamily and overexpression of *SSU1* increases selenite tolerance (Pinson et al. 2000). Ssu1 has also been implicated in sulphite efflux from yeast cells (Park and Bakalinsky 2000) and due to analogous structures, both selenite and sulphite are likely to be recognised by Ssu1 for export. However, expression of *SSU1* is not induced by selenite and the primary physiological substrate of this transporter has not been determined. Moreover, selenite can efficiently be reduced by glutathione to less toxic elemental selenium that precipitates as red granules within the cell (Pinson et al. 2000). Hence, the mechanism by which Ssu1 affects selenite tolerance remains unclear.

PCA1 encodes a P-type metal-transporting ATPase with a role in copper and iron homeostasis (Rad et al. 1994; De Freitas et al. 2004) and a single amino acid substitution in *PCA1* (Arg970Gly) has been reported to increase cadmium tolerance (Shiraishi et al. 2000). Cells expressing *PCA1*-Arg970Gly accumulated less cadmium than either wild type or *pca1Δ* cells. Overexpression of *PCA1*-Arg970Gly further increased cadmium tolerance by lowering the intracellular cadmium level compared to cells with a single copy of this allele. Collectively, Pca1-Arg970Gly appears to contribute to cadmium tolerance, possibly by increasing cadmium export (Shiraishi et al. 2000). However, how this is achieved remains to be revealed.

4.2 Vacuolar sequestration of toxic metals

In *S. cerevisiae*, the yeast cadmium factor 1 (Ycf1) constitutes the major pathway of toxic metal sequestration in the vacuole. The *YCF1* gene was isolated in a

screen for genes conferring cadmium resistance to cells when present in multiple copies. Cells that overexpress *YCF1* exhibit elevated cadmium resistance while the *ycf1Δ* mutant is hypersensitive to this metal (Szczypka et al. 1994). Ycf1 is an ATP-binding cassette (ABC) transporter and it shares strong sequence similarity to the human cystic fibrosis conductance regulator (CFTR) and to the multidrug-associated proteins MRP1 and MRP2 (Szczypka et al. 1994; Buchler et al. 1996). Ycf1 is a 171 kDa protein present in the vacuolar membrane and it catalyses ATP-dependent uptake of a range of glutathione-conjugated metals and xenobiotics into the vacuole (Li et al. 1996, 1997; Tommasini et al. 1996). Other MRP family members involved in metal detoxification includes *Leishmania* PgpA that transports $As(GS)_3$ into intracellular vesicles (Légaré et al. 2001), mammalian MRP2 that, in the human liver, catalyses the extrusion of arsenic-glutathione complexes into bile (Kala et al. 2000) and human MRP1 that mediates export of $As(GS)_3$ in several tissues (Leslie et al. 2004).

In addition to its role in cadmium detoxification, Ycf1 has been shown to confer resistance to the metalloids arsenic and antimony. The *ycf1Δ* mutant is moderately sensitive to arsenite whereas it displays very strong antimonite sensitivity (Ghosh et al. 1999; Wysocki et al. 2001). In contrast, cells lacking *ACR3* are As(III) and As(V) sensitive but do not show any antimonite sensitivity. Deletion of both *ACR3* and *YCF1* genes results in additive arsenite sensitivity indicating that yeast cells possess two distinct metalloid detoxification pathways with different specificities (Ghosh et al. 1999).

Transport of $As(GS)_3$ into vacuoles is inhibited not only by cadmium and antimony but also by mercury, suggesting an additional role of Ycf1 in mercury tolerance (Ghosh et al. 1999). In agreement with this hypothesis, deletion of *YCF1* sensitises cells to mercury and active transport of $Hg(GS)_2$ across the membrane of vesicles isolated from *YCF1*-overexpressing cells has been demonstrated (Gueldry et al. 2003). Ycf1 also confers resistance to lead; the *ycf1Δ* mutant is Pb(II)-sensitive whereas cells overexpressing *YCF1* are more Pb(II)-resistant (Song et al. 2003).

How Ycf1 is regulated by various metals and xenobiotics is not fully understood. Expression of the *YCF1* gene is not induced to any large extent by metal treatment; cadmium exposure results in a twofold increase in *YCF1* mRNA levels (Li et al. 1997; Sharma et al. 2002), a twofold increase under selenite exposure (Pinson et al. 2000) whereas arsenite and antimonite exposure does not seem to affect *YCF1* expression (Wysocki et al. 2004). It is possible that the basal level of Ycf1 in the vacuolar membrane is sufficient to mediate metal tolerance. Thus, conjugation of metals to GSH may represent the rate-limiting step in tolerance acquisition. In that case, increased GSH synthesis might be sufficient to promote increased vacuolar sequestration of GSH-conjugated metals by the action of Ycf1 (Wysocki et al. 2004). A similar mechanism of arsenic tolerance has been proposed in *Leishmania*, where increased synthesis of trypanothione, the major source of reduced thiols in trypanosomatidae, is required to produce resistance as a result of formation and extrusion of metalloid-thiol complexes (Mukhopadhyay et al. 1996). It has been shown that Ycf1 is phosphorylated at two residues, Ser908 and Thr911, and that mutation of these sites severely impairs its transport activity

(Szczypka et al. 1994; Eraso et al. 2004). Neither the kinase responsible for Ycf1 phosphorylation nor whether phosphorylation affects Ycf1 activity under metal exposure is known. Finally, Ycf1 is also controlled at the level of proteolytic processing and intracellular trafficking (Mason and Michaelis 2002; Mason et al. 2003). Again, whether these processes are regulated under metal exposure is unknown.

Besides Ycf1, five additional *S. cerevisiae* members of the ABC transporter superfamily have been identified based on sequence similarities: Bpt1, Ybt1/Bat1, Yor1, Vmr1 (Yhl035), and Nft1 (Ykr103/Ykr104w) (Decottignies and Goffeau 1997; Mason et al. 2003). Bpt1 is localised in the vacuolar membrane and mediates transport of unconjugated bilirubin and glutathione conjugates into the vacuole (Petrovic et al. 2000; Klein et al. 2002; Sharma et al. 2002). In addition, Bpt1 plays a role in cadmium detoxification. However, the contribution of Bpt1 in vacuolar sequestration of cadmium is less important than that of Ycf1. In fact, the function of Bpt1 in cadmium resistance is only detectable in cells lacking *YCF1* (Sharma et al. 2002). Ybt1/Bat1 transports free bile acid (Ortiz et al. 1997) and, together with Bpt1 and Ycf1, Ybt1/Bat1 contributes to vacuolar accumulation of the toxic red pigment that is produced by adenine biosynthetic mutants (Sharma et al. 2002, 2003). Yor1 is a plasma membrane protein that participates in the detoxification of several unrelated compounds, like oligomycin, cadmium and a wide spectrum of organic anions (Cui et al. 1996; Decottignies et al. 1998; Katzmann et al. 1999). The function and cellular localisation of Vma1 and Nft1 remain to be characterized (Mason et al. 2003), although Vma1 appears to be in the vacuolar membrane and to mediate cadmium resistance (D. Wawrzycka, personal communication). Since these proteins share structural similarities and overlapping substrate specificities, it is reasonable to assume that the yeast MRP1-like ABC transporters provide resistance to a wide range of toxic metals and metalloids. Detailed phenotypic analysis of metal tolerance in various multiple deletion mutants in conjunction with transport studies will be required to support this hypothesis.

Two additional transporters appear to have a function in vacuolar sequestration of toxic metals; Zrc1 and Cot1. Zrc1 and Cot1 are both vacuolar membrane proteins (Li and Kaplan 1998) belonging to the CDF (cation diffusion facilitator) family of transporters (Paulsen and Saier 1997). Overexpression of *ZRC1* or *COT1* confers zinc tolerance, suggesting an increased capacity to sequester zinc in the vacuole (MacDiarmid et al. 2000, 2002). The *COT1* gene was originally isolated as a dosage-dependent suppressor of cobalt toxicity and overexpression of *COT1* increases cobalt and rhodium tolerance (Conklin et al. 1992). In fact, Cot1 and Zrc1 share significant sequence similarity and Zrc1 was originally isolated as a high-copy suppressor of zinc and cadmium toxicity (Kamizono et al. 1989). Hence, in addition to zinc, these transporters are also capable of mediating vacuolar cobalt and cadmium uptake.

Cadmium detoxification involves an ABC-type transporter also in *S. pombe*. Fission yeast Hmt1 is located in the vacuolar membrane and contributes to cadmium tolerance (Ortiz et al. 1992). In contrast to *S. cerevisiae* Ycf1, *S. pombe* Hmt1 does not transport cadmium-glutathione conjugates, but instead catalyses

the transport of glutathione-derived phytochelatins and phytochelatin-cadmium complexes into the vacuole (Ortiz et al. 1995).

4.3 Metal-binding peptides and proteins: metallothioneins and phytochelatins

Toxic metals may inactivate cellular proteins by reacting with sulphhydryl groups of cysteine residues and thiol-mediated defence mechanisms are commonly employed by eukaryotic cells. These include small cysteine-rich proteins known as metallothioneins and metal-binding peptides such as phytochelatins and glutathione (see Section 5).

Yeast metallothioneins encoded by *CUP1* were shown to maintain copper homeostasis and to confer cadmium tolerance by directly chelating metal ions (Winge et al. 1985). In contrast to mammalian metallothioneins, yeast Cup1 does not seem to protect cells against mercury, zinc, or arsenic toxicity (Ecker et al. 1986; Wysocki and Tamás: unpublished data). Moreover, deletion of the second metallothionein gene *CRS5*, exhibiting high similarity to mammalian metallothioneins and to a lesser extent to *CUP1*, renders cells sensitive only to copper (Culotta et al. 1994). In support of these findings, Crs5 was reported to sequester copper ions in a similar manner as mammalian metallothioneins (Jensen et al. 1996). Taken together, the contribution of metallothioneins to metal tolerance in yeast appears to be limited to copper and possibly to cadmium. However, although overexpression of *CUP1* increases cadmium tolerance, *cup1Δ* cells are not cadmium sensitive indicating a minor contribution of metallothioneins to cadmium detoxification. Furthermore, expression of *CUP1* is not induced in response to cadmium (Vido et al. 2001) suggesting that cadmium detoxification by chelation to metallothionein may not be a physiologically relevant mechanism in *S. cerevisiae*.

In *S. pombe*, resistance to a wide range of metals is associated with phytochelatins (Cobbett 2000b, 2000a). The general structure of these polypeptides is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2-11) and their synthesis from glutathione is mediated by the enzyme phytochelatin synthase (*PCS*). Production of phytochelatins is induced in response to cadmium, copper, arsenic, zinc, silver, and nickel exposure (Clemens et al. 2001). The enzymatic activity of phytochelatin synthase is furthermore stimulated by cadmium, copper, silver, zinc, lead, and mercury (Ha et al. 1999). However, only phytochelatin complexed with cadmium, copper, silver, and arsenic have been detected *in vitro* (Maitani et al. 1996; Schmoger et al. 2000).

Identification of the *PCS* gene encoding phytochelatin synthase in *S. pombe* and plants was an important step in understanding the physiological function of phytochelatins (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999). Phenotypic analysis of *S. pombe* cells lacking the *PCS* gene showed that the mutant was sensitised to cadmium, arsenate, arsenite, and copper (Clemens et al. 1999; Ha et al. 1999; Wysocki et al. 2003). Heterologous expression of the *S. pombe PCS* gene in *S. cerevisiae* wild type and the *acr3Δ ycf1Δ* double mutant not only confirmed an important role of phytochelatins in cadmium and arsenite de-

toxification but also revealed their ability to confer antimonite resistance (Clemens et al. 1999; Ha et al. 1999; Wysocki et al. 2003).

Chelation of cadmium by phytochelatins in the *S. pombe* cytosol is followed by the sequestration of phytochelatin-Cd complexes in the vacuole by the ABC transporter Hmt1 (Ortiz et al. 1992, 1995). Moreover, the existence of an additional phytochelatin-independent pathway of cadmium accumulation into the vacuole based on a $Cd(II)/H^+$ antiporter activity has been proposed (Ortiz et al. 1995). Sulphite metabolism also contributes to phytochelatin-mediated cadmium tolerance. It is believed that sulphite incorporation into phytochelatin-Cd complexes leads to their stabilisation and increases the number of cadmium molecules per complex (Reese and Winge 1988).

4.4 Arsenate reduction – a pathway leading to tolerance and drug activation

The initial step in arsenate detoxification in most organisms is the enzymatic reduction of As(V) to As(III). Two distinct prokaryotic families of arsenate reductases have been identified and extensively studied at the molecular level; one family includes the ArsC from the *E. coli* plasmid pR773 and the other one is represented by the ArsC encoded by the plasmid pI258 from *S. aureus* (Mukhopadhyay and Rosen 2002). In contrast, no arsenate reductase was known in eukaryotic organisms until the isolation of the *ACR* gene cluster in *S. cerevisiae* (see above; Bobrowicz et al. 1997). Overexpression of the arsenite export protein-encoding gene *ACR3* conferred resistance only to As(III), while the presence of both *ACR2* and *ACR3* genes on a multicopy plasmid resulted in increased tolerance to As(III) and As(V). This observation suggested that *ACR2* might encode an arsenate reductase (Bobrowicz et al. 1997). Indeed, *ACR2* deletion sensitises cells only to the pentavalent form of arsenic (Mukhopadhyay and Rosen 1998) and the purified homodimeric protein exhibits As(V) reductase activity (Mukhopadhyay et al. 2000).

Despite the lack of sequence identity, Acr2 and the *E. coli* R773 arsenate reductase share functional and mechanistic similarities. Both enzymes use glutathione and glutaredoxin as electron donors for arsenate reduction, and Acr2 complements the arsenate sensitivity of *E. coli* cells bearing an *arsC* deletion (Mukhopadhyay et al. 2000). Furthermore, a single cysteine residue (Cys12 in ArsC and Cys76 in Acr2) is required for catalytic activity (Mukhopadhyay and Rosen 2001). This may suggest that, as in the case of *E. coli* R773 ArsC, a mixed disulphide between the Acr2 enzyme and glutathione is formed, followed by glutaredoxin binding associated with reduction of arsenate to the dihydroxy monothiol As(III) intermediate; the two last steps in this reaction would involve the formation of a monohydroxy intermediate containing positively charged arsenic and the final release of free $As(OH)_3$, as determined for the crystal structure of *E. coli* R773 ArsC (DeMel et al. 2004).

Cys76 of Acr2 is a part of the $HC(X)_5R$ motif that is conserved in the Cdc25 family of dual-specific protein phosphatases (PTPs) (Fauman et al. 1998) and in

rhodanase, a thiosulphate sulphurtransferase (Hofmann et al. 1998). In PTPases, the $HC(X)_5R$ consensus, called the P-loop, serves as a phosphate binding pocket, where the active-site cysteine forms the phosphoenzyme intermediate that is stabilized by the adjacent arginine residue (Jackson and Denu 2001). The Cys76Ala and Arg82Ala mutations in the $HC(X)_5R$ consensus site of Acr2 resulted in loss of arsenate resistance and loss of reductase activity *in vitro*, suggesting that a phosphatase-active site is used as the catalytic centre for arsenate reduction in yeast (Mukhopadhyay and Rosen 2001).

Despite having the phosphatase active site, Acr2 does not exhibit phosphatase activity towards the substrate *p*-nitrophenyl phosphate (Mukhopadhyay and Rosen 2001). However, the P-loop in Acr2 lacks the GXGXXG motif that is found in a variety of PTPs (Mukhopadhyay et al. 2003). Interestingly, Acr2 was converted into a phosphatase at the expense of its arsenate reductase activity when three glycine residues were introduced at positions 79, 81, and 84 within the phosphatase motif (Mukhopadhyay et al. 2003). The conserved cysteine and arginine residues of the $HC(X)_5R$ were also required for the acquired phosphatase activity of the mutated Acr2 suggesting that both PTPs and Acr2 share a similar mechanism of catalysis. Based on these results and sequence similarities of Acr2 and I258 ArsC family to phosphatases, Mukhopadhyay et al. (2003) hypothesised that all three families of arsenate reductases, including *E. coli* R773 ArsC, *S. aureus* I258 ArsC, and *S. cerevisiae* Acr2, may have evolved independently for at least three times from an ancestral phosphatase (Mukhopadhyay et al. 2003). Due to the anaerobic atmosphere, the dominant form of arsenic in water was originally a trivalent arsenite. Thus, microorganisms first developed specific arsenite exporters to cope with arsenic toxicity. Next, when the atmosphere became oxidizing, the phosphatase would have been converted by only a couple of simple point mutations into arsenate reductases, which could produce substrates for the existing arsenite transporters (Rosen 1999; Mukhopadhyay et al. 2003).

Until recently, Acr2 was the only known eukaryotic arsenate reductase. The sequence of Acr2 was used to find an arsenate reductase-encoding gene in *Leishmania major* (Zhou et al. 2004). *LmACR2* is able to restore arsenate tolerance to *E. coli* and *S. cerevisiae* cells lacking arsenate reductases. Furthermore, the purified *Lm*Acr2 reduces not only arsenate but also antimonate [Sb(V)] in the presence of glutathione and glutaredoxin. Most importantly, expression of *LmACR2* in *L. infantum* amastigotes resulted in increased sensitivity to the Sb(V)-containing drug Pentostam (Zhou et al. 2004). Pentavalent antimony-containing drugs are used in the treatment of protozoan infections and reduction of Sb(V) to the more toxic Sb(III) is believed to be a pathway to activate the drug in *Leishmania* cells (dos Santos Ferreira et al. 2003; Wyllie et al. 2004; Zhou et al. 2004). Identification of an arsenate/antimonate reductase in *Leishmania* could be of major importance for predicting treatment efficacy of patients with leishmaniasis as well as in designing new strategies to sensitise pathogenic protozoa to available drugs.

Chromate (VI) is believed to be reduced non-enzymatically to the more stable trivalent form (Arslan et al. 1987). However, a chromate-resistance phenotype in *Candida maltosa* strain has recently been associated with active NADPH-dependent chromate reduction (Ramirez-Ramirez et al. 2004). The chromate re-

ductase enzyme remains to be identified. In most prokaryota, Hg(II) is reduced enzymatically to an inert monoatomic form Hg(0) by a MerA mercuric reductase located in the plasmid-encoded *mer* operon (reviewed in: Nies 1999; Brown et al. 2002). No mercuric reductase is known in eukaryotic organisms and the Acr2 arsenate reductase seems to be the sole example of toxic metal-detoxification reductase in lower eukaryota.

5 Sulphur and glutathione metabolism

A major chemical property of many toxic metals including Cd(II), As(III), Hg(II), and Pb(II) is their capacity to strongly bind to thiol residues. This property is used by most organisms for chelation, sequestration, and detoxification. For detoxification of nonessential toxic metals, yeasts overexpress the thiol-containing peptides glutathione and/or phytochelatins depending on the species (see above).

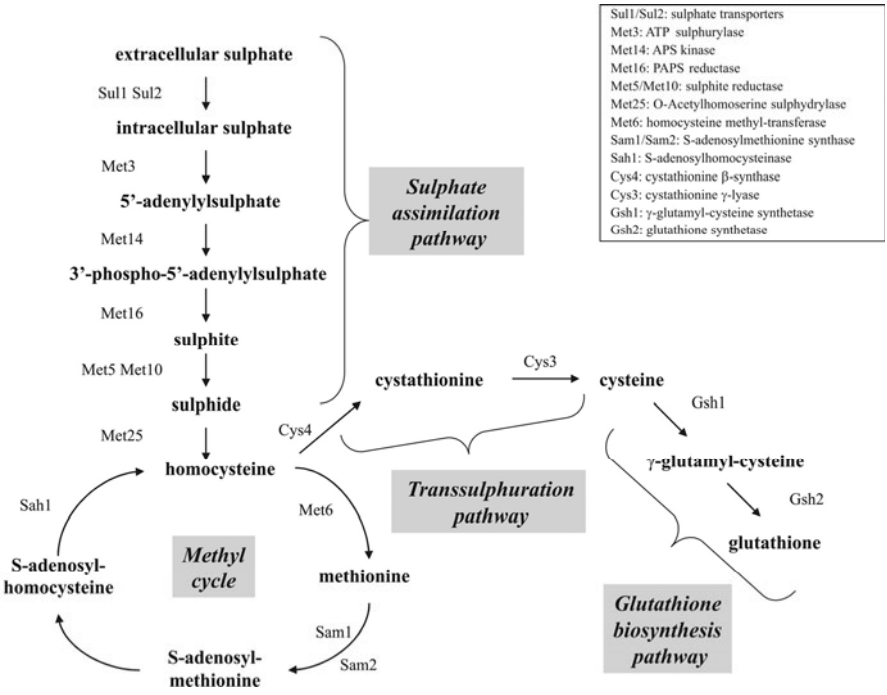


Fig. 2. Sulphur and glutathione metabolism in *Saccharomyces cerevisiae*. The sulphur pathway can be divided into three parts: the sulphate assimilation pathway, the methyl cycle, and the branch leading to cysteine and glutathione synthesis. See the text for details.

5.1 The sulphur pathway of *S. cerevisiae*

The sulphur pathway is composed of the sulphate assimilation pathway, the methyl cycle, the transsulphuration pathway and the glutathione biosynthesis pathway (Fig. 2). Inorganic sulphate (SO_4^{2-}) or sulphite (SO_3^{2-}) is reduced through the assimilation pathway resulting in production of sulphide (S^{2-}) and its incorporation into homocysteine or cysteine. Most yeast species are able to incorporate sulphide into both homocysteine and cysteine (Paszewski 2001). In *S. cerevisiae*, however, sulphide cannot be incorporated into cysteine leaving homocysteine the only precursor of the two sulphur amino acids; methionine through the methyl cycle and cysteine through the transsulphuration pathway (Thomas and Surdin-Kerjan 1997). Cysteine or a derivative of cysteine is probably the sensor of the metabolic state in the pathway (Hansen and Johannsen 2000; Paszewski 2001). Cysteine is also required for glutathione synthesis. Another essential function of the sulphur pathway is its involvement, through S-adenosylmethionine, in the biosynthesis of polyamines, biotin, and for almost all transmethylation reactions in the cell. The *S. cerevisiae* sulphur pathway and its regulation has been reviewed in detail by Thomas and Surdin-Kerjan (1997).

5.2 Toxic metals induce the synthesis of glutathione

Genome-wide transcriptional analyses in *S. cerevisiae* show that Cd(II), As(III) and Hg(II) induce the expression of genes of the sulphate assimilation and of the glutathione biosynthesis pathways (Momose and Iwahashi 2001; Vido et al. 2001; Fauchon et al. 2002; Haugen et al. 2004; Thorsen et al. in preparation). Proteome analysis also evidenced strong induction of the enzymes of the sulphur pathway in response to cadmium (Vido et al. 2001). Consistent with these data and the importance of glutathione for cadmium detoxification, the glutathione synthesis rate, and the flux in the pathway are strongly increased in response to this metal (Vido et al. 2001; Fauchon et al. 2002). In addition, all the intermediate metabolites of the glutathione pathway increase under cadmium exposure (Lafaye et al. 2005a, 2005b). Importantly, the cadmium response has been analysed at the level of the transcriptome, proteome and metabolome and the data correlate nicely (Vido et al. 2001; Fauchon et al. 2002; Lafaye et al. 2005a, 2005b).

Arsenite treatment also induces expression of genes and enzymes of the sulphur pathway (Haugen et al. 2004) but the induction levels seem less pronounced than with cadmium. The flux in the sulphur pathway and the glutathione synthesis rate are also strongly increased (Thorsen et al. in preparation). Reduced glutathione forms a complex with Cd(II), As(III) and other toxic metals which are transported into the vacuole by the ABC transporter Ycf1 as outlined above.

Interestingly, the two major transcriptional activators of the cadmium response, Met4 and Yap1 (see Section 6), control different parts of the detoxification pathway. Met4 controls the first part (*CYS4*, *CYS3*, *GSH1*) (Dormer et al. 2000; Fauchon et al. 2002) whereas Yap1 regulates the second part of the glutathione biosynthesis pathway (*GSH1*, *GSH2*). Yap1 also controls expression of glutathione

metabolism genes (*GTT2*, *GLR1*, *YCF1*) (Wemmie et al. 1994b; Wu and Moye-Rowley 1994; Grant et al. 1996; Gasch et al. 2000; Sugiyama et al. 2000). Both factors contribute to induced *GSH1* expression (Wheeler et al. 2003).

5.3 Sulphur sparing in proteins

Cadmium detoxification by GSH necessitates high amounts of sulphur. To cope with this vital sulphur requirement, the sulphur metabolism, normally directed to the production of methionine and cysteine for protein synthesis, is redirected to glutathione biosynthesis (Fig. 3; Fauchon et al. 2002). For optimal sulphur sparing, yeast cells both decrease their global protein synthesis rate (Lafaye et al. 2005a) and reduce the sulphur amino acid composition of the newly synthesized proteome. Remarkably, some abundant glycolytic enzymes rich in sulphur amino acids are replaced by sulphur-depleted isoenzymes. For example, the pyruvate decarboxylase enzyme Pdc1 containing 16 sulphur amino acids is strongly repressed whereas the sulphur-poor isoenzyme Pdc6 (5 sulphur amino acids) is dramatically induced. This global change in protein expression allows an overall sulphur amino acid saving of 30%. Interestingly, Met4, the main transcriptional activator of the sulphate assimilation pathway, is responsible for the isoenzyme switches and

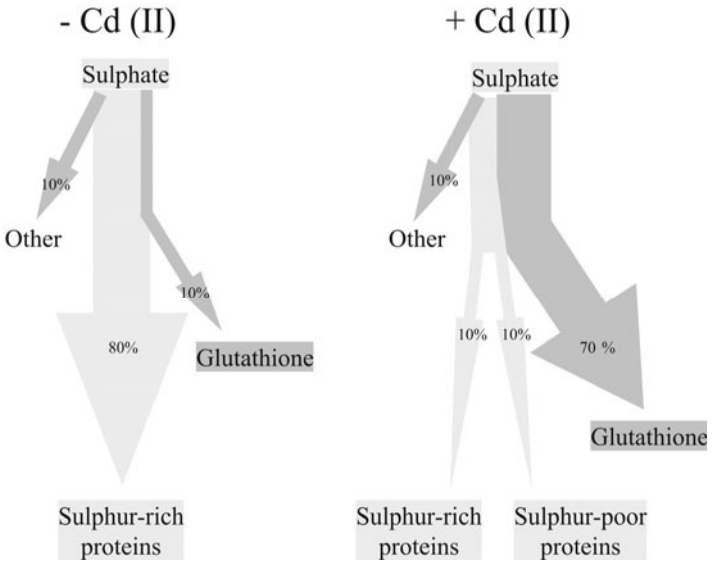


Fig. 3. Sulphur sparing in response to cadmium. Cd(II) exposure leads to a strong increase in glutathione levels, a decreased synthesis of many sulphur-rich proteins and the replacement of these proteins by sulphur-poor proteins.

plays a major role in the sulphur-sparing response, indicating that the same regulator controls both glutathione synthesis and the mechanisms to save sulphur in order to produce more glutathione (Fauchon et al. 2002). This regulation emphasizes the importance of sulphur sources in detoxification and indicates that a selective pressure may act on the atomic composition of proteins as also showed by Baudouin-Cornu et al. (2001).

5.4 Other yeasts

Induction of the (genes in the) glutathione biosynthesis pathway could also be expected in *S. pombe* since the main detoxification mechanism in this yeast involves chelation of Cd(II) to glutathione and to phytochelatins (PC). However, transcriptome and proteome analyses indicated that enzymes of the sulphate assimilation, glutathione and the PC synthesis pathways are not induced upon exposure to this metal (Chen et al. 2003; Bae and Chen 2004). Only some transporters of sulphur compounds are transcriptionally induced by Cd(II) (Chen et al. 2003), particularly a sulphate transporter, and this induction is dependent on the bZIP transcription factor Zip1 (Harrison et al. 2005). Although expression of genes encoding enzymes of the sulphur metabolic pathway are not induced, a strong increase in PC synthesis is observed in response to cadmium as well as to many other metals including Hg(II) (Ow et al. 1994). Binding of Cd(II) to PC synthase strongly activates the enzyme (Grill et al. 1991; Vatamaniuk et al. 2000; Maier et al. 2003). Sulphide synthesis is also increased without any transcriptional induction of enzymes in the sulphur assimilation pathway except the sulphate transporter-encoding gene (Bae and Chen 2004). Sulphide participates in the production of the high molecular weight PC-Cd-S complex with high cadmium binding capacity (Ow et al. 1994). A similar mechanism has been described in *Candida glabrata* (Dameron et al. 1989). Detoxification of Cd(II), As(III) and Sb(III) through PC-based chelation seems to be more efficient than the GSH-based chelation mechanism operating in *S. cerevisiae* since the expression of *S. pombe* or *A. thaliana* PC synthase in *S. cerevisiae* results in a strong increase in tolerance to these metals (Clemens et al. 1999; Wysocki et al. 2003).

5.5 Selenate and chromate interferes with the sulphate assimilation pathway

Selenate and chromate salts probably enter cells through sulphate transporters (Marzluf 1970; Breton and Surdin-Kerjan 1977). Interestingly, mutants resistant to selenate or chromate (*sul1Δ*, *sul2Δ*, *met3Δ*, *met14Δ*, and *met16Δ*) are all mutants of the sulphate assimilation pathway (Cherest et al. 1997) suggesting that metabolization of chromate and selenate through this pathway is necessary to cause toxicity. It is conceivable that selenate [Se(VI)] is toxic due to its conversion to selenite [Se(III)]. Another possibility to explain the phenotype of these mutants is that a sulphur-precursor metabolite accumulates in the mutant cells as a consequence of

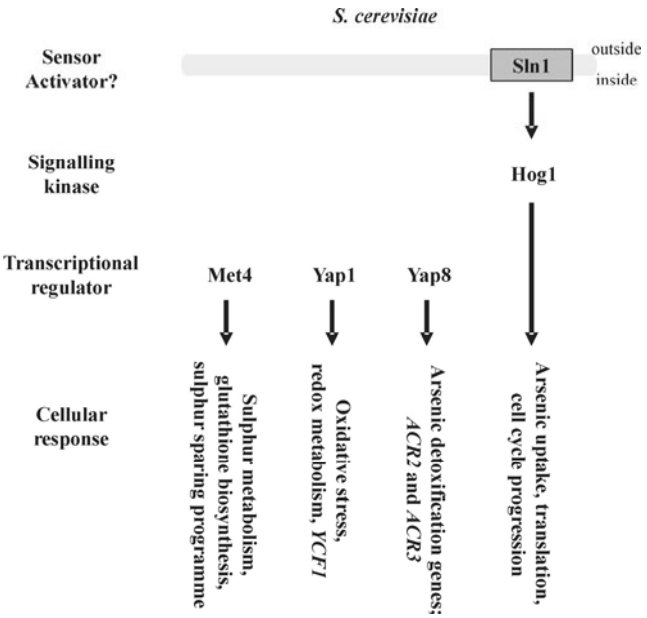


Fig. 4. Signalling proteins and transcriptional regulators involved in metal tolerance.

the lacking enzyme activity and causes a repression/inhibition of sulphate transporters and then resistance to the toxic analogues selenate and chromate.

6 Signalling and transcriptional regulation

The activity of various detoxification systems that contribute to cellular tolerance is controlled by signalling proteins and transcriptional regulators; yet, our understanding of the mechanisms by which eukaryotic cells sense the presence of non-essential metals, and activate such tolerance systems is rudimentary. However, recent work has identified a number of players involved (Fig. 4). Below, we will outline what is known about the mechanisms through which these transcription factors and signalling proteins are activated and contribute to tolerance.

6.1 Yap1 protects cells from a variety of oxidants and metals

One of the most well-characterised transcription factors involved in metal tolerance acquisition is the *S. cerevisiae* transcription factor Yap1 (yeast AP-1). Yap1 regulates the main peroxide (H_2O_2 and organic peroxides) metabolism pathway and contributes to stress responses elicited by specific metals and by several chemicals with electrophilic properties. Over the last few years, Yap1 has provided a unique model to understand how cells specifically sense and respond to

oxidants and to molecules of seemingly different chemical nature. Moreover, identification of the Yap1 gene-targets has provided basic insights into the mechanisms of oxidative, metal and chemical stress and the specific cellular defences used to counter these threats. The extended role of Yap1 in stress tolerance acquisition can be rationalised as an important cellular device for survival in soil habitats or during successful host invasion, *i.e.*, for providing protection against hostile environments and host-defence mechanisms that include both oxidative stress and a variety of fungicidal molecules. The recent description of a Yap1-operated indoleacetic response (Prusty et al. 2004), a plant hormone that cause yeast to differentiate into an invasive form, highlights the role of Yap1 and more generally of fungal stress responses in plant-pathogen interactions. True functional homologues of Yap1 have now been identified in many different fungi, including *S. pombe* Pap1 (Toone et al. 2001), *Candida albicans* Cap1 (Alarco and Raymond 1999), *Kluyveromyces lactis* KLYap1 (Billard et al. 1997) and Chap1 of the maize pathogen *Cochliobolus heterostrophus* (Lev et al. 2005). Genes homologous to *YAP1* are also present in *Aspergillus nidulans* (GeneBank accession no EAA62093) and *Neurospora crassa* (CAB91681). In addition to Yap1, *S. cerevisiae* contains another seven AP-1-like proteins: Yap2 to Yap8 (Fernandes et al. 1997; Toone et al. 2001).

6.1.1 Yap1, an AP-1-like bZIP transcription factor

Yap1 was initially identified as a protein capable of activating the SV40 AP-1 recognition-element (ARE) and purified by virtue of its ARE-specific DNA-binding affinity as a 90 kDa protein (Harshman et al. 1988). The cloning of the *YAP1* gene revealed the presence of a basic-leucine zipper (bZIP)-domain, which in contrast to other bZIP family members is amino- rather than carboxy-terminal (Moye-Rowley et al. 1989). This domain also differs from AP-1 factors at two of the five highly conserved residues that contact DNA, conferring a distinctive DNA-binding specificity to Yap1 (Fernandes et al. 1997). The identification of several natural Yap1 binding sites (Kuge and Jones 1994; Wu and Moye-Rowley 1994) and analysis of Yap1 DNA-binding properties (Fernandes et al. 1997) have established the Yap1 recognition element (YRE) as either TTACTAA or TGACTAA, which appear distinct from the canonical AP-1 recognition site (TGACTCA). There may be one or more additional Yap1 recognition sites since many *bona fide* Yap1 target gene promoters (*e.g. TSA1*) lack a YRE, although they directly bind Yap1 (Lee et al. 1999). Whether Yap1 homodimerizes to bind DNA as most other bZIP proteins is not established with certainty (Fernandes et al. 1997).

6.1.2 Yap1 regulates the yeast peroxide detoxification pathway in conjunction with Skn7

Yap1 regulates the yeast peroxide detoxification pathway. Such regulation meant to prevent oxidative stress-induced cellular damage, is essential for aerobic life and has the hallmarks of a homeostatic control. This function, first observed by

Schnell et al. (1992) on the basis of the hypersensitivity of *yap1Δ* cells to H_2O_2, *tert*-butylhydroperoxide (*t*-BOOH), cumene hydroperoxide and to redox cycling drugs (Schnell et al. 1992), was initially missed due to lack of a *yap1Δ* phenotype under normal laboratory growth conditions. Kuge and Jones (1994) subsequently established this function by identifying *TRX2,* encoding one of the two cytoplasmic thioredoxins, as a Yap1-dependent gene induced by H_2O_2, *t*-BOOH, diamide and diethylmaleate and by showing that *yap1Δ* is hypersensitive to diamide and diethylmaleate in addition to peroxide (Kuge and Jones 1994). Importantly, the response to peroxide and to the prooxidant drugs diamide and diethylmaleate relates to distinct modes of Yap1 activation (see further). Since then, an important number of peroxide-inducible Yap1-dependent genes have been identified that comprise most cellular antioxidants, catalases, superoxide dismutases, peroxiredoxins, glutathione peroxidases, cytochrome c peroxidase, activities of the glutathione, thioredoxin and pentose phosphate pathways, and other reductase systems such as the ATP-dependent sulphinic reductase sulphiredoxin (Lee et al. 1999; Gasch et al. 2000; Biteau et al. 2003). The nature of the peroxide-inducible Yap1 regulon fully explains the inability of *yap1Δ* to adapt to and to grow in the presence of peroxides (Stephen et al. 1995). The *yap1Δ* mutant is also hypersensitive to the lipid peroxidation by-products malondialdehyde (Turton et al. 1997) and linoleic acid hydroperoxide (LoaOOH) (Evans et al. 1998). The crucial function of Yap1 in peroxide detoxification is further supported by its identification in a genome-wide screen for genes that suppress the accumulation of mutations (Huang et al. 2003) and by the effect of *YAP1* overexpression in delaying chronological aging (Herker et al. 2004); two cellular functions largely affected by oxygen stress.

Yap1 controls the peroxide response in cooperation with Skn7, a transcription factor sharing similarities with the receiver domain of prokaryotic two-component systems (Brown et al. 1993) and to the helix-turn-helix DNA-binding domain of the heat shock transcription factor Hsf1 (Brown et al. 1994). Skn7 was identified in a genetic screen for peroxide sensitive (POS) mutants (Krems et al. 1995, 1996) and was later shown to be required for Yap1-dependent activation of *TRX2* and *TRR1* in response to H_2O_2 (Morgan et al. 1997; Lee et al. 1999). Skn7 actually only operates on a subset of the peroxide-inducible Yap1 regulon that includes mainly antioxidants and activities of the thioredoxin pathway (Lee et al. 1999). Interestingly, Yap1-dependent genes that do not require Skn7 often coincide with those needed for metal and chemical tolerance such as those encoding activities of the GSH pathway and efflux pumps (see below). Skn7 may cooperate with Yap1 through formation of a heteromeric transcription factor, based on the simultaneously binding of the two factors at target promoters (Morgan et al. 1997; Lee et al. 1999). The gene-promoter information that discriminates between Yap1 regulation versus Yap1-Skn7 co-regulation is not clearly established.

Skn7 also operates in a Yap1-independent peroxide-inducible pathway through interaction with Hsf1 (Raitt et al. 2000) and has ramifications in several other cellular pathways, including cell cycle control, cell wall synthesis, osmotic stress, and the calcineurin pathway. Together, these data suggest that one function of Skn7 might be to coordinate the responses elicited by these multiple pathways (reviewed in: Toledano et al. 2003).

6.1.3 Role of Yap1 in the tolerance to metal and chemical stress

Yap1 is also required for cellular tolerance to metals and to unrelated chemicals having in common, at least for most of them, demonstrated electrophilic properties. The very early identification of the *YAP1* gene in several independent high-copy plasmid screens for resistance to several unrelated drugs [4-nitroquinoline-N-oxide (4-NQO), N-methyl-N'-nitro-N-nitrosoguanine (MNNG), triaziquone, sulphomethuron methyl, cycloheximide, the iron chelators o-phenanthroline, 1-nitroso-2-naphtol] (Leppert et al. 1990; Hertle et al. 1991; Hussain and Lenard 1991; Schnell and Entian 1991) strongly hinted to a function of Yap1 in some aspects of drug-stress responses. Hypersensitivity of *yap1Δ* to methylglyoxal (Wu et al. 1993), 4-NQO and to a lesser extent to cycloheximide, MNNG and sulphomethuron methyl (Hertle et al. 1991) further supported this notion. Finally, this assumption gained full credence upon the observation that Yap1 is activated by several of the toxic chemicals for which it confers tolerance, including benomyl and MMS (Nguyen et al. 2001), and upon the identification of several Yap1 target genes that operate in the multiple drug resistance mechanism. These genes include those encoding the membrane-associated transporters *YCF1*, *ATR1*, and *FLR1* that operate as drug-efflux pumps. As already described, Ycf1 functions as a GSH-conjugate pump in the detoxification of several metals and of diazaborine (Jungwirth et al. 2000). Atr1 and Flr1 are transporters of the major facilitator family. Atr1 is involved in resistance to 4-NQO and aminotriazole (Coleman et al. 1997) and Flr1 in the resistance to fluconazole (Alarco et al. 1997; Alarco and Raymond 1999), the pro-oxidant drugs diamide, diethylmaleate and menadione (Nguyen et al. 2001), cerulenin (Oskouian and Saba 1999), benomyl, methotrexate (Broco et al. 1999; Tenreiro et al. 2001), and diazaborine (Jungwirth et al. 2000).

Yap1 regulates an integral cadmium detoxification pathway distinct from but overlapping with the bZIP transcription factor Met4 (see below) at several target genes (Dormer et al. 2000; Fauchon et al. 2002; Wheeler et al. 2003). This function was initially identified by the cadmium hypersensitivity of *yap1Δ* (Hirata et al. 1994; Wemmie et al. 1994b) and by the identification of *GSH1,* encoding γ-glutamyl synthase, as a Yap1 target (Wu and Moye-Rowley 1994). Several other Yap1-dependent cadmium inducible genes important for cadmium tolerance have been identified, including the already mentioned Ycf1 (Wemmie et al. 1994a), other GSH biosynthesis and sulphur amino acid metabolism genes (Hirata et al. 1994; Stephen and Jamieson 1997; Takeuchi et al. 1997; Vido et al. 2001). Yap1 also plays an important role in arsenite and antimonite tolerance; Yap1 is activated by these metalloids and is responsible for induced expression of GSH biosynthesis and sulphur amino acid metabolism genes and genes with oxidative stress defence and detoxification function (Haugen et al. 2004; Wysocki et al. 2004; Thorsen et al. in preparation). Moreover, growth of *yap1Δ* is impaired in the presence of these metalloids. Yap1 is also involved in mercury tolerance (Westwater et al. 2002).

The function of Yap1 in cadmium and, more generally, chemical stress tolerance does not require Skn7; the *skn7Δ* mutant is not sensitive but rather more resistant to diamide and cadmium (Morgan et al. 1997; Lee et al. 1999). Such resistance could be explained by the selection of genes operated by Skn7 in the Yap1

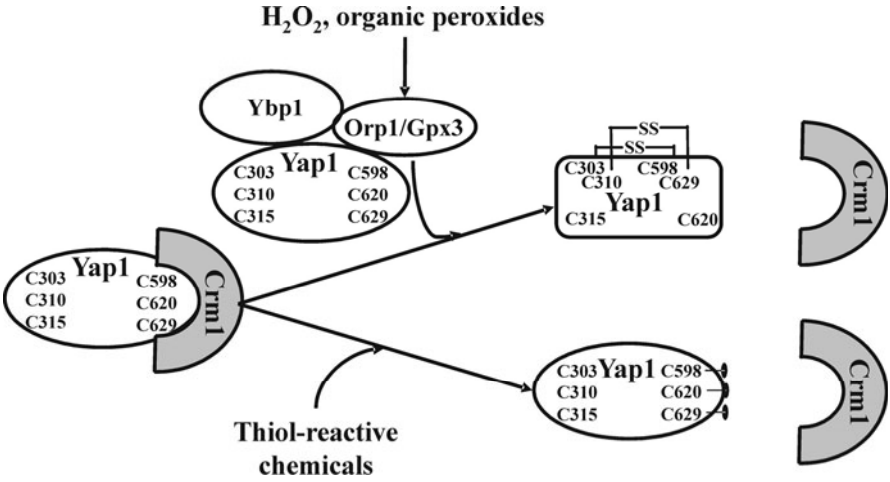


Fig. 5. A model of the activation of Yap1 by peroxides (H_2O_2 and organic peroxide) and thiol reactive chemicals (cadmium, mercury, diethylmaleate, N-ethylmaleimide etc.) that highlights the existence of two redox centres within the protein (adapted from: Azevedo et al. 2003; Wood et al. 2004). Unmodified Yap1 is recognized by Crm1 and permanently exported out of the nucleus. Peroxide will lead to Yap1 intramolecular disulphide bond formation through an Orp1 and Ybp1-dependent mechanism (see text), resulting in a change of conformation concealing the Yap1 nuclear export signal (NES) and, hence, its interaction with Crm1 and export out of the nucleus. Thiol reactive agents, by modifying Yap1 C-terminal cysteines lead to a presumable modification of the Yap1 NES. The later mechanism is Orp1 and presumably also Ybp1-independent.

regulon (see above) (Lee et al. 1999; Vido et al. 2001). Moreover, Skn7 has a negative effect on this response, based on the super-induced levels of some Yap1 target genes in cadmium treated *skn7Δ* cells (Vido et al. 2001).

6.1.4 Molecular control of Yap1

An intriguing feature of Yap1 resides in its activation by a multitude of stress signals, including peroxides (H_2O_2, *t*-BOOH), diamide (Kuge and Jones 1994), menadione (Stephen et al. 1995; Stephen and Jamieson 1997), the electrophiles diethylmaleate (Kuge and Jones 1994), benomyl and MMS (Nguyen et al. 2001), cadmium (Hirata et al. 1994; Stephen and Jamieson 1997), arsenite (Menezes et al. 2004; Wysocki et al. 2004), antimonite (Wysocki et al. 2004), and mercury (Westwater et al. 2002). In fact, all these signals control Yap1 through regulated nuclear export by stress-induced post-transcriptional modification(s).

Under non-stress conditions, Yap1 is restricted to the cytoplasm (Kuge et al. 1997) by virtue of rapid nuclear export by the nuclear export receptor Crm1/Xpo1 (Fig. 5). Crm1 recognises and non-covalently interacts with a non-canonical hydrophobic leucine-rich nuclear export signal (NES) located in a carboxy-terminal domain, named C-terminal cysteine rich domain (cCRD) that also carries three re-

peats of the cysteine motif CSE (Kuge et al. 1997, 1998; Yan et al. 1998). Upon exposure to stress signals, Yap1 accumulates in the nucleus due to loss of the Yap1-Crm1 interaction (Kuge et al. 1998; Yan et al. 1998). In contrast to its regulated nuclear export, Yap1 import into the nucleus is constitutive and is mediated by the nuclear import receptor Pse1/Kap121 (Isoyama et al. 2001). Hence, a key step in Yap1 activation resides in the regulation of a Crm1-dependent nuclear export controlling subcellular location. Stress-induced redox or chemical modification of Yap1 cysteines appeared as the likely mechanism disrupting the interaction between the Yap1-NES and Crm1. Although this early model was correct, it did not consider how peroxides, metal or chemical signals reach Yap1, a process actually more complex than initially thought, which underlie the true sensing function operated at the level of Yap1 and its specificity.

6.1.5 The S. cerevisiae peroxide sensor

In response to H_2O_2, Yap1 is activated by oxidation to an intramolecular disulphide bond formed between the cCRD Cys598 and Cys303 located within a second CRD at the N-terminal portion of the protein (nCRD) (Delaunay et al. 2000; for a detailed review see Toledano et al. 2004). A second intra-molecular disulphide bond between Cys310 and Cys629 has been identified in purified and air-oxidized Yap1 (Wood et al. 2003). Disulphide linkage between N- and C-terminal Yap1 cysteine residues promotes a conformational change concealing the Yap1 C-terminal NES, as recently established by a detailed NMR structural analysis of an oxidized truncated form of Yap1 encompassing both N- and C-terminal CRDs (Wood et al. 2004).

Yap1 oxidation by H_2O_2 is not direct, involving Orp1 (Oxidant Receptor Peroxidase) that acts as the actual H_2O_2 sensor of the pathway (Delaunay et al. 2002). Yap1 oxidation involves a third subunit Ybp1 (Yap1-Binding Protein) (Veal et al. 2003; Gulshan et al. 2004), a protein with no discernable functional domain, likely acting by chaperoning the Orp1-Yap1 interaction. Orp1, also known as Hyr1/Gpx3, is a 20 kDa protein with sequence homology to glutathione peroxidases (GPx) (Inoue et al. 1999; Avery and Avery 2001). Orp1 has *in vitro* peroxidase activity operated by a mechanism distinct from classical GPxs, involving a catalytic disulphide between the conserved peroxidatic Cys36 and Cys82 and a reduction by thioredoxin and not by GSH (Delaunay et al. 2002). Although the Orp1 disulphide forms in cells exposed to H_2O_2, Orp1 only contributes to H_2O_2 tolerance by regulating Yap1 but not by acting as an H_2O_2 peroxide reductase. However, Orp1 is active for the detoxification of lipid peroxides *in vivo* (Avery and Avery 2001). Further, in some strain backgrounds, *orp1Δ* is hypersensitive to cadmium (Avery et al. 2004), a result taken as an indication that the toxicity of this metal is, at least in part, due to production of lipid hydroperoxides.

The Orp1-Yap1 H_2O_2 sensor operates as a cysteine-redox relay (Delaunay et al. 2002; Toledano et al. 2004). The Orp1 catalytic Cys36 senses the H_2O_2 signal and oxidizes to a Cys-SOH. Oxidized Orp1 transduces this signal to Yap1 by engaging the latter into a Cys36-Cys598 intermolecular disulphide, which is then converted to the intramolecular Cys303-Cys598 disulphide of active Yap1. The second Yap1

intramolecular disulphide bond probably forms similarly through recruitment of a second Orp1 oxidized molecule. Importantly, since both alternate Orp1 intra- and intermolecular disulphides are observed in H_2O_2-treated cells, the nascent Orp1 Cys36 sulphenic acid is poised to react with either Orp1 Cys82 to complete its peroxidatic cycle or with Yap1 Cys598. In this process, Ybp1, the absence of which prevents Orp1-Yap1 disulphide linkage, may act as a scaffold bringing Orp1 and Yap1 into a non-redox complex, and may also chaperon mixed-disulphide formation by guiding Orp1 Cys36-SOH towards Yap1 Cys598 and/or preventing formation of the competing Orp1 Cys36-Cys82 disulphide bond.

Yap1 oxidation occurs rapidly, within 1 minute and transiently, lasting about 30-45 minutes, indicating that it is deactivated by an efficient reductase system (Delaunay et al. 2000). This system is probably thioredoxin as suggests the partial Yap1 oxidation in cells lacking this reductase (Izawa et al. 1999; Delaunay et al. 2000; Carmel-Harel et al. 2001) although *in vivo* evidence of a Yap1-thioredoxin interaction could not be obtained (Izawa et al. 1999; Delaunay et al. 2000). Alternatively, lack of thioredoxin may maintain Yap1 activation by the increase in intracellular H_2O_2 resulting from impairment of the H_2O_2 scavenging capacity of thioredoxin-dependent peroxiredoxins (Carmel-Harel et al. 2001). In contrast to thioredoxin, the GSH pathway does not seem to interfere with Yap1 regulation, as attests lack of a Yap1 phenotype upon inactivation of GSH reductase or of both dithiol glutaredoxins (Izawa et al. 1999; Delaunay et al. 2000). However, GSH depletion caused by *GSH1* inactivation results, to some extent, in Yap1 activation as observed by some (Wheeler et al. 2003) but not by other authors (Izawa et al. 1999; Delaunay et al. 2000). This difference may either be due to the paucity of the effect or to the difficulty of total cellular depletion of GSH, an essential molecule in yeast.

6.1.6 A second Yap1 redox centre for metals and electrophiles

Although metals and chemicals also activate Yap1 by regulating its nuclear export (Kuge et al. 1997), these compounds are sensed through a distinct mechanism (Azevedo et al. 2003). This distinction was initially suggested by Wemmie et al. (1997) who observed that Yap1 activation by diamide only relied on cCRD cysteines, whereas peroxide required additional cysteines in the nCRD (Wemmie et al. 1997; Coleman et al. 1999). In addition, all known non-peroxide Yap1 inducers tested were unable to promote the oxidation of Yap1 to the intramolecular disulphides characteristic of its activation by peroxides (Delaunay et al. 2000; Azevedo et al. 2003). Further, Yap1-activation by these compounds by-passed the requirement of Orp1 (Delaunay et al. 2000; Azevedo et al. 2003) and Ybp1 (Veal et al. 2003; Gulshan et al. 2004). The observation that most, if not all, non-peroxide Yap1 inducers had thiol-reactive properties in common suggested that they could directly activate Yap1 by binding to critical NES vicinal cysteines, either covalently as in the case of electrophiles or non-covalently as in the case of metals, thereby altering the Yap1-Crm1 interaction. This hypothesis was confirmed using the model electrophile N-ethylmaleimide, shown to activate Yap1 by covalent modification of the Yap1 C-terminal Cys598, Cys620 and Cys629 (Azevedo et al.

2003). Activation of *S. pombe* Pap1 by diethylmaleate has also been proposed to operate through covalent cysteine adduct formation (Castillo et al. 2002). Yet, one more distinct mechanism has been proposed for Yap1 activation by diamide, involving formation of either one of the three possible disulphide bonds within C-terminal cysteines (Kuge et al. 2001). However, this model seems unlikely since Yap1 activation by diamide only requires either one of the Yap1 C-terminal cysteines (Kuge et al. 1997, 1998; Yan et al. 1998), which rather suggests covalent modification of single cysteines, as diamide may also form stable cysteine adducts. Interestingly, the two modes of Yap1 sensing were shown to operate simultaneously with the quinone menadione (Azevedo et al. 2003), a drug that is both a superoxide anion generator and a highly reactive electrophile. Here, superoxide anions are believed to activate the Orp1-dependent activation pathway through their dismutation to H_2O_2.

6.2 Yap8: a specific arsenic resistance factor

Yap8 is a 33 kDa protein of the yeast AP-1 family of transcription factors and exhibits about 15% identity to Yap1. The *YAP8* (also called *ACR1*/*ARR1*) gene was isolated together with *ACR2* and *ACR3* on a genomic DNA fragment that increased arsenic tolerance when expressed from a multicopy plasmid. Based on genetic data and on the similarity of Yap8 to other yeast AP-1-like proteins, it was suggested that Yap8 is a transcriptional regulator of the *ACR3* gene (Bobrowicz et al. 1997). Later, several labs provided evidence that Yap8 contributes to arsenic tolerance by controlling expression of the *ACR2* and *ACR3* genes (Bobrowicz and Ulaszewski 1998; Bouganim et al. 2001; Haugen et al. 2004; Maciaszczyk et al. 2004; Menezes et al. 2004; Wysocki et al. 2004).

Although Yap8 is a member of the yeast AP-1 protein family and shares sequence homology with other fungal AP-1-like transcription factors, it appears to behave differently from the most well-characterised yeast AP-1 protein Yap1. Firstly, deletion of *YAP8* produces hypersensitivity to As(III), As(V) and a weak Sb(III) sensitivity whereas growth of the *yap8Δ* mutant is unaffected by the presence of oxidants including methyl viologen (paraquat), *t*-BOOH, menadione and diamide (Wysocki et al. 2004). Secondly, Wysocki et al. (2004) showed that Yap8 resides predominantly in the nucleus by monitoring a GFP-Yap8 fusion protein as well as by detecting a genomic copy of a myc-tagged Yap8 in nuclear extracts. Chromatin immunoprecipitation (ChIP) assays further strengthened the notion that at least a portion of Yap8 is nuclear since Yap8 was found to be associated with the *ACR3* promoter in both untreated as well as As(III)-exposed cells (Wysocki et al. 2004). In contrast to Wysocki et al. (2004), Menezes et al. (2004) reported that GFP-Yap8 is regulated at the level of nuclear export in a Crm1-dependent fashion, suggesting that Yap8 is controlled in a similar way as Yap1. Whether the discrepancy between these two studies is due to the use of different *Saccharomyces* strains has yet to be clarified. Thirdly, Yap8 may bind to a DNA sequence that is different from that of Yap1 (Wysocki et al. 2004). Arsenic-induced expression of *ACR2* and *ACR3* requires the presence of a TTAATAA promoter sequence (Yap1

binds to and regulates expression primarily through a TTACTAA sequence) as well as Yap8: deletion of TTAATAA from the *ACR2*/*ACR3* promoter or deletion of *YAP8* reduces induction of *ACR2* and *ACR3* expression in response to As(III) exposure. In contrast, *ACR2* and *ACR3* expression is largely unaffected by *YAP1* deletion (Wysocki et al. 2004) although other studies suggested that Yap1 may affect expression under certain conditions (Bouganim et al. 2001; Haugen et al. 2004; Menezes et al. 2004). Since ChIP assays evidenced the presence of Yap8 on the *ACR3* promoter (Wysocki et al. 2004), it is tempting to speculate that Yap8 binds to the TTAATAA sequence. However, whether this is indeed the case has yet to be demonstrated. It is important to note that Yap8 was not found to be bound to its own promoter despite the presence of a TTAATAA element (Wysocki et al. 2004). The nucleotides flanking the TTAATAA element are different for the two promoters; TTGATTAATAATCAA in the *ACR3* promoter and TTCTTAATAAATT in the *YAP8* promoter, and it is possible that this difference affects DNA binding and expression of target genes (Wysocki et al. 2004). The fact that mutations of nucleotides flanking the Yap1 and Yap2 DNA binding-sites decreased expression of their respective target genes (Cohen et al. 2002) would support this notion.

The finding that Yap8 is permanently associated with the *ACR3* promoter suggests that arsenic-induced activation of Yap8 is neither exerted at the level of localisation nor at the level of As(III)-stimulated binding to the *ACR3* promoter (Wysocki et al. 2004). Yap8 activation requires a number of cysteine residues (Cys132 and Cys274) that are conserved in several fungal AP-1-like proteins; the Yap8 Cys132Ala and Yap8 Cys274Ala mutants were unable to stimulate induction of *ACR3* expression and, consequently, failed to promote cellular arsenic tolerance (Wysocki et al. 2004). In contrast to *yap1Δ*, *yap8Δ* cells are not sensitive to chemical oxidants. Moreover, overexpression of *YAP8* does not restore H_2O_2 tolerance to *yap1Δ* cells. Although Yap8 activation by metalloid-induced oxidative modifications cannot be excluded solely based on these data, such an activation mechanism may appear less likely. Instead, it is conceivable that these Yap8 cysteines directly bind to As(III) inducing a conformational change such that the modified Yap8 can trigger gene expression (Wysocki et al. 2004). The exact mechanism of arsenic-mediated Yap8 activation remains to be unveiled.

6.3 Hog1 mediates As(III) tolerance through multiple mechanisms

Mitogen-activated protein kinase (MAPK) pathways are of central importance for all eukaryotic cells since they are critically involved in controlling cell growth, differentiation as well as in establishing stress responses. MAPKs are activated by dual phosphorylation of adjacent threonine and tyrosine residues by a wide variety of environmental cues (Widmann et al. 1999; Kyriakis and Avruch 2001). For instance, As(III) exposure results in activation of mammalian p38, *S. pombe* Sty1 and *S. cerevisiae* Hog1 (Tamás and Wysocki 2001; Qian et al. 2003). However, the molecular mechanisms through which these proteins mediate As(III) tolerance are not fully understood.

Hog1 is the MAPK of the high osmolarity glycerol (HOG) pathway that mediates the response to high osmolarity in *S. cerevisiae* (reviewed in: Hohmann 2002; Tamás and Hohmann 2003). Increased extracellular osmolarity leads to activation of Hog1 through two independent upstream branches, the Sln1-Ssk1 branch and the Sho1 branch, converging at the MAPKK Pbs2 (Brewster et al. 1993). In turn, Hog1 activates a number of targets including various transcription factors (Rep et al. 1999; Alepuz et al. 2001; Proft et al. 2001; de Nadal et al. 2003) and the MAPK activated protein kinase (MAPKAPK) Rck2 involved in translation control (Bilsland-Marchesan et al. 2000; Teige et al. 2001). Hog1 has an additional role in controlling cell cycle progression under osmotic stress (Alexander et al. 2001; Belli et al. 2001; Yaakov et al. 2003; Escoté et al. 2004).

Besides its role in osmoprotection, Hog1 has an important function in mediating arsenic and antimony tolerance; cells lacking the *HOG1* gene or that express *HOG1* alleles that cannot be phosphorylated or are deficient in kinase activity, are highly sensitive to As(III) and Sb(III). In line with the observed phenotypes, Hog1 is phosphorylated in As(III) or Sb(III) exposed cells. Interestingly, metalloid-induced phosphorylation, and hence activation of Hog1, displays both quantitative and qualitative differences to osmotic activation: phosphorylation is clearly weaker, reaches its maximum at a later time point and lasts longer. As(III)-stimulated Hog1 phosphorylation is furthermore fully dependent on Pbs2 and Ssk1; Hog1 is not phosphorylated in As(III)-exposed *pbs2Δ* and *ssk1Δ* cells and these mutants are basically as As(III) sensitive as the *hog1Δ* mutant. In contrast, mutants in the Sho1 branch of the pathway affect metalloid signalling and tolerance only to a minor extent (Wysocki, Van der Does, Johansson, and Tamás: unpublished data). Collectively, these data suggest that As(III)-stimulated activation of Hog1 mainly occurs through the Sln1 branch of the pathway.

A striking feature of As(III)-induced activation of Hog1 is the lack of nuclear accumulation of the MAPK and an almost complete absence of Hog1-dependent transcriptional responses (Thorsen, Kristiansson, Nerman, and Tamás: unpublished data). Hog1 controls expression of about 150 genes under osmotic stress (Posas et al. 2000; Rep et al. 2000) whereas only four genes display Hog1-dependent expression changes under As(III) exposure. Strains lacking these genes display wild type As(III)-tolerance and are therefore not likely to play a major role in metalloid protection (Thorsen, Kristiansson, Nerman, and Tamás: unpublished data). Instead, Hog1 appears to mediate As(III) tolerance by other mechanisms.

Firstly, Hog1 contributes to As(III)-tolerance by affecting Fps1-mediated transport: *hog1Δ* cells accumulate more arsenic than wild type cells whereas *hog1Δ fps1Δ* accumulate little As(III). Additional deletion of *FPS1* in a *hog1Δ* background suppresses the As(III) and also the Sb(III) sensitivity of this mutant (Thorsen, Tängemo, Wagner, Wysocki, Boman, and Tamás: unpublished data). It was previously reported that osmotic inactivation of Fps1 is independent of Hog1 (see Section 3.1) (Luyten et al. 1995; Tamás et al. 1999). Similarly, *FPS1* expression in metalloid-treated cells is reduced to similar extents both in wild type and *hog1Δ* cells. However, the basal Fps1-dependent transport rate is elevated in a *hog1Δ* mutant: deletion of *HOG1* increases Fps1-dependent uptake of both glyc-

erol and As(III). Hence, metalloid sensitivity of *hog1Δ* cells can largely be attributed to increased metalloid influx through Fps1 (Thorsen, Tängemo, Wagner, Wysocki Boman, and Tamás: unpublished data).

Secondly, Hog1 may activate the MAPKAPK Rck2 under As(III) exposure. Rck2 is phosphorylated in a Hog1-dependent manner both upon osmotic (Bilsland-Marchesan et al. 2000; Teige et al. 2001) and oxidative (Bilsland et al. 2004) stress treatments. Rck2 is also required for optimal As(III) tolerance and genetic evidence suggests that the protein is a Hog1 target also under these conditions; growth of *rck2Δ* is impaired in the presence of As(III) and *RCK2* overexpression partially suppressed the As(III) sensitivity of *hog1Δ* (Wysocki and Tamás: unpublished data). Hence, epistasis analysis indicates that the two proteins act in the same pathway. The molecular function of Rck2 is not well understood although the protein has been implicated in downregulation of protein synthesis under osmotic stress by affecting phosphorylation of elongation factor 2 (Teige et al. 2001). Regulation of protein synthesis appears to be an important response to environmental stresses (Sheikh and Fornace 1999). Although the effect of arsenic on protein synthesis has not been explored, the arsenic-sensitive phenotype of mutants lacking regulators of protein synthesis suggests that this may be the case.

Thirdly, Hog1 affects cell cycle progression under As(III) exposure. A temporary cell cycle arrest is part of the physiological response to various environmental stress conditions since such a delay provides time to repair damage and to change metabolism so that cells can adapt to new growth conditions (Mendenhall and Hodge 1998; Wilson and Roach 2002). It was recently reported that Hog1 phosphorylates the CDK-inhibitor protein Sic1 in response to osmotic stress, resulting in Sic1 stabilisation and inhibition of the cell cycle at the G_1/S phase (Escoté et al. 2004). After short-time exposure to arsenite, however, loss of *HOG1* and *SIC1* does not affect an initial cell cycle arrest in G_1. Interestingly, Hog1 and Sic1 seem to be required for maintaining G_1 arrest as both *hog1*Δ and *sic1*Δ mutants resume cell cycle faster then wild type cells. In addition, the double *hog1*Δ *sic1*Δ mutant displays higher arsenite sensitivity and a stronger checkpoint defect (Wysocki and Tamás: unpublished data). These results suggest that Hog1 and Sic1 may have independent roles in cell cycle regulation after short-time arsenic treatment.

Surprisingly, in asynchronous cultures containing As(III), *hog1*Δ cells accumulated with 1N DNA content, while wild type preferentially arrested in G_2/M phase. Analysis of cells synchronized in G_1 and cultivated continuously in the presence of As(III) confirmed that the *hog1*Δ mutant was defective in abrogation of the As(III)-induced G_1 arrest (Wysocki and Tamás: unpublished data). This observation suggests that Hog1 plays an additional role in adaptation to long-term metalloid stress. Interestingly, Hog1 functions in recovery from G_1 arrest under higher concentrations of salt (Belli et al. 2001). The possible mechanism of cell division resumption under arsenic and osmotic stresses might include restoring transcription and/or translation leading to the production of factors required for the relief of inhibitory mechanisms or stimulation of G_1/S transition. The *hog1*Δ mutant is defective in translation initiation after osmotic stress-induced inhibition (Uesono and Toh 2002). Thus, lack of adaptation to arsenic-induced G_1 delay could be ex-

plained by a failure to re-start translation. Since *rck2Δ* did not show any cell cycle defect, Rck2 is not likely to be involved in this process (Wysocki and Tamás: unpublished data).

Hog1 is also required for optimal cadmium tolerance and Hog1 is phosphorylated in cadmium exposed cells (Bilsland et al. 2004). However, the targets of Hog1 under cadmium stress as well as the mechanisms by which Hog1 controls cadmium tolerance are unknown.

6.4 Met4

The *de novo* synthesis of sulphur amino acids and S-adenosylmethionine from inorganic sulphur source, sulphate, or sulphite (Fig. 2) is controlled by the bZIP transcriptional activator Met4. This metabolic pathway is tightly regulated probably because sulphate reduction necessitates high levels of energy (ATP). Met4 activity strongly decreases when methionine is added into the culture medium. This regulation is the result of methionine conversion into cysteine which is the probable sensor of the pathway (Hansen and Johannsen 2000). Different factors modulate the activity of Met4 including Met28, Met31, Met32, Cbf1, and Met30 (Thomas and Surdin-Kerjan 1997). Met30 is the F subunit of the SCF^{Met30} ubiquitin ligase. SCF ubiquitin ligases are multisubunit complexes and the budding yeast SCF consists of Skp1, the scaffold protein Cdc53, the RING-finger protein Rbx1/Roc1/Hrt1 and one member of the family of F-box proteins (Deshaies 1999). In minimal medium, the function of Met30 is to target the ubiquitylation and the degradation of Met4 when the genes of the sulphur pathway should not be expressed, *i.e.*, upon addition of methionine to the medium (Rouillon et al. 2000). In rich medium, Met4 is oligo-ubiquitinated but is not degraded. This modification alleviates transactivation properties for most *MET* genes but gives transcriptional activation specificity to the *SAM1* and *SAM2* genes (Kuras et al. 2002). In response to Cd(II), both mechanisms of inhibition are over-ridden to enable rapid Met4-dependent induction of the sulphur metabolic pathway necessary to increase glutathione production (Barbey et al. 2005). In rich medium, a deubiquitylation step rapidly removes inhibitory ubiquitin moieties from Met4, which fully activates the transactivator. In minimal medium, Cd(II) inhibits cysteine-dependent degradation of Met4 through dissociation of the Skp1-Met30 interaction (Barbey et al. 2005).

Although Met4 contains a bZIP domain, the transactivator is not able to bind DNA without association to its cofactors, Cbf1, Met28, Met31, and Met32, which in different combinations tether Met4 to DNA. A first complex (Cbf1-Met4-Met28) contains the bZIP factor Met28 and the centromere factor Cbf1 (Thomas and Surdin-Kerjan 1997). This complex recognizes a specific DNA sequence (TCACGTG) also present in yeast centromeres and recognized by Cbf1 at the centromeres. Moreover, this sequence is found in most *MET* genes and is necessary for activation of *MET16* and *MET25* (Kuras et al. 1997; Thomas and Surdin-Kerjan 1997). Alternatively, Met4 and Met28 can associate to the homologous factors Met31 or Met32. These complexes recognize the DNA sequence

AAACTGTG (Blaiseau and Thomas 1998) found in the promoter of many *MET* genes (Thomas and Surdin-Kerjan 1997). These factors are dispensable for *MET25* expression but required for expression of both *MET3* and *MET14* (Blaiseau et al. 1997). The variety of transactivating complexes acting on different DNA sequences suggests the flexibility of Met4 to modulate expression of its target genes in response to different environmental conditions.

The two DNA binding sites cited above were also found at proximity (both 370 to 350 nt upstream from ATG) in the promoter region of *GSH1*. This DNA sequence as well as the presence of Met4 and Met31/Met32 is important for Cd(II)-induced *GSH1-lacZ* reporter gene expression (Dormer et al. 2000). Thus, the Met4-Met28-Met31/Met32 complex is thought to play an important role in the transcriptional induction of *GSH1* expression by cadmium. The contribution of the different Met4 complexes in the expression of the numerous other Met4-dependent genes that are induced by cadmium (Fauchon et al. 2002) is still unknown.

The Met4-dependent genes induced by cadmium can be classified into three different groups according to their function: (1) enzymes of the sulphur metabolic pathway; (2) transporters of sulphur compounds and; (3) other proteins with functions unrelated to sulphur metabolism. This latter group includes sulphur depleted proteins and particularly isoenzymes of some glycolysis enzymes. Interestingly, the most abundant proteins induced in a Met4-dependent way have a particularly low sulphur content indicating that Met4 controls the global saving of sulphur in proteins in response to Cd(II) (Fauchon et al. 2002).

In *S. pombe*, among the enzymes involved in sulphur metabolism, only the transporters of sulphur compounds are induced upon Cd(II) exposure (Chen et al. 2003). Induction of these genes is controlled by the bZIP transcription factor Zip1 (Harrison et al. 2005). The level of Zip1 in the cell is tightly controlled by the SCF^{pof1} ubiquitin ligase by a mechanism very similar to the regulated degradation of Met4 by SCF^{Met30} ubiquitin ligase in *S. cerevisiae*. Upon cadmium exposure, Zip1 is stabilized and expression of Zip1-dependent genes is induced (Harrison et al. 2005). However, the functions of Met4 and Zip1 present some differences; Zip1 controls the expression of only a small number of genes compared to Met4. Moreover, Met4 is important both for the synthesis of sulphur amino acids in unstressed conditions and for glutathione synthesis in response to metals whereas Zip1 seems to be required only for the Cd(II) response. However, it cannot be excluded that Zip1 controls the detoxification pathways of other toxic compounds. Strikingly, this mechanism of regulation seems conserved in mammals; the SCF ubiquitin ligase complex Keap1/Cul3/Rbx1 controls the degradation of the bZIP transcription factor Nrf2. This factor is stabilized by Cd(II) and other oxidative stress conditions (Stewart et al. 2003; Kobayashi et al. 2004) and it also controls glutathione synthesis (Chan and Kwong 2000; Sun et al. 2005) indicating an evolutionary conservation of this feature among eukaryotes.

6.5 Other transcriptional regulators

Genome-wide transcriptional and subsequent bioinformatics analysis has identified additional proteins that may affect transcription under metal exposure. Network mapping of As(III) exposed cells resulted in the identification of a number of proteins that are likely to mediate part of the transcriptional response to As(III) (Haugen et al. 2004). The proteins identified include the general stress-responsive transcription factors Msn2 and Msn4, the heat shock factor Hsf1, the transcriptional activator of the ubiquitin-proteasome pathway Rpn4 as well as Fhl1 involved in the control of rRNA processing (Haugen et al. 2004). The molecular details of As(III)-induced activation of these proteins and the details of how the systems whose expression they regulate contribute to tolerance remain unknown. Among the yeast AP-1-like proteins, Yap2 is most closely related to Yap1. Although overexpression of *YAP2* confers increased Cd(II) tolerance (Wu et al. 1993; Hirata et al. 1994) and Cd(II) exposure results in Yap2 nuclear retention (Bilsland et al. 2004), *yap2Δ* has no Cd(II) sensitive phenotype. Similarly, Yap2 does not appear to have a role in As(III) tolerance (Haugen et al. 2004; Wysocki et al. 2004). Hence, besides Yap1 and Yap8, the function of the yeast AP-1-like proteins is unknown.

7 Conclusions and future perspectives

S. cerevisiae is extensively used as a eukaryotic model organism for both fundamental and applied studies. From genetic and physiological points of view, yeast is a favourite organism for many molecular cell biologists. Also with respect to the cellular defence mechanisms against toxic metals described in this review, yeast appears to be a very suitable model system. For instance, the proteins mediating metal influx and efflux seem to be conserved in various eukaryotic organisms. Similarly, one can predict that sulphur metabolism and glutathione biosynthesis will play an equally important role in other eukaryotes as it does in yeast. Finally, the signalling and regulatory mechanisms that the cell utilises to control the tolerance systems have counterparts in higher organisms. However, it is important to keep in mind that, while general strategies employed by cells to evade metal toxicity may be conserved through evolution, it is likely that the molecular details differ between organisms. One such example is the usage of different thiol-based detoxification systems by *S. cerevisiae* and *S. pombe*: while the former employs glutathione the latter utilises phytochelatin for detoxification purposes. In any case, the study of metal responses in budding and fission yeast are complementary and have revealed important information with relevance to plant and mammalian cells.

Although our understanding of how eukaryotic cells respond to nonessential toxic metals has increased considerably in recent years, there is still a long way to go before we have a comprehensive insight into the molecular details of such defence systems. Firstly, we have not yet identified all the players involved in metal

tolerance acquisition. Moreover, we need to know the exact mechanism by which these proteins mediate tolerance. For instance, what is the nature of the (metal-induced) signal that activates transcription factors and signalling proteins? How do these proteins, in turn, activate their respective targets? What is the substrate (metal) specificity of plasma membrane permeases and ABC-transporters that are involved in metal tolerance? Secondly, the dynamic nature of signalling events and transcriptional responses during metal exposure remains poorly explored. For instance, the mechanisms controlling the subcellular localisation of individual signalling proteins and transcriptional regulators are only starting to emerge. Moreover, how these proteins interact in space and time as they orchestrate the cellular response during metal exposure remains to be investigated. One should also keep in mind that cells are likely to respond differently under acute and chronic exposure and/or use different mechanisms to cope with sudden or chronic exposure. Thirdly, a global whole-cell or organism-level understanding of metal responses requires integration of various 'omic' approaches including transcriptomic, proteomic and metabolic profiling. In this way, it is possible to achieve a systems-level comprehension of the protective mechanisms used by cells or organisms during exposure to various cytotoxic agents and drugs. Fourthly, there is a clear need for technological developments that will allow the measurement of metal concentrations in different organelles or accurate measurements of ROS in the cell and/or organelles. A detailed understanding of metal tolerance mechanisms in yeast may prove of value for identifying similar mechanisms in other organisms and have important implications for the use of these metals in medical therapy.

Acknowledgments

The authors acknowledge funding from the following sources: the Swedish Research Council (VR) and the Swedish National Research School in Genomics and Bioinformatics to M.J.T.; the National Institutes of Health (grant GM60443) to R.W. and the Programme de Toxicologie Nucléaire to J.L.

References

Abedin MJ, Feldmann J, Meharg AA (2002) Uptake kinetics of arsenic species in rice plants. Plant Physiol 128:1120-1128

Alarco AM, Balan I, Talibi D, Mainville N, Raymond M (1997) AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. J Biol Chem 272:19304-19313

Alarco AM, Raymond M (1999) The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. J Bacteriol 181:700-708

Alepuz PM, Jovanovic A, Reiser V, Ammerer G (2001) Stress-induced MAP kinase Hog1 is part of transcription activation complexes. Mol Cell 7:767-777

Alexander MR, Tyers M, Perret M, Craig BM, Fang KS, Gustin MC (2001) Regulation of cell cycle progression by Swe1p and Hog1p following hypertonic stress. Mol Biol Cell 12:53-62

Alkorta I, Hernandez-Allica J, Garbisu C (2004) Plants against the global epidemic of arsenic poisoning. Environ Int 30:949-951

Arslan P, Beltrame M, Tomasi A (1987) Intracellular chromium reduction. Biochim Biophys Acta 931:10-15

Avery AM, Avery SV (2001) *Saccharomyces cerevisiae* expresses three phospholipid hydroperoxide glutathione peroxidases. J Biol Chem 276:33730-33735

Avery AM, Willetts SA, Avery SV (2004) Genetic dissection of the phospholipid hydroperoxidase activity of yeast gpx3 reveals its functional importance. J Biol Chem 279:46652-46658

Azevedo D, Tacnet F, Delaunay A, Rodrigues-Pousada C, Toledano MB (2003) Two redox centers within Yap1 for H_2O_2 and thiol-reactive chemicals signaling. Free Radic Biol Med 35:889-900

Bae W, Chen X (2004) Proteomic study for the cellular responses to Cd^{2+} in *Schizosaccharomyces pombe* through amino acid-coded mass tagging and liquid chromatography tandem mass spectrometry. Mol Cell Proteomics 3:596-607

Baes CFJ, Mesmer RE (1976) The hydrolysis of cations. John Wiley and Sons, New York, NY

Bai M, Zhou JM, Perrett S (2004) The yeast prion protein Ure2 shows glutathione peroxidase activity in both native and fibrillar forms. J Biol Chem 279:50025-50030

Ballatori N (2002) Transport of toxic metals by molecular mimicry. Environ Health Perspect 110 Suppl 5:689-694

Banerjee S, Flores-Rozas H (2005) Cadmium inhibits mismatch repair by blocking the ATPase activity of the MSH2-MSH6 complex. Nucleic Acids Res 33:1410-1419

Barbey R, Baudouin-Cornu P, Lee TA, Rouillon A, Zarzov P, Tyers M, Thomas D (2005) Inducible dissociation of SCF(Met30) ubiquitin ligase mediates a rapid transcriptional response to cadmium. EMBO J 24:521-532

Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, Krishna S (2003) The trypanosomiases. Lancet 362:1469-1480

Belli G, Gari E, Aldea M, Herrero E (2001) Osmotic stress causes a G_1 cell cycle delay and downregulation of Cln3/Cdc28 activity in *Saccharomyces cerevisiae*. Mol Microbiol 39:1022-1035

Bennett RL, Malamy MH (1970) Arsenate resistant mutants of *Escherichia coli* and phosphate transport. Biochem Biophys Res Commun 40:496-503

Berman J (2003) Current treatment approaches to leishmaniasis. Curr Opin Infect Dis 16:397-401

Bhattacharjee H, Carbrey J, Rosen BP, Mukhopadhyay R (2004) Drug uptake and pharmacological modulation of drug sensitivity in leukemia by AQP9. Biochem Biophys Res Commun 322:836-841

Bhattacharjee H, Ho YS, Rosen BP (2001) Genomic organization and chromosomal localization of the Asna1 gene, a mouse homologue of a bacterial arsenic-translocating ATPase gene. Gene 272:291-299

Billard P, Dumond H, Bolotin-Fukuhara M (1997) Characterization of an AP-1-like transcription factor that mediates an oxidative stress response in *Kluyveromyces lactis*. Mol Gen Genet 257:62-70

Bilsland E, Molin C, Swaminathan S, Ramne A, Sunnerhagen P (2004) Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. Mol Microbiol 53:1743-1756

Bilsland-Marchesan E, Arino J, Saito H, Sunnerhagen P, Posas F (2000) Rck2 kinase is a substrate for the osmotic stress-activated mitogen-activated protein kinase Hog1. Mol Cell Biol 20:3887-3895

Biteau B, Labarre J, Toledano MB (2003) ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. Nature 425:980-984

Blaiseau PL, Isnard AD, Surdin-Kerjan Y, Thomas D (1997) Met31p and Met32p, two related zinc finger proteins, are involved in transcriptional regulation of yeast sulfur amino acid metabolism. Mol Cell Biol 17:3640-3648

Blaiseau PL, Thomas D (1998) Multiple transcriptional activation complexes tether the yeast activator Met4 to DNA. EMBO J 17:6327-6336

Bobrowicz P, Ulaszewski S (1998) Arsenical-induced transcriptional activation of the yeast *Saccharomyces cerevisiae ACR2* and *ACR3* genes requires the presence of the *ACR1* gene product. Cell Mol Biol Lett 3:13-20

Bobrowicz P, Wysocki R, Owsianik G, Goffeau A, Ulaszewski S (1997) Isolation of three contiguous genes, *ACR1*, *ACR2* and *ACR3*, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. Yeast 13:819-828

Bouganim N, David J, Wysocki R, Ramotar D (2001) Yap1 overproduction restores arsenite resistance to the ABC-transporter deficient mutant *ycf1* by activating *ACR3* expression. Biochem Cell Biol 79:441-448

Brennan RJ, Schiestl RH (1996) Cadmium is an inducer of oxidative stress in yeast. Mutat Res 356:171-178

Breton A, Surdin-Kerjan Y (1977) Sulfate uptake in *Saccharomyces cerevisiae*: biochemical and genetic study. J Bacteriol 132:224-232

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993) An osmosensing signal transduction pathway in yeast. Science 259:1760-1763

Broco N, Tenreiro S, Viegas CA, Sa-Correia I (1999) *FLR1* gene (ORF YBR008c) is required for benomyl and methotrexate resistance in *Saccharomyces cerevisiae* and its benomyl-induced expression is dependent on pdr3 transcriptional regulator. Yeast 15:1595-1608

Brown JL, Bussey H, Stewart RC (1994) Yeast Skn7p functions in a eukaryotic two-component regulatory pathway. EMBO J 13:5186-5194

Brown JL, North S, Bussey H (1993) *SKN7*, a yeast multicopy suppressor of a mutation affecting cell wall beta-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock transcription factors. J Bacteriol 175:6908-6915

Brown NL, Shih YC, Leang C, Glendinning KJ, Hobman JL, Wilson JR (2002) Mercury transport and resistance. Biochem Soc Trans 30:715-718

Buchler M, Konig J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem 271:15091-15098

Bun-ya M, Shikata K, Nakade S, Yompakdee C, Harashima S, Oshima Y (1996) Two new genes, *PHO86* and *PHO87*, involved in inorganic phosphate uptake in *Saccharomyces cerevisiae*. Curr Genet 29:344-351

Carmel-Harel O, Stearman R, Gasch AP, Botstein D, Brown PO, Storz G (2001) Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in *Saccharomyces cerevisiae*. Mol Microbiol 39:595-605

Castillo EA, Ayte J, Chiva C, Moldon A, Carrascal M, Abian J, Jones N, Hidalgo E (2002) Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. Mol Microbiol 45:243-254

Cellier M, Prive G, Belouchi A, Kwan T, Rodrigues V, Chia W, Gros P (1995) Nramp defines a family of membrane proteins. Proc Natl Acad Sci USA 92:10089-10093

Chan JY, Kwong M (2000) Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. Biochim Biophys Acta 1517:19-26

Chen D, Toone WM, Mata J, Lyne R, Burns G, Kivinen K, Brazma A, Jones N, Bahler J (2003) Global transcriptional responses of fission yeast to environmental stress. Mol Biol Cell 14:214-229

Chen F, Shi X (2002) Intracellular signal transduction of cells in response to carcinogenic metals. Crit Rev Oncol Hematol 42:105-121

Chen XZ, Peng JB, Cohen A, Nelson H, Nelson N, Hediger MA (1999) Yeast SMF1 mediates $H(^{+})$-coupled iron uptake with concomitant uncoupled cation currents. J Biol Chem 274:35089-35094

Cherest H, Davidian JC, Thomas D, Benes V, Ansorge W, Surdin-Kerjan Y (1997) Molecular characterization of two high affinity sulfate transporters in *Saccharomyces cerevisiae*. Genetics 145:627-635

Chou WC, Jie C, Kenedy AA, Jones RJ, Trush MA, Dang CV (2004) Role of NADPH oxidase in arsenic-induced reactive oxygen species formation and cytotoxicity in myeloid leukemia cells. Proc Natl Acad Sci USA 101:4578-4583

Chrestensen CA, Starke DW, Mieyal JJ (2000) Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. J Biol Chem 275:26556-26565

Clemens S, Antosiewicz DM, Ward JM, Schachtman DP, Schroeder JI (1998) The plant cDNA *LCT1* mediates the uptake of calcium and cadmium in yeast. Proc Natl Acad Sci USA 95:12043-12048

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 18:3325-3333

Clemens S, Schroeder JI, Degenkolb T (2001) *Caenorhabditis elegans* expresses a functional phytochelatin synthase. Eur J Biochem 268:3640-3643

Cobbett CS (2000a) Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr Opin Plant Biol 3:211-216

Cobbett CS (2000b) Phytochelatins and their roles in heavy metal detoxification. Plant Physiol 123:825-832

Cohen BA, Pilpel Y, Mitra RD, Church GM (2002) Discrimination between paralogs using microarray analysis: application to the Yap1p and Yap2p transcriptional networks. Mol Biol Cell 13:1608-1614

Coleman ST, Epping EA, Steggerda SM, Moye-Rowley WS (1999) Yap1p activates gene transcription in an oxidant-specific fashion. Mol Cell Biol 19:8302-8313

Coleman ST, Tseng E, Moye-Rowley WS (1997) *Saccharomyces cerevisiae* basic region-leucine zipper protein regulatory networks converge at the *ATR1* structural gene. J Biol Chem 272:23224-23230

Conklin DS, McMaster JA, Culbertson MR, Kung C (1992) *COT1*, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. Mol Cell Biol 12:3678-3688

Cui Z, Hirata D, Tsuchiya E, Osada H, Miyakawa T (1996) The multidrug resistance-associated protein (MRP) subfamily (Yrs1/Yor1) of *Saccharomyces cerevisiae* is important for the tolerance to a broad range of organic anions. J Biol Chem 271:14712-14716

Culotta VC, Howard WR, Liu XF (1994) *CRS5* encodes a metallothionein-like protein in *Saccharomyces cerevisiae*. J Biol Chem 269:25295-25302

Dameron CT, Smith BR, Winge DR (1989) Glutathione-coated cadmium-sulfide crystallites in *Candida glabrata*. J Biol Chem 264:17355-17360

De Freitas JM, Kim JH, Poynton H, Su T, Wintz H, Fox T, Holman P, Loguinov A, Keles S, van der Laan M, Vulpe C (2004) Exploratory and confirmatory gene expression profiling of *mac1Δ*. J Biol Chem 279:4450-4458

de Nadal E, Casadome L, Posas F (2003) Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. Mol Cell Biol 23:229-237

Decottignies A, Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nat Genet 15:137-145

Decottignies A, Grant AM, Nichols JW, de Wet H, McIntosh DB, Goffeau A (1998) ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem 273:12612-12622

Delaunay A, Isnard AD, Toledano MB (2000) H_2O_2 sensing through oxidation of the Yap1 transcription factor. EMBO J 19:5157-5166

Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB (2002) A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. Cell 111:471-481

DeMel S, Shi J, Martin P, Rosen BP, Edwards BF (2004) Arginine 60 in the ArsC arsenate reductase of *E. coli* plasmid R773 determines the chemical nature of the bound As(III) product. Protein Sci 13:2330-2340

Deshaies RJ (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol 15:435-467

Desoize B (2004) Metals and metal compounds in cancer treatment. Anticancer Res 24:1529-1544

Dix D, Bridgham J, Broderius M, Eide D (1997) Characterization of the FET4 protein of yeast. Evidence for a direct role in the transport of iron. J Biol Chem 272:11770-11777

Dix DR, Bridgham JT, Broderius MA, Byersdorfer CA, Eide DJ (1994) The *FET4* gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae*. J Biol Chem 269:26092-26099

Dormer UH, Westwater J, McLaren NF, Kent NA, Mellor J, Jamieson DJ (2000) Cadmium-inducible expression of the yeast *GSH1* gene requires a functional sulfur-amino acid regulatory network. J Biol Chem 275:32611-32616

dos Santos Ferreira C, Martins PS, Demicheli C, Brochu C, Ouellette M, Frezard F (2003) Thiol-induced reduction of antimony(V) into antimony(III): a comparative study with trypanothione, cysteinyl-glycine, cysteine and glutathione. Biometals 16:441-446

Ecker DJ, Butt TR, Sternberg EJ, Neeper MP, Debouck C, Gorman JA, Crooke ST (1986) Yeast metallothionein function in metal ion detoxification. J Biol Chem 261:16895-16900

Eraso P, Martinez-Burgos M, Falcon-Perez JM, Portillo F, Mazon MJ (2004) Ycf1-dependent cadmium detoxification by yeast requires phosphorylation of residues Ser(908) and Thr(911). FEBS Lett 577:322-326

Ercal N, Gurer-Orhan H, Aykin-Burns N (2001) Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr Top Med Chem 1:529-539

Escoté X, Zapater M, Clotet J, Posas F (2004) Hog1 mediates cell-cycle arrest in G_1 phase by the dual targeting of Sic1. Nat Cell Biol 6:997-1002

Evans MV, Turton HE, Grant CM, Dawes IW (1998) Toxicity of linoleic acid hydroperoxide to *Saccharomyces cerevisiae*: involvement of a respiration-related process for maximal sensitivity and adaptive response. J Bacteriol 180:483-490

Evens AM, Tallman MS, Gartenhaus RB (2004) The potential of arsenic trioxide in the treatment of malignant disease: past, present, and future. Leuk Res 28:891-900

Faller P, Kienzler K, Krieger-Liszkay A (2005) Mechanism of Cd^{2+} toxicity: Cd^{2+} inhibits photoactivation of Photosystem II by competitive binding to the essential Ca^{2+} site. Biochim Biophys Acta 1706:158-164

Fauchon M, Lagniel G, Aude JC, Lombardia L, Soularue P, Petat C, Marguerie G, Sentenac A, Werner M, Labarre J (2002) Sulfur sparing in the yeast proteome in response to sulfur demand. Mol Cell 9:713-723

Fauman EB, Cogswell JP, Lovejoy B, Rocque WJ, Holmes W, Montana VG, Piwnica-Worms H, Rink MJ, Saper MA (1998) Crystal structure of the catalytic domain of the human cell cycle control phosphatase, Cdc25A. Cell 93:617-625

Fernandes L, Rodrigues-Pousada C, Struhl K (1997) Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. Mol Cell Biol 17:6982-6993

Forbes JR, Gros P (2001) Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. Trends Microbiol 9:397-403

Frisbie SH, Ortega R, Maynard DM, Sarkar B (2002) The concentrations of arsenic and other toxic elements in Bangladesh's drinking water. Environ Health Perspect 110:1147-1153

Gaither LA, Eide DJ (2001) Eukaryotic zinc transporters and their regulation. Biometals 14:251-270

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241-4257

Gatti D, Mitra B, Rosen BP (2000) *Escherichia coli* soft metal ion-translocating ATPases. J Biol Chem 275:34009-34012

Gebel T (1997) Arsenic and antimony: comparative approach on mechanistic toxicology. Chem Biol Interact 107:131-144

Ghosh M, Shen J, Rosen BP (1999) Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 96:5001-5006

Gitan RS, Eide DJ (2000) Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter. Biochem J 346:329-336

Gitan RS, Luo H, Rodgers J, Broderius M, Eide D (1998) Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. J Biol Chem 273:28617-28624

Gitan RS, Shababi M, Kramer M, Eide DJ (2003) A cytosolic domain of the yeast Zrt1 zinc transporter is required for its post-translational inactivation in response to zinc and cadmium. J Biol Chem 278:39558-39564

Gomes DS, Fragoso LC, Riger CJ, Panek AD, Eleutherio EC (2002) Regulation of cadmium uptake by *Saccharomyces cerevisiae*. Biochim Biophys Acta 1573:21-25

Gourbal B, Sonuc N, Bhattacharjee H, Legare D, Sundar S, Ouellette M, Rosen BP, Mukhopadhyay R (2004) Drug uptake and modulation of drug resistance in leishmania by an aquaglyceroporin. J Biol Chem 279:31010-31017

Goyer RA, Clarkson TW (2001) Toxic effects of metals. In: Klaassen CD (ed) Casarett and Doull's Toxicology: the basic science of poisons. McGraw-Hill, New York, pp 811-867

Grant CM, Maciver FH, Dawes IW (1996) Stationary-phase induction of *GLR1* expression is mediated by the yAP-1 transcriptional regulatory protein in the yeast *Saccharomyces cerevisiae*. Mol Microbiol 22:739-746

Grill E, Winnacker EL, Zenk MH (1991) Phytochelatins. Methods Enzymol 205:333-341

Gueldry O, Lazard M, Delort F, Dauplais M, Grigoras I, Blanquet S, Plateau P (2003) Ycf1p-dependent Hg(II) detoxification in *Saccharomyces cerevisiae*. Eur J Biochem 270:2486-2496

Guerinot ML (2000) The ZIP family of metal transporters. Biochim Biophys Acta 1465:190-198

Gulshan K, Rovinsky SA, Moye-Rowley WS (2004) *YBP1* and its homologue *YBP2*/*YBH1* influence oxidative-stress tolerance by nonidentical mechanisms in *Saccharomyces cerevisiae*. Eukaryot Cell 3:318-330

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388:482-488

Ha SB, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. Plant Cell 11:1153-1164

Halliwell B, Gutteridge JM (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1-14

Handel ML, Watts CK, deFazio A, Day RO, Sutherland RL (1995) Inhibition of AP-1 binding and transcription by gold and selenium involving conserved cysteine residues in Jun and Fos. Proc Natl Acad Sci USA 92:4497-4501

Hansen J, Johannsen PF (2000) Cysteine is essential for transcriptional regulation of the sulfur assimilation genes in *Saccharomyces cerevisiae*. Mol Gen Genet 263:535-542

Harris GK, Shi X (2003) Signaling by carcinogenic metals and metal-induced reactive oxygen species. Mutat Res 533:183-200

Harrison C, Katayama S, Dhut S, Chen D, Jones N, Bahler J, Toda T (2005) SCF(Pof1)-ubiquitin and its target Zip1 transcription factor mediate cadmium response in fission yeast. EMBO J 24:599-610

Harshman KD, Moye-Rowley WS, Parker CS (1988) Transcriptional activation by the SV40 AP-1 recognition element in yeast is mediated by a factor similar to AP-1 that is distinct from GCN4. Cell 53:321-330

Hartwig A (2001) Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antioxid Redox Signal 3:625-634

Hassett R, Dix DR, Eide DJ, Kosman DJ (2000) The Fe(II) permease Fet4p functions as a low affinity copper transporter and supports normal copper trafficking in *Saccharomyces cerevisiae*. Biochem J 351 Pt 2:477-484

Haugen AC, Kelley R, Collins JB, Tucker CJ, Deng C, Afshari CA, Brown JM, Ideker T, Van Houten B (2004) Integrating phenotypic and expression profiles to map arsenic-response networks. Genome Biol 5:R95

Hei TK, Filipic M (2004) Role of oxidative damage in the genotoxicity of arsenic. Free Radic Biol Med 37:574-581

Herker E, Jungwirth H, Lehmann KA, Maldener C, Frohlich KU, Wissing S, Buttner S, Fehr M, Sigrist S, Madeo F (2004) Chronological aging leads to apoptosis in yeast. J Cell Biol 164:501-507

Hertle K, Haase E, Brendel M (1991) The *SNQ3* gene of *Saccharomyces cerevisiae* confers hyper-resistance to several functionally unrelated chemicals. Curr Genet 19:429-433

Hettema EH, Valdez-Taubas J, Pelham HR (2004) Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. EMBO J 23:1279-1288

Himeno S, Yanagiya T, Enomoto S, Kondo Y, Imura N (2002) Cellular cadmium uptake mediated by the transport system for manganese. Tohoku J Exp Med 196:43-50

Hirata D, Yano K, Miyakawa T (1994) Stress-induced transcriptional activation mediated by *YAP1* and *YAP2* genes that encode the Jun family of transcriptional activators in *Saccharomyces cerevisiae*. Mol Gen Genet 242:250-256

Hobman JL, Brown NL (1996) Overexpression of MerT, the mercuric ion transport protein of transposon Tn501, and genetic selection of mercury hypersensitivity mutations. Mol Gen Genet 250:129-134

Hofmann K, Bucher P, Kajava AV (1998) A model of Cdc25 phosphatase catalytic domain and Cdk-interaction surface based on the presence of a rhodanese homology domain. J Mol Biol 282:195-208

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66:300-372

Hohmann S, Bill RM, Kayingo G, Prior BA (2000) Microbial MIP channels. Trends Microbiol 8:33-38

Howlett NG, Avery SV (1997) Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and influence of plasma membrane fatty acid unsaturation. Appl Environ Microbiol 63:2971-2976

Hu J, Fang J, Dong Y, Chen SJ, Chen Z (2005) Arsenic in cancer therapy. Anticancer Drugs 16:119-127

Huang C, Ke Q, Costa M, Shi X (2004) Molecular mechanisms of arsenic carcinogenesis. Mol Cell Biochem 255:57-66

Huang ME, Rio AG, Nicolas A, Kolodner RD (2003) A genomewide screen in *Saccharomyces cerevisiae* for genes that suppress the accumulation of mutations. Proc Natl Acad Sci USA 100:11529-11534

Huang RN, Lee TC (1996) Cellular uptake of trivalent and pentavalent arsenate in KB cells cultured in phosphate-free medium. Toxicol Appl Pharmacol 136:243-249

Hussain M, Lenard J (1991) Characterization of *PDR4*, a *Saccharomyces cerevisiae* gene that confers pleiotropic drug resistance in high-copy number: identity with *YAP1*, encoding a transcriptional activator. Gene 101:149-152

Inoue Y, Matsuda T, Sugiyama K, Izawa S, Kimura A (1999) Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. J Biol Chem 274:27002-27009

Isoyama T, Murayama A, Nomoto A, Kuge S (2001) Nuclear import of the yeast AP-1-like transcription factor Yap1p is mediated by transport receptor Pse1p, and this import step is not affected by oxidative stress. J Biol Chem 276:21863-21869

Izawa S, Maeda K, Sugiyama K, Mano J, Inoue Y, Kimura A (1999) Thioredoxin deficiency causes the constitutive activation of Yap1, an AP-1-like transcription factor in *Saccharomyces cerevisiae*. J Biol Chem 274:28459-28465

Jackson MD, Denu JM (2001) Molecular reactions of protein phosphatases--insights from structure and chemistry. Chem Rev 101:2313-2340

Jensen LT, Ajua-Alemanji M, Culotta VC (2003) The *Saccharomyces cerevisiae* high affinity phosphate transporter encoded by *PHO84* also functions in manganese homeostasis. J Biol Chem 278:42036-42040

Jensen LT, Culotta VC (2002) Regulation of *Saccharomyces cerevisiae FET4* by oxygen and iron. J Mol Biol 318:251-260

Jensen LT, Howard WR, Strain JJ, Winge DR, Culotta VC (1996) Enhanced effectiveness of copper ion buffering by *CUP1* metallothionein compared with *CRS5* metallothionein in *Saccharomyces cerevisiae*. J Biol Chem 271:18514-18519

Jin YH, Clark AB, Slebos RJ, Al-Refai H, Taylor JA, Kunkel TA, Resnick MA, Gordenin DA (2003) Cadmium is a mutagen that acts by inhibiting mismatch repair. Nat Genet 34:326-329

Jungwirth H, Wendler F, Platzer B, Bergler H, Hogenauer G (2000) Diazaborine resistance in yeast involves the efflux pumps Ycf1p and Flr1p and is enhanced by a gain-of-function allele of gene *YAP1*. Eur J Biochem 267:4809-4816

Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW (2000) The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic. J Biol Chem 275:33404-33408

Kamizono A, Nishizawa M, Teranishi Y, Murata K, Kimura A (1989) Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. Mol Gen Genet 219:161-167

Katzmann DJ, Epping EA, Moye-Rowley WS (1999) Mutational disruption of plasma membrane trafficking of *Saccharomyces cerevisiae* Yor1p, a homologue of mammalian multidrug resistance protein. Mol Cell Biol 19:2998-3009

Kim IY, Stadtman TC (1997) Inhibition of NF-kappaB DNA binding and nitric oxide induction in human T cells and lung adenocarcinoma cells by selenite treatment. Proc Natl Acad Sci USA 94:12904-12907

King LS, Kozono D, Agre P (2004) From structure to disease: the evolving tale of aquaporin biology. Nat Rev Mol Cell Biol 5:687-698

Klein M, Mamnun YM, Eggmann T, Schuller C, Wolfger H, Martinoia E, Kuchler K (2002) The ATP-binding cassette (ABC) transporter Bpt1p mediates vacuolar sequestration of glutathione conjugates in yeast. FEBS Lett 520:63-67

Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M (2004) Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol 24:7130-7139

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB (1999) The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Mol Biol 40:37-44

Krems B, Charizanis C, Entian KD (1995) Mutants of *Saccharomyces cerevisiae* sensitive to oxidative and osmotic stress. Curr Genet 27:427-434

Krems B, Charizanis C, Entian KD (1996) The response regulator-like protein Pos9/Skn7 of *Saccharomyces cerevisiae* is involved in oxidative stress resistance. Curr Genet 29:327-334

Kuge S, Arita M, Murayama A, Maeta K, Izawa S, Inoue Y, Nomoto A (2001) Regulation of the yeast Yap1p nuclear export signal is mediated by redox signal-induced reversible disulfide bond formation. Mol Cell Biol 21:6139-6150

Kuge S, Jones N (1994) YAP1 dependent activation of *TRX2* is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. EMBO J 13:655-664

Kuge S, Jones N, Nomoto A (1997) Regulation of yAP-1 nuclear localization in response to oxidative stress. EMBO J 16:1710-1720

Kuge S, Toda T, Iizuka N, Nomoto A (1998) Crm1 (XpoI) dependent nuclear export of the budding yeast transcription factor yAP-1 is sensitive to oxidative stress. Genes Cells 3:521-532

Kuras L, Barbey R, Thomas D (1997) Assembly of a bZIP-bHLH transcription activation complex: formation of the yeast Cbf1-Met4-Met28 complex is regulated through Met28 stimulation of Cbf1 DNA binding. EMBO J 16:2441-2451

Kuras L, Rouillon A, Lee T, Barbey R, Tyers M, Thomas D (2002) Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. Mol Cell 10:69-80

Kurdi-Haidar B, Heath D, Aebi S, Howell SB (1998) Biochemical characterization of the human arsenite-stimulated ATPase (hASNA-I). J Biol Chem 273:22173-22176

Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81:807-869

Lafaye A, Junot C, Pereira Y, Lagniel G, Tabet JC, Ezan E, Labarre J (2005a) Combined proteome and metabolite-profiling analyses reveal surprising insights into yeast sulfur metabolism. J Biol Chem 280:24723-24730

Lafaye A, Labarre J, Tabet JC, Ezan E, Junot C (2005b) Liquid chromatography-mass spectrometry and 15N metabolic labeling for quantitative metabolic profiling. Anal Chem 77:2026-2033

Lau WT, Howson RW, Malkus P, Schekman R, O'Shea EK (2000) Pho86p, an endoplasmic reticulum (ER) resident protein in *Saccharomyces cerevisiae*, is required for ER exit of the high-affinity phosphate transporter Pho84p. Proc Natl Acad Sci USA 97:1107-1112

Lee J, Godon C, Lagniel G, Spector D, Garin J, Labarre J, Toledano MB (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J Biol Chem 274:16040-16046

Légaré D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulou B, Ouellette M (2001) The *Leishmania* ABC protein PGPA is an intracellular metal-thiol transporter ATPase. J Biol Chem 276:26301-26307

Leppert G, McDevitt R, Falco SC, Van Dyk TK, Ficke MB, Golin J (1990) Cloning by gene amplification of two loci conferring multiple drug resistance in *Saccharomyces*. Genetics 125:13-20

Leslie EM, Haimeur A, Waalkes MP (2004) Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. J Biol Chem 279:32700-32708

Lesuisse E, Labbe P (1995) Effects of cadmium and of *YAP1* and *CAD1*/*YAP2* genes on iron metabolism in the yeast *Saccharomyces cerevisiae*. Microbiology 141 (Pt 11):2937-2943

Lev S, Hadar R, Amedeo P, Baker SE, Yoder OC, Horwitz BA (2005) Activation of an AP1-like transcription factor of the maize pathogen *Cochliobolus heterostrophus* in response to oxidative stress and plant signals. Eukaryot Cell 4:443-454

Li L, Kaplan J (1998) Defects in the yeast high affinity iron transport system result in increased metal sensitivity because of the increased expression of transporters with a broad transition metal specificity. J Biol Chem 273:22181-22187

Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, Rea PA (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc Natl Acad Sci USA 94:42-47

Li ZS, Szczypka M, Lu YP, Thiele DJ, Rea PA (1996) The yeast cadmium factor protein (*YCF1*) is a vacuolar glutathione S- conjugate pump. J Biol Chem 271:6509-6517

Liu KJ, Shi X, Dalal NS (1997a) Synthesis of Cr(IV)-GSH, its identification and its free hydroxyl radical generation: a model compound for Cr(VI) carcinogenicity. Biochem Biophys Res Commun 235:54-58

Liu SX, Athar M, Lippai I, Waldren C, Hei TK (2001) Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. Proc Natl Acad Sci USA 98:1643-1648

Liu XF, Culotta VC (1994) The requirement for yeast superoxide dismutase is bypassed through mutations in *BSD2*, a novel metal homeostasis gene. Mol Cell Biol 14:7037-7045

Liu XF, Culotta VC (1999) Post-translation control of Nramp metal transport in yeast. Role of metal ions and the *BSD2* gene. J Biol Chem 274:4863-4868

Liu XF, Supek F, Nelson N, Culotta VC (1997b) Negative control of heavy metal uptake by the *Saccharomyces cerevisiae BSD2* gene. J Biol Chem 272:11763-11769

Liu Z, Boles E, Rosen BP (2004a) Arsenic trioxide uptake by hexose permeases in *Saccharomyces cerevisiae*. J Biol Chem 279:17312-17318

Liu Z, Carbrey JM, Agre P, Rosen BP (2004b) Arsenic trioxide uptake by human and rat aquaglyceroporins. Biochem Biophys Res Commun 316:1178-1185

Liu Z, Shen J, Carbrey JM, Mukhopadhyay R, Agre P, Rosen BP (2002) Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci USA 99:6053-6058

Luk EE, Culotta VC (2001) Manganese superoxide dismutase in *Saccharomyces cerevisiae* acquires its metal co-factor through a pathway involving the Nramp metal transporter, Smf2p. J Biol Chem 276:47556-47562

Luyten K, Albertyn J, Skibbe WF, Prior BA, Ramos J, Thevelein JM, Hohmann S (1995) Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. EMBO J 14:1360-1371

MacDiarmid CW, Gaither LA, Eide D (2000) Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*. EMBO J 19:2845-2855

MacDiarmid CW, Milanick MA, Eide DJ (2002) Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. J Biol Chem 277:39187-39194

Macek T, Francova K, Kochankova L, Lovecka P, Ryslava E, Rezek J, Sura M, Triska J, Demnerova K, Mackova M (2004) Phytoremediation: biological cleaning of a polluted environment. Rev Environ Health 19:63-82

Maciaszczyk E, Wysocki R, Golik P, Lazowska J, Ulaszewski S (2004) Arsenical resistance genes in *Saccharomyces douglasii* and other yeast species undergo rapid evolution involving genomic rearrangements and duplications. FEMS Yeast Res 4:821-832

Maier T, Yu C, Kullertz G, Clemens S (2003) Localization and functional characterization of metal-binding sites in phytochelatin synthases. Planta 218:300-308

Maitani T, Kubota H, Sato K, Yamada T (1996) The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. Plant Physiol 110:1145-1150

Marzluf GA (1970) Genetic and metabolic controls for sulfate metabolism in *Neurospora crassa*: isolation and study of chromate-resistant and sulfate transport-negative mutants. J Bacteriol 102:716-721

Mason DL, Mallampalli MP, Huyer G, Michaelis S (2003) A region within a lumenal loop of *Saccharomyces cerevisiae* Ycf1p directs proteolytic processing and substrate specificity. Eukaryot Cell 2:588-598

Mason DL, Michaelis S (2002) Requirement of the N-terminal extension for vacuolar trafficking and transport activity of yeast Ycf1p, an ATP-binding cassette transporter. Mol Biol Cell 13:4443-4455

Maurel C, Javot H, Lauvergeat V, Gerbeau P, Tournaire C, Santoni V, Heyes J (2002) Molecular physiology of aquaporins in plants. Int Rev Cytol 215:105-148

McMurray CT, Tainer JA (2003) Cancer, cadmium and genome integrity. Nat Genet 34:239-241

Meharg AA, Macnair MR (1990) An altered phosphate uptake system in arsenate tolerant *Holcus lanatus*. New Phytol 116:29-35

Meharg AA, Macnair MR (1992) Suppression of the high affinity phosphate uptake system: a mechanism of arsenate tolerance in *Holcus lanatus* L. J Exp Bot 43:519-524

Mendenhall MD, Hodge AE (1998) Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 62:1191-1243

Menezes RA, Amaral C, Delaunay A, Toledano M, Rodrigues-Pousada C (2004) Yap8p activation in *Saccharomyces cerevisiae* under arsenic conditions. FEBS Lett 566:141-146

Meng YL, Liu Z, Rosen BP (2004) As(III) and Sb(III) uptake by GlpF and efflux by ArsB in *Escherichia coli*. J Biol Chem 279:18334-18341

Momose Y, Iwahashi H (2001) Bioassay of cadmium using a DNA microarray: genome-wide expression patterns of *Saccharomyces cerevisiae* response to cadmium. Environ Toxicol Chem 20:2353-2360

Morgan BA, Banks GR, Toone WM, Raitt D, Kuge S, Johnston LH (1997) The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. EMBO J 16:1035-1044

Moye-Rowley WS, Harshman KD, Parker CS (1989) Yeast *YAP1* encodes a novel form of the jun family of transcriptional activator proteins. Genes Dev 3:283-292

Mukhopadhyay R, Dey S, Xu N, Gage D, Lightbody J, Ouellette M, Rosen BP (1996) Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. Proc Natl Acad Sci USA 93:10383-10387

Mukhopadhyay R, Rosen BP (1998) *Saccharomyces cerevisiae ACR2* gene encodes an arsenate reductase. FEMS Microbiol Lett 168:127-136

Mukhopadhyay R, Rosen BP (2001) The phosphatase C(X)5R motif is required for catalytic activity of the *Saccharomyces cerevisiae* Acr2p arsenate reductase. J Biol Chem 276:34738-34742

Mukhopadhyay R, Rosen BP (2002) Arsenate reductases in prokaryotes and eukaryotes. Environ Health Perspect 110 Suppl 5:745-748

Mukhopadhyay R, Shi J, Rosen BP (2000) Purification and characterization of Acr2p, the *Saccharomyces cerevisiae* arsenate reductase. J Biol Chem 275:21149-21157

Mukhopadhyay R, Zhou Y, Rosen BP (2003) Directed evolution of a yeast arsenate reductase into a protein-tyrosine phosphatase. J Biol Chem 278:24476-24480

Murray HW (2001) Clinical and experimental advances in treatment of visceral leishmaniasis. Antimicrob Agents Chemother 45:2185-2197

Naganuma A, Miura N, Kaneko S, Mishina T, Hosoya S, Miyairi S, Furuchi T, Kuge S (2000) GFAT as a target molecule of methylmercury toxicity in *Saccharomyces cerevisiae*. FASEB J 14:968-972

Nguyen DT, Alarco AM, Raymond M (2001) Multiple Yap1p-binding sites mediate induction of the yeast major facilitator *FLR1* gene in response to drugs, oxidants, and alkylating agents. J Biol Chem 276:1138-1145

Nies DH (1999) Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51:730-750

Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27:313-339

Nordstrom DK (2002) Public health. Worldwide occurrences of arsenic in ground water. Science 296:2143-2145

Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J 11:3491-3499

Ortiz DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of metal-binding peptides by *HMT1*, a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270:4721-4728

Ortiz DF, St Pierre MV, Abdulmessih A, Arias IM (1997) A yeast ATP-binding cassette-type protein mediating ATP-dependent bile acid transport. J Biol Chem 272:15358-15365

Oskouian B, Saba JD (1999) *YAP1* confers resistance to the fatty acid synthase inhibitor cerulenin through the transporter Flr1p in *Saccharomyces cerevisiae*. Mol Gen Genet 261:346-353

Ow DW, Ortiz DF, Speiser DM, Mc Cue KF (1994) Molecular genetic analysis of cadmium tolerance in *Schizosaccharomyces pombe*. In: Winkelmann G, Winge DR (eds) Metal ions in fungi. Marcel Dekker, Inc, New York, pp 339-359

Park H, Bakalinsky AT (2000) *SSU1* mediates sulphite efflux in *Saccharomyces cerevisiae*. Yeast 16:881-888

Park HS, Park E, Kim MS, Ahn K, Kim IY, Choi EJ (2000) Selenite inhibits the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) through a thiol redox mechanism. J Biol Chem 275:2527-2531

Paszewski A (2001) Sulphur metabolism in fungi: pathways and regulation. Recent Res Devel Microbiology 5:223-234

Paulsen IT, Saier MH Jr (1997) A novel family of ubiquitous heavy metal ion transport proteins. J Membr Biol 156:99-103

Petrovic S, Pascolo L, Gallo R, Cupelli F, Ostrow JD, Goffeau A, Tiribelli C, Bruschi CV (2000) The products of *YCF1* and *YLL015w* (*BPT1*) cooperate for the ATP-dependent vacuolar transport of unconjugated bilirubin in *Saccharomyces cerevisiae*. Yeast 16:561-571

Pinson B, Sagot I, Daignan-Fornier B (2000) Identification of genes affecting selenite toxicity and resistance in *Saccharomyces cerevisiae*. Mol Microbiol 36:679-687

Portnoy ME, Liu XF, Culotta VC (2000) *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the Nramp family of metal transporters. Mol Cell Biol 20:7893-7902

Portnoy ME, Schmidt PJ, Rogers RS, Culotta VC (2001) Metal transporters that contribute copper to metallochaperones in *Saccharomyces cerevisiae*. Mol Genet Genomics 265:873-882

Posas F, Chambers JR, Heyman JA, Hoeffler JP, de Nadal E, Arino J (2000) The transcriptional response of yeast to saline stress. J Biol Chem 275:17249-17255

Proft M, Pascual-Ahuir A, de Nadal E, Arino J, Serrano R, Posas F (2001) Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J 20:1123-1133

Prusty R, Grisafi P, Fink GR (2004) The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 101:4153-4157

Qian H, Sahlman L, Eriksson PO, Hambraeus C, Edlund U, Sethson I (1998) NMR solution structure of the oxidized form of MerP, a mercuric ion binding protein involved in bacterial mercuric ion resistance. Biochemistry 37:9316-9322

Qian Y, Castranova V, Shi X (2003) New perspectives in arsenic-induced cell signal transduction. J Inorg Biochem 96:271-278

Rad MR, Kirchrath L, Hollenberg CP (1994) A putative P-type Cu(2+)-transporting ATPase gene on chromosome II of *Saccharomyces cerevisiae*. Yeast 10:1217-1225

Rai R, Tate JJ, Cooper TG (2003) Ure2, a prion precursor with homology to glutathione S-transferase, protects *Saccharomyces cerevisiae* cells from heavy metal ion and oxidant toxicity. J Biol Chem 278:12826-12833

Raitt DC, Johnson AL, Erkine AM, Makino K, Morgan B, Gross DS, Johnston LH (2000) The Skn7 response regulator of *Saccharomyces cerevisiae* interacts with Hsf1 *in vivo* and is required for the induction of heat shock genes by oxidative stress. Mol Biol Cell 11:2335-2347

Ramirez-Ramirez R, Calvo-Mendez C, Avila-Rodriguez M, Lappe P, Ulloa M, Vazquez-Juarez R, Gutierrez-Corona JF (2004) Cr(VI) reduction in a chromate-resistant strain of *Candida maltosa* isolated from the leather industry. Antonie Van Leeuwenhoek 85:63-68

Ramirez-Solis A, Mukopadhyay R, Rosen BP, Stemmler TL (2004) Experimental and theoretical characterization of arsenite in water: insights into the coordination environment of As-O. Inorg Chem 43:2954-2959

Ravandi F (2004) Arsenic trioxide: expanding roles for an ancient drug? Leukemia 18:1457-1459

Rayman MP (2000) The importance of selenium to human health. Lancet 356:233-241

Reese RN, Winge DR (1988) Sulfide stabilization of the cadmium-gamma-glutamyl peptide complex of *Schizosaccharomyces pombe*. J Biol Chem 263:12832-12835

Rep M, Krantz M, Thevelein JM, Hohmann S (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock: Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J Biol Chem 275:8290-8300

Rep M, Reiser V, Gartner U, Thevelein JM, Hohmann S, Ammerer G, Ruis H (1999) Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p. Mol Cell Biol 19:5474-5485

Rodriguez-Manzaneque MT, Tamarit J, Belli G, Ros J, Herrero E (2002) Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. Mol Biol Cell 13:1109-1121

Rogers EE, Eide DJ, Guerinot ML (2000) Altered selectivity in an *Arabidopsis* metal transporter. Proc Natl Acad Sci USA 97:12356-12360

Rollin-Genetet F, Berthomieu C, Davin AH, Quemeneur E (2004) *Escherichia coli* thioredoxin inhibition by cadmium: two mutually exclusive binding sites involving Cys32 and Asp26. Eur J Biochem 271:1299-1309

Rosen BP (1999) Families of arsenic transporters. Trends Microbiol 7:207-212

Rosen BP (1999b) The role of efflux in bacterial resistance to soft metals and metalloids. Essays Biochem 34:1-15

Rosen BP (2002) Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. Comp Biochem Physiol A Mol Integr Physiol 133:689-693

Rouillon A, Barbey R, Patton EE, Tyers M, Thomas D (2000) Feedback-regulated degradation of the transcriptional activator Met4 is triggered by the SCF(Met30)complex. EMBO J 19:282-294

Sahlman L, Wong W, Powlowski J (1997) A mercuric ion uptake role for the integral inner membrane protein, MerC, involved in bacterial mercuric ion resistance. J Biol Chem 272:29518-29526

Sato T, Kobayashi Y (1998) The ars operon in the skin element of *Bacillus subtilis* confers resistance to arsenate and arsenite. J Bacteriol 180:1655-1661

Schmoger ME, Oven M, Grill E (2000) Detoxification of arsenic by phytochelatins in plants. Plant Physiol 122:793-801

Schnell N, Entian KD (1991) Identification and characterization of a *Saccharomyces cerevisiae* gene (*PAR1*) conferring resistance to iron chelators. Eur J Biochem 200:487-493

Schnell N, Krems B, Entian KD (1992) The *PAR1* (*YAP1*/*SNQ3*) gene of *Saccharomyces cerevisiae*, a c-jun homologue, is involved in oxygen metabolism. Curr Genet 21:269-273

Schützendübel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351-1365

Seko Y, Imura N (1997) Active oxygen generation as a possible mechanism of selenium toxicity. Biomed Environ Sci 10:333-339

Sharma KG, Kaur R, Bachhawat AK (2003) The glutathione-mediated detoxification pathway in yeast: an analysis using the red pigment that accumulates in certain adenine biosynthetic mutants of yeasts reveals the involvement of novel genes. Arch Microbiol 180:108-117

Sharma KG, Mason DL, Liu G, Rea PA, Bachhawat AK, Michaelis S (2002) Localization, regulation, and substrate transport properties of Bpt1p, a *Saccharomyces cerevisiae* MRP-type ABC transporter. Eukaryot Cell 1:391-400

Sheikh MS, Fornace AJ Jr (1999) Regulation of translation initiation following stress. Oncogene 18:6121-6128

Shen J, Hsu CM, Kang BK, Rosen BP, Bhattacharjee H (2003) The *Saccharomyces cerevisiae* Arr4p is involved in metal and heat tolerance. Biometals 16:369-378

Shi H, Shi X, Liu KJ (2004) Oxidative mechanism of arsenic toxicity and carcinogenesis. Mol Cell Biochem 255:67-78

Shi X, Mao Y, Knapton AD, Ding M, Rojanasakul Y, Gannett PM, Dalal N, Liu K (1994) Reaction of Cr(VI) with ascorbate and hydrogen peroxide generates hydroxyl radicals and causes DNA damage: role of a Cr(IV)-mediated Fenton-like reaction. Carcinogenesis 15:2475-2478

Shiraishi E, Inouhe M, Joho M, Tohoyama H (2000) The cadmium-resistant gene, *CAD2,* which is a mutated putative copper-transporter gene (*PCA1*), controls the intracellular cadmium-level in the yeast *S. cerevisiae*. Curr Genet 37:79-86

Silver S (1998) Genes for all metals - a bacterial view of the periodic table. The 1996 Thom Award Lecture. J Ind Microbiol Biotechnol 20:1-20

Silver S (2003) Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev 27:341-353

Smith AH, Hopenhayn-Rich C, Bates MN, Goeden HM, Hertz-Picciotto I, Duggan HM, Wood R, Kosnett MJ, Smith MT (1992) Cancer risks from arsenic in drinking water. Environ Health Perspect 97:259-267

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y (2003) Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nat Biotechnol 21:914-919

Sorgel F (2004) The return of Ehrlich's 'Therapia magna sterilisans' and other Ehrlich concepts? Series of papers honoring Paul Ehrlich on the occasion of his 150th birthday. Chemotherapy 50:6-10

Stadtman TC (1996) Selenocysteine. Annu Rev Biochem 65:83-100

Stephen DW, Jamieson DJ (1997) Amino acid-dependent regulation of the *Saccharomyces cerevisiae GSH1* gene by hydrogen peroxide. Mol Microbiol 23:203-210

Stephen DW, Rivers SL, Jamieson DJ (1995) The role of the *YAP1* and *YAP2* genes in the regulation of the adaptive oxidative stress responses of *Saccharomyces cerevisiae*. Mol Microbiol 16:415-423

Stewart D, Killeen E, Naquin R, Alam S, Alam J (2003) Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. J Biol Chem 278:2396-2402

Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 18:321-336

Sugiyama K, Izawa S, Inoue Y (2000) The Yap1p-dependent induction of glutathione synthesis in heat shock response of *Saccharomyces cerevisiae*. J Biol Chem 275:15535-15540

Sun X, Erb H, Murphy TH (2005) Coordinate regulation of glutathione metabolism in astrocytes by Nrf2. Biochem Biophys Res Commun 326:371-377

Sundar S, Rai M (2002) Advances in the treatment of leishmaniasis. Curr Opin Infect Dis 15:593-598

Supek F, Supekova L, Nelson H, Nelson N (1996) A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. Proc Natl Acad Sci USA 93:5105-5110

Szczypka MS, Wemmie JA, Moye-Rowley WS, Thiele DJ (1994) A yeast metal resistance protein similar to human cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance- associated protein. J Biol Chem 269:22853-22857

Takeuchi T, Miyahara K, Hirata D, Miyakawa T (1997) Mutational analysis of Yap1 protein, an AP-1-like transcriptional activator of *Saccharomyces cerevisiae*. FEBS Lett 416:339-343

Tamás MJ, Hohmann S (2003) The osmotic stress response in *Saccharomyces cerevisiae*. In: Hohmann S, Mager WH (eds) Yeast stress responses. Springer Verlag, Heidelberg, pp 121-200

Tamás MJ, Karlgren S, Bill RM, Hedfalk K, Allegri L, Ferreira M, Thevelein JM, Rydström J, Mullins JG, Hohmann S (2003) A short regulatory domain restricts glycerol transport through yeast Fps1p. J Biol Chem 278:6337-6345

Tamás MJ, Luyten K, Sutherland FC, Hernandez A, Albertyn J, Valadi H, Li H, Prior BA, Kilian SG, Ramos J, Gustafsson L, Thevelein JM, Hohmann S (1999) Fps1p controls

the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol Microbiol 31:1087-1104

Tamás MJ, Wysocki R (2001) Mechanisms involved in metalloid transport and tolerance acquisition. Curr Genet 40:2-12

Teige M, Scheikl E, Reiser V, Ruis H, Ammerer G (2001) Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast. Proc Natl Acad Sci USA 98:5625-5630

Tenreiro S, Fernandes AR, Sa-Correia I (2001) Transcriptional activation of *FLR1* gene during *Saccharomyces cerevisiae* adaptation to growth with benomyl: role of Yap1p and Pdr3p. Biochem Biophys Res Commun 280:216-222

Thomas D, Surdin-Kerjan Y (1997) Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 61:503-532

Thomine S, Wang R, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes. Proc Natl Acad Sci USA 97:4991-4996

Toledano MB, Delaunay A, Biteau B, Spector D, Azevedo D (2003) Oxidative stress responses in yeast. In: Hohmann S, Mager WH (eds) Yeast stress responses. Springer Verlag, Heidelberg, pp 241-303

Toledano MB, Delaunay A, Monceau L, Tacnet F (2004) Microbial H_2O_2 sensors as archetypical redox signaling modules. Trends Biochem Sci 29:351-357

Tommasini R, Evers R, Vogt E, Mornet C, Zaman GJ, Schinkel AH, Borst P, Martinoia E (1996) The human multidrug resistance-associated protein functionally complements the yeast cadmium resistance factor 1. Proc Natl Acad Sci USA 93:6743-6748

Toone WM, Morgan BA, Jones N (2001) Redox control of AP-1-like factors in yeast and beyond. Oncogene 20:2336-2346

Turner RJ, Weiner JH, Taylor DE (1998) Selenium metabolism in *Escherichia coli*. Biometals 11:223-227

Turton HE, Dawes IW, Grant CM (1997) *Saccharomyces cerevisiae* exhibits a yAP-1-mediated adaptive response to malondialdehyde. J Bacteriol 179:1096-1101

Uesono Y, Toh EA (2002) Transient inhibition of translation initiation by osmotic stress. J Biol Chem 277:13848-13855

Vatamaniuk OK, Mari S, Lu YP, Rea PA (1999) *AtPCS1*, a phytochelatin synthase from *Arabidopsis*: isolation and *in vitro* reconstitution. Proc Natl Acad Sci USA 96:7110-7115

Vatamaniuk OK, Mari S, Lu YP, Rea PA (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase: blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. J Biol Chem 275:31451-31459

Veal EA, Ross SJ, Malakasi P, Peacock E, Morgan BA (2003) Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor. J Biol Chem 278:30896-30904

Vido K, Spector D, Lagniel G, Lopez S, Toledano MB, Labarre J (2001) A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. J Biol Chem 276:8469-8474

Waalkes MP (2003) Cadmium carcinogenesis. Mutat Res 533:107-120

Wang J, Zhao FJ, Meharg AA, Raab A, Feldmann J, McGrath SP (2002) Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. Plant Physiol 130:1552-1561

Waters BM, Eide DJ (2002) Combinatorial control of yeast *FET4* gene expression by iron, zinc, and oxygen. J Biol Chem 277:33749-33757

Waxman S, Anderson KC (2001) History of the development of arsenic derivatives in cancer therapy. Oncologist 6 Suppl 2:3-10

Wemmie JA, Steggerda SM, Moye-Rowley WS (1997) The *Saccharomyces cerevisiae* AP-1 protein discriminates between oxidative stress elicited by the oxidants H_2O_2 and diamide. J Biol Chem 272:7908-7914

Wemmie JA, Szczypka MS, Thiele DJ, Moye-Rowley WS (1994a) Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. J Biol Chem 269:32592-32597

Wemmie JA, Wu AL, Harshman KD, Parker CS, Moye-Rowley WS (1994b) Transcriptional activation mediated by the yeast AP-1 protein is required for normal cadmium tolerance. J Biol Chem 269:14690-14697

Westwater J, McLaren NF, Dormer UH, Jamieson DJ (2002) The adaptive response of *Saccharomyces cerevisiae* to mercury exposure. Yeast 19:233-239

Wheeler GL, Trotter EW, Dawes IW, Grant CM (2003) Coupling of the transcriptional regulation of glutathione biosynthesis to the availability of glutathione and methionine via the Met4 and Yap1 transcription factors. J Biol Chem 278:49920-49928

Widmann C, Gibson S, Jarpe MB, Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 79:143-180

Willsky GR, Malamy MH (1980) Effect of arsenate on inorganic phosphate transport in *Escherichia coli*. J Bacteriol 144:366-374

Wilson WA, Roach PJ (2002) Nutrient-regulated protein kinases in budding yeast. Cell 111:155-158

Winge DR, Nielson KB, Gray WR, Hamer DH (1985) Yeast metallothionein. Sequence and metal-binding properties. J Biol Chem 260:14464-14470

Wood MJ, Andrade EC, Storz G (2003) The redox domain of the Yap1p transcription factor contains two disulfide bonds. Biochemistry 42:11982-11991

Wood MJ, Storz G, Tjandra N (2004) Structural basis for redox regulation of Yap1 transcription factor localization. Nature 430:917-921

Wu A, Wemmie JA, Edgington NP, Goebl M, Guevara JL, Moye-Rowley WS (1993) Yeast bZip proteins mediate pleiotropic drug and metal resistance. J Biol Chem 268:18850-18858

Wu AL, Moye-Rowley WS (1994) *GSH1*, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. Mol Cell Biol 14:5832-5839

Wyllie S, Cunningham ML, Fairlamb AH (2004) Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. J Biol Chem 279:39925-39932

Wysocki R, Bobrowicz P, Ulaszewski S (1997) The *Saccharomyces cerevisiae ACR3* gene encodes a putative membrane protein involved in arsenite transport. J Biol Chem 272:30061-30066

Wysocki R, Chéry CC, Wawrzycka D, Van Hulle M, Cornelis R, Thevelein JM, Tamás MJ (2001) The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. Mol Microbiol 40:1391-1401

Wysocki R, Clemens S, Augustyniak D, Golik P, Maciaszczyk E, Tamás MJ, Dziadkowiec D (2003) Metalloid tolerance based on phytochelatins is not functionally equivalent to the arsenite transporter Acr3p. Biochem Biophys Res Commun 304:293-300

Wysocki R, Fortier PK, Maciaszczyk E, Thorsen M, Leduc A, Odhagen A, Owsianik G, Ulaszewski S, Ramotar D, Tamás MJ (2004) Transcriptional activation of metalloid tolerance genes in *Saccharomyces cerevisiae* requires the AP-1-like proteins Yap1p and Yap8p. Mol Biol Cell 15:2049-2060

Yaakov G, Bell M, Hohmann S, Engelberg D (2003) Combination of two activating mutations in one *HOG1* gene forms hyperactive enzymes that induce growth arrest. Mol Cell Biol 23:4826-4840

Yan C, Lee LH, Davis LI (1998) Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. EMBO J 17:7416-7429

Yompakdee C, Bun-ya M, Shikata K, Ogawa N, Harashima S, Oshima Y (1996a) A putative new membrane protein, Pho86p, in the inorganic phosphate uptake system of *Saccharomyces cerevisiae*. Gene 171:41-47

Yompakdee C, Ogawa N, Harashima S, Oshima Y (1996b) A putative membrane protein, Pho88p, involved in inorganic phosphate transport in *Saccharomyces cerevisiae*. Mol Gen Genet 251:580-590

Zhao H, Eide D (1996a) The yeast *ZRT1* gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc Natl Acad Sci USA 93:2454-2458

Zhao H, Eide D (1996b) The *ZRT2* gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*. J Biol Chem 271:23203-23210

Zhou Y, Messier N, Ouellette M, Rosen BP, Mukhopadhyay R (2004) *Leishmania major* LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug pentostam. J Biol Chem 279:37445-37451

Zhu J, Chen Z, Lallemand-Breitenbach V, de The H (2002) How acute promyelocytic leukaemia revived arsenic. Nat Rev Cancer 2:705-713

Zuniga S, Boskovic J, Jimenez A, Ballesta JP, Remacha M (1999) Disruption of six *Saccharomyces cerevisiae* novel genes and phenotypic analysis of the deletants. Yeast 15:945-953

Labarre, Jean
Service de Biochimie et de Génétique Moléculaire, DBJC, CEA/Saclay, 91191 Gif-sur-Yvette, France

Tamás, Markus J.
Department of Cell and Molecular Biology/Microbiology, Göteborg University, S-405 30 Göteborg, Sweden
markus.tamas@gmm.gu.se

Toledano, Michel B.
Laboratoire Stress Oxydants et Cancer, Service de Biologie Moléculaire Systemique, DBJC, DSV, CEA-Saclay, 91191 Gif-sur-Yvette, France

Wysocki, Robert
Center for Molecular Oncology and Department of Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, USA, and Institute of Genetics and Microbiology, University of Wroclaw, 51-148 Wroclaw, Poland

Transport of nonessential metals across mammalian cell membranes

Nazzareno Ballatori and Michael S. Madejczyk

Abstract

Nonessential metals are opportunistic. They compete with essential metals for binding to various cofactors, receptors, transcription factors, transporters, and other metalloproteins, and in doing so they gain access to various cellular and subcellular compartments, and interfere in the functions of the essential metals. Because of their high chemical reactivities, nonessential metals also interact nonspecifically with a multitude of other cellular ligands, and interfere with many other cellular processes. In general, nonessential metals cross biological membranes by three mechanisms. First, as indicated above, they often compete with endogenous metals for transport on the various metal ion transporters, pumps, and channels. Alternatively, they may form complexes that are then substrates for other ion and organic solute transporters and pumps. A third general mode of transport involves both signal-induced (e.g. receptor-mediated) and constitutive endocytosis of metals ions and metal complexes. In contrast with these mediated transport pathways, simple diffusion appears to play only a minor role in metal transport. Collectively, these permeation pathways allow toxic metals to reach diverse cellular and subcellular targets, but can also be exploited in the design of therapeutic strategies aimed at accelerating the removal of these toxic elements from the body.

1 Introduction

Metal ions are unusually reactive species: they can participate in reduction or oxidation reactions, acid-base chemistry, or ligand coordination reactions. These chemical properties allow the essential metals to catalyze a variety of biochemical reactions and physiological processes. However, both the essential and nonessential metals have comparable reactivities, and thus their concentrations must be maintained below a certain level to avoid nonspecific or toxic reactions. As described in other chapters in this series, many of the genes and gene products that regulate the concentrations and chemical activities of essential metals have now been identified and characterized, including metal-sensitive transcription factors, chaperones, cofactors, transport proteins, and enzymes. Genetic or acquired defects in many of these genes are associated with a variety of human diseases (Andrews 2002; Chung and Wessling-Resnick 2004; Cox and Moore 2002; Eide

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_102 / Published online: 11 August 2005

2004; Mackenzie and Hediger 2004; McKie and Barlow 2004; Miyajima 2002; Nittis and Gitlin 2002; Petris 2004; Pietrangelo 2004; Schaefer and Gitlin 1999; Vulpe et al. 1999).

In contrast with the essential metals, the nonessential metals generally lack these regulatory controls, and thus these elements are particularly hazardous to living organisms. The present report provides an overview over the general mechanisms by which nonessential metals permeate cellular and subcellular compartments. The elucidation of these pathways should help to define their mechanism of action and toxicity in living organisms; it should facilitate the discovery of novel biomarkers of exposure and dose, and may identify novel therapeutic strategies for metal intoxication.

2 Metal ion interactions with biological molecules

Transport of a given nonessential metal is dependent to a large extent on its chemical form in biological fluids. Because formation of metal complexes is highly favored thermodynamically, most heavy metals are present in biological tissues and fluids as complexes, rather than as the free cations. Although the thermodynamic stability of coordinate-covalent bonds is typically quite high, they are kinetically labile, so that a given metal may exchange rapidly from one ligand to another. This kinetic lability has proven to be the greatest stumbling block to the isolation and identification of metal complexes in biological fluids and tissues. For example, during tissue homogenization or chromatographic separation, additional binding sites may be exposed (or some eliminated), thus altering the distribution of the metal. Reactivity varies between metals, and is influenced by the nature of the ligand, whether mono- or multidentate, and the pH and ionic strength of the media. Copper, for example, forms relatively low affinity complexes with albumin or amino acids, but is tightly bound to ceruloplasmin. Similarly, mercury and cadmium form kinetically labile complexes with amino acids, glutathione, or albumin, but more stable complexes with metallothionein. Chelating agents normally display low specificity for metals, and bind a wide range of metals.

For the heavy metals, detoxification or protection from toxicity usually involves binding to specific proteins, including metallothioneins which form complexes with copper, zinc, cadmium, mercury, and other metals; ferritin, transferring, and hemosiderin, which are predominantly iron binding proteins, but also have some affinity for other metals; and ceruloplasmin, which chelates copper and possibly other metals. Similarly, the biological activity of the essential trace metals is due to their ability to attach to specific prosthetic groups on proteins. Manganese may be an exception to this generalization, since at least some of its biological functions are related to the free divalent metal.

Conversely, metal-induced toxicity is usually attributed to the reactivity of the "free" metal, and is most often observed in tissues involved in their transport, such as the intestine, liver, and kidney. Toxic metals, or an excess of essential metals

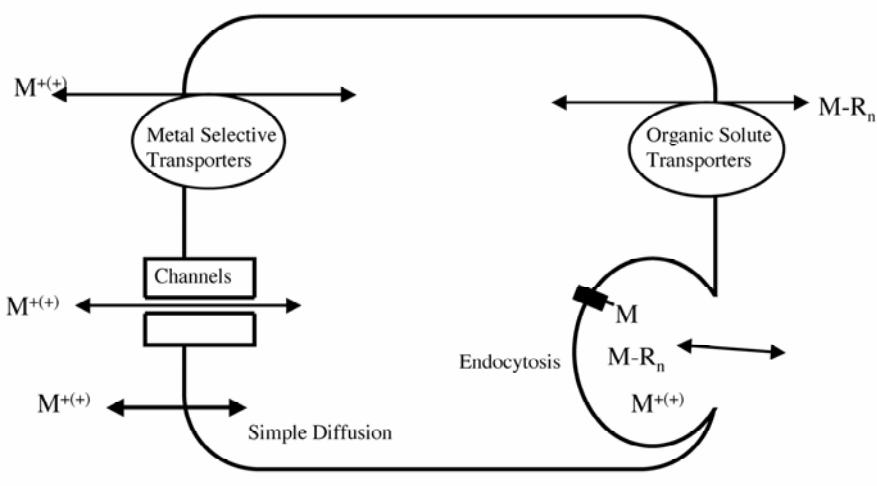


Fig. 1. General modes of metal transport across cell membranes. Metals can cross the plasma membrane via transport on either metal-selective transporters, channels, and pumps; other ion and organic solute transporters and pumps; by endocytosis/exocytosis; or by simple diffusion.

cause injury by binding to and perturbing the functions of cellular ligands. For example, mercury binds avidly to reduced sulfhydryl groups on cysteine moieties, and interferes in the function of all sulfhydryl-dependent proteins that have been examined (Clarkson 1972). Because of the shared physicochemical properties between Ca^{2+}, Pb^{2+} and Cd^{2+}, lead and cadmium probably exert some of their toxic effects by replacing Ca^{2+} on essential proteins or lipids. Similarly, vanadate and arsenate are structurally similar to phosphate and are able to displace phosphate from critical binding sites.

As illustrated in Figure 1, metals can cross the plasma membrane via transport on either, a) metal-selective transporters, channels, and pumps; b) other ion and organic solute transporters and pumps; c) endocytosis/exocytosis; or d) by simple diffusion. The following sections describe these major pathways, with an emphasis on the ability of nonessential metals to be transported by proteins that carry essential metals or organometallic complexes across cell membranes.

3 Metal-selective transporters, pumps, and channels

Much exciting progress has been made over the past decade in the molecular identification and functional characterization of metal-selective membrane transport proteins, and some of the major transport systems are illustrated in Figure 2. These studies have resolved many long-standing questions regarding permeation pathways of both essential and nonessential metals, and have elucidated the molecular basis of some disease states.

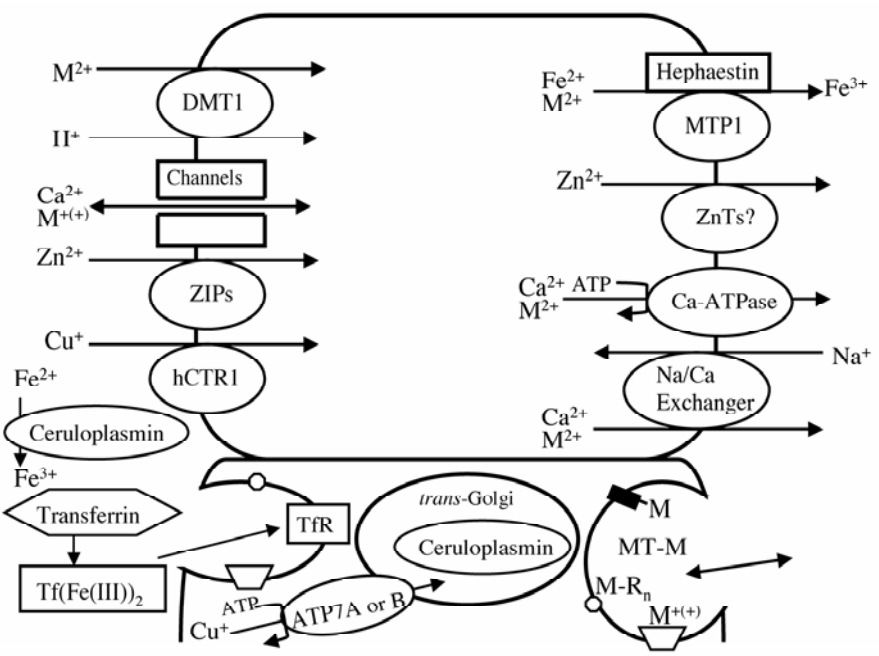


Fig. 2. Metal-selective membrane transport proteins. These proteins mediate uptake and efflux of metals from the extracellular space, and some are involved in transport in intracellular organelles.

3.1 The SLC11A family of H^+-coupled metal ion transporters: NRAMP1/SLC11A1 and NRAMP2/SLC11A2 (the latter is also known as DMT1 and DCT1)

The mouse natural resistance-associated macrophage proteins-1 and –2 (Nramp1 and Nramp2) were identified in 1993 and 1994 as the proteins that affect the capacity of macrophages to fight bacterial pathogens, but their molecular mechanisms of action was unknown. Studies by Gunshin and coworkers (1997) and others (Atkinson et al. 1997; Cellier et al. 1995; Fleming et al. 1997; Supek et al. 1996, 1997) provided important insight into this mechanism, and at the same time identified a major pathway for metal transport across cell membranes, including the transport of nonessential metals. Gunshin and colleagues (1997) used expression cloning in *Xenopus laevis* oocytes to identify a mammalian iron and divalent cation transporter-1 (Dct1, encoded by *Nramp2/Slc11A2*, but later renamed divalent metal transporter-1, Dmt1) that is expressed in a number of tissues. Fleming et al. (1997) used a positional cloning strategy to identify *Nramp1/Slc11a1* as the defective gene in the microcystic anemia (*mk*) inbred strain of mice. The observation that Nramp2 is an iron transporter provided an explanation for the phenotype in the Nramp1-defective *mk* mouse strain, and provided clues as to how Nramp1 may confer resistance to bacterial infection.

The Nramp family of membrane-associated proteins displays high sequence conservation from yeast to humans, with many species expressing at least two discrete gene copies (Cellier et al. 1995). Human NRAMP1 and NRAMP2/DMT1 share 61% predicted amino acid sequence identity. Nramp1 is expressed in only a few cell types, namely macrophages and neutrophils, whereas Nramp2 is expressed in many tissues, including intestine, erythroid cells, kidney, lung, brain, testes, and thymus (Mackenzie and Hediger 2004). At the cellular level, Nramp1 is localized to the phagolysosomal membrane of macrophages and neutrophils, where it most likely contributes to macrophage antimicrobial function by extruding essential metal ions (including Mn^{2+}) from the phagolysosome via H^+/metal-ion cotransport. In contrast to Nramp1, Nramp2/Dmt1 is localized to the plasma membrane and to endosomes. In the intestine and the renal tubule, Nramp2/Dmt1 is present on the apical plasma membrane and on recycling endosomes. The plasma membrane-associated Nramp2/Dmt1 mediates metal uptake into the cell from the apical compartment (intestinal lumen and renal tubular fluid), whereas the Nramp2/Dmt1 localized to recycling endosomes is thought to mediate transport of metals from acidified endosomes into the cytosol. Thus, any nonessential metal that is a substrate for Nramp2/Dmt1 and is taken up into the cell by endocytosis may then be delivered into the cytosol via this cotransporter.

Although the precise substrate specificity of Nramp2/Dmt1 remains to be established, it is clear that this is a multispecific carrier that can accept a variety of metal substrates, including many nonessential metals. Nramp2/Dmt1 displays a high affinity for Fe^{2+}, with a Km value of approximately 2 μM at a pH of 5.5 (Gunshin et al. 1997); however, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, and to a lesser extent Ni^{2+} and Pb^{2+}, also evoke strong currents in oocytes expressing the protein, suggesting that each of these metals are substrates (Gunshin et al. 1997).

More recent studies have provided direct evidence for the broad substrate specificity of the transporter, and for its ability to mediate transport of both essential and nonessential metals. Picard et al. (2000) expressed Nramp2/Dmt1 in CHO cells and demonstrated that it can transport Co^{2+} and Cd^{2+} but not Mg^{2+} into the calcein-accessible, or labile iron pool, and Sacher et al. (2001) demonstrated that $^{60}Co^{2+}$ and $^{54}Mn^{2+}$ are taken up by Nramp2/Dmt1-expressing *Xenopus* oocytes. Copper(I) and Ni^{2+} also appear to be substrates for this protein (Arredondo et al. 2003; Tallkvist et al. 2003). Several other studies have also documented a role for Nramp2/Dmt1 in Cd^{2+} uptake (Barbier et al. 2004; Bressler et al. 2004; Himeno et al. 2002; Kwong et al. 2004; Okubo et al. 2003; Olivi et al. 2001; Park et al. 2002; Ryu et al. 2004; Tallkvist et al. 2001). Collectively, these studies provide convincing evidence that Nramp2/Dmt1 is a major factor in Cd transport across the cell membrane in several tissues.

Although some investigators have suggested that Nramp2/Dmt1 may also mediate Pb^{2+} transport into cells (Bressler et al. 2004; Kwong et al. 2004; Okubo et al. 2003), this conclusion is based largely on indirect evidence, and the physiological significance of the observed transport is undefined (Mackenzie and Hediger 2004; Bannon et al. 2002, 2003). For example, Bannon et al. (2002) reported that human embryonic kidney fibroblasts (HEK293 cells) overexpressing DMT1 show a higher uptake of lead than control cells. However, this same group of in-

vestigators found that lead failed to inhibit iron uptake in control human Caco-2 cells expressing endogenous DMT1, and that Caco-2 cells whose DMT1 mRNA was knocked down exhibited no difference in lead uptake, but did show a 50% decrease in iron and cadmium uptake (Bannon et al. 2003).

Nevertheless, the observation that Nramp2/Dmt1 can transport cadmium and likely other divalent toxic metals has important consequences for human health. As reviewed by Bressler et al. (2004) and Kwong et al. (2004), experimental and epidemiological studies indicate that diets low in iron result in increased absorption of Pb and Cd, suggesting common molecular mechanisms of Cd and Pb transport. Thus, because DMT1 is regulated in part by dietary iron, an iron-deficient diet may be a significant risk factor for Pb and Cd poisoning. On the other hand, recent human data suggest that high iron intake and sufficient iron stores may reduce the risk of lead poisoning (Kwong et al. 2004).

3.2 MTP1/SLC40A1 and hephaestin in cellular iron export

Cellular export of iron appears to be mediated by the complementary action of two proteins, a ferroxidase enzyme called hephaestin, and the metal transporter protein-1 (MTP1/SLC40A1; also called ferroportin or iron-regulated transporter-1, IREG1) (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000, 2001; McKie and Barlow 2004; Vulpe et al. 1999). Although the role of MTP1 in the export of other metals has not yet been examined, it is likely that it will also be able to transport other metals based on findings with MTP1 orthologues in non-mammalian species (Rolfs and Hediger 2001).

MTP1 is expressed in tissues involved in body iron homeostasis, including the developing and mature reticuloendothelial system, the duodenum, and the pregnant uterus (Abboud and Haile 2000). It has also recently been identified in cells of the central nervous system including those of the blood-brain barrier, choroid plexus, neurons, oligodendrocytes, astrocytes, and retina (Hahn et al. 2004; Wu et al. 2004). MTP1 is localized to the basolateral membrane of the duodenal epithelial cells, and its overexpression in tissue culture cells results in intracellular iron depletion. In the adult mouse, MTP1 expression in the liver and duodenum are reciprocally regulated (Abboud and Haile 2000). To date, the mechanism of MTP1-mediated transport has not been elucidated, and no information is available on the role of other ions or of energy source in transport (McKie and Barlow 2004).

Hephaestin is a transmembrane-bound ceruloplasmin homologue that functions as a multicopper ferroxidase. All residues involved in copper binding and disulfide bond formation in ceruloplasmin are conserved in hephaestin; however, unlike ceruloplasmin, hephaestin is an integral membrane protein with a single transmembrane domain. Hephaestin is mutated in the sex-linked anemic mouse (*sla* mouse), is highly expressed in the intestine, and is necessary for iron egress from intestinal enterocytes into the circulation (Vulpe et al. 1999). The mechanism by which hephaestin and MTP1 interact to mediate cellular metal export has not yet been defined.

3.3 The ZNT/SLC30 and ZIP/SLC39 families of zinc transporters

Movement of zinc into and out of cells, and into various subcellular compartments is mediated in large part by the ZNT/SLC30 and ZIP/SLC39 transporter families (Eide 2004; Liuzzi and Cousins 2004; Palmiter and Huang 2004). There are at least 9 *ZNT* and 15 *ZIP* genes in the human genome. The ZIP proteins function largely for zinc uptake into cells (from both the extracellular space, and possibly from endocytosed vesicles), whereas the ZNT proteins function primarily in exporting zinc from cells or in sequestering zinc in intracellular compartments, including endosomes, secretory granules, synaptic vesicles, Golgi apparatus, or trans-Golgi network. The ZNT and ZIP transporter families exhibit unique tissue-specific expression, differential responsiveness to dietary zinc deficiency and excess, and differential responsiveness to physiologic stimuli via hormones and cytokines. However, their biochemical mechanisms of substrate transport are not yet known.

Although the ZNT and ZIP proteins are relatively selective for zinc, there is some evidence that they may also transport iron and manganese, indicating that they may provide a permeation pathway for nonessential metals (Eide 2004); however, additional studies are needed to test this possibility.

3.4 The hCTR1/SLC31A1 and hCTR2/SLC31A2 copper uptake transporters

Although it has long been recognized that intracellular copper concentrations are tightly regulated, the molecular basis for this regulation remained a mystery until the mid-1990s (Bull and Cox 1994; Vulpe et al. 1993; Vulpe and Packman 1995; Yamaguchi et al. 1996; Zhou and Gitschier 1997). Important insight into these mechanisms was provided by studies in several laboratories, many of which took advantage of the remarkable similarities between yeast and mammalian cells in terms of copper and iron metabolism, and the relative ease with which genetic manipulations can be carried out in yeast.

Using a genetic approach, Zhou and Gitschier (1997) isolated a human gene involved in copper uptake by complementation of the yeast high-affinity copper uptake mutant, *ctr1*. The human gene product (hCTR1) exhibits 29% amino acid identity with yeast CTR1. A database search by Zhou and Gitschier (1997) revealed an additional human gene that was named *hCTR2*. By Northern blot analysis, *hCTR1* and *hCTR2* were expressed in all tissues examined, but the liver exhibited the highest level of expression.

CTR orthologues are present in all eukaryotes, and their discovery has provided new insights into how cells acquire and regulate this essential metal (Petris 2004). These studies also indicate that although hCTR1 is a relatively selective, high-affinity copper uptake transporter, it may also mediate cellular uptake of cisplatin, carboplatin, and oxaliplatin (Holzer et al. 2004; Safaei and Howell 2005). This observation provides insight into the mechanism of action and possible loss of efficacy for this important family of anticancer drugs.

3.5 ATP7A and ATP7B, and other ATPases

Copper imported by the plasma membrane CTR proteins is immediately bound to intracellular copper chaperone proteins, which then deliver the copper to various intracellular sites. One of these copper chaperones, Atox1, delivers copper to the endosomal/lysosomal compartment by interacting with the copper-transporting P-type ATPases ATP7A (the Menkes disease protein) and ATP7B (the Wilson's disease protein). ATP7B is expressed predominantly in the liver, and delivers the copper to ceruloplasmin or to biliary excretion in concert with a newly discovered chaperone, Murr1, the protein missing in canine copper toxicosis (Gitlin 2003; Wijmenga and Klomp 2004). Within this intracellular compartment, copper is then used for the synthesis of copper-containing proteins such as ceruloplasmin, or it is stored for subsequent excretion (Davis et al. 1996). Some of the intravesicular copper is presumably sorted into vesicles destined for the lysosomal-biliary excretory pathway. According to this model, fusion of exocytic vesicles with the canalicular membrane delivers copper into bile (Fig. 2); however, neither the site nor the mechanism by which this vesicular sorting occurs is known.

In tissues other than the liver, a comparable P-type ATPase (ATP7A) pumps copper into endosomal/lysosomal compartments (Cox and Moore 2002; Voskoboinik and Camakaris 2002). ATP7A delivers copper into the trans-golgi network for synthesis of the proteins dopamine beta-monooxgenase, peptidylglycine alpha-amidating monooxygenase, lysyl oxidase, and tyrosinase, depending on the cell type. Mutations in *ATP7A* leads to significant copper accumulation in intestinal mucosa, kidney, and selected other tissues. This inability to deliver copper from sites of its absorption and storage results in a systemic copper insufficiency in Menkes patients. In contrast with the copper insufficiency of Menkes patients, Wilson's disease patients accumulate excess copper in many tissues (e.g. liver, brain, kidney, cornea) due to the inability to excrete copper into bile, the main route of its elimination. At the cellular level, copper accumulates in the cytosol and the cell eventually succumbs to copper toxicity.

In addition to copper, recent studies indicate that ATP7A and ATP7B also mediate cellular efflux of cisplatin, carboplatin, and oxaliplatin (Safaei and Howell 2005), suggesting that they may also participate in the export of other nonessential metals.

Nonessential metals also interact with plasma membrane Ca^{2+}-ATPases, the predominant proteins for cellular Ca^{2+} efflux (Blitzer et al. 1989; Pavoine et al. 1987; Suzuki and Kawakita 1993), raising the possibility that nonessential metals may utilize these ATPases to traverse cell membranes.

3.6 Channels

Ion channels are involved in a number of important cellular functions, including regulation of the membrane potential, cation and anion homeostasis, and cellular signaling. Channel opening can be regulated by intracellular or extracellular ion concentrations, changes in membrane potential (voltage-dependent), or receptor

binding either directly or via G-proteins (Atchison 2003; Fleig and Penner 2004). Recent studies demonstrate that many of the substances that were originally thought to enter the cell by simple diffusion are actually transported by membrane proteins, including channels (Khademi et al. 2004; King et al. 2004).

Ion channels also appear to play a significant role in the transport of nonessential metals. Several studies have shown that the uptake of Cd^{2+} is sensitive to commonly used channel blockers (Blazka and Shaikh 1991; Hinkle et al. 1987; Souza et al. 1997). For example, in excitable tissues Cd^{2+} and Pb^{2+} may enter cells via voltage-sensitive channels (Atchison 2003; Hinkle et al. 1987; Reuter 1983; Simons and Pocock 1987). Studies by Hinkle and coworkers (1987) in a pituitary cell line demonstrate that one route of cadmium uptake in these cells is via voltage-gated dihydropyridine-sensitive calcium channels. The voltage-gated calcium channels also admit Ba^{2+} and Sr^{2+}, and are inhibited by a number of divalent metal cations.

Calcium uptake in many cells may also occur through receptor-activated calcium channels, which could theoretically allow other divalent cations to enter the cell. Support for this possibility is provided by the observation that hepatocyte receptor-activated calcium channels are inhibited by Zn, Cd, Ni, Co, and Mn (Hughes and Barritt 1989); however, the nature of the inhibition by the metals is unknown. Crofts and Barritt (1990) indicate that Mn^{2+} can move into hepatocytes through the receptor-activated Ca^{2+} inflow system, identifying a potential regulated mechanism for hepatic manganese uptake. Data by Baker et al. (2003) also suggest that the higher susceptibility of female rats to Cd induced hepatotoxicity may be due to the activation of Ca channels by progesterone. However, some investigators have failed to see an increase in Cd transport in the absence of Ca, or a decrease in uptake with Ca channel blockers (Endo et al. 2002; Jumarie et al. 1997; Pham et al. 2004; Templeton 1990).

A major contributor to metal transport may be from the transient receptor potential (TRP) superfamily of channels. This superfamily consists of a number cation-selective channels, some of which are known to be involved in Ca^{2+} and Mg^{2+} transport (Huang 2004). Of particular importance may be members of the TRPM family. Many members of this family are thought to participate in cation adsorption and homeostasis. TRPM7 is the most ubiquitously expressed member and is permeable to a number of divalent metal ions including Zn^{2+}, Ni^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Cd^{2+}, and Ca^{2+} (Fleig and Penner 2004).

Other channels may also participate in metal transport. Aquaglyceroporins, which allow for the movement of uncharged solutes such as glycerol and urea down their concentration gradients, have recently been shown to also facilitate the movement of arsenic. Liu et al. (2004) has provided evidence that two members of the human aquaglyceroporin family, AQP9 and AQP7, allow movement of aqueous arsenic trioxide (in the form of $As(OH)_3$) when expressed in both *S. cerevisiea* and *Xenopus laevis* oocytes. This raises the possibility that other metal oxides may be transported in this manner.

4 Other ion and organic solute transporters

Because metals are often present in biological fluids as complexes with amino acids, peptides, proteins, lipids, and other macromolecules, they are also likely to permeate cell membranes as these complexes. Some of the ion and organic solute transporters that may play a role in this process are illustrated in Figure 3, and are described in more detail below.

4.1 Amino acid and peptide transporters

Amino acid transporters are suspected to be major contributors to the endogenous transport and rapid distribution of toxic metals (Ballatori 2002). In general, amino acid carriers are widely expressed in tissues, and have been classically described according to their substrate specificity and driving force (Boll et al. 2004; Gasnier 2004; Kanai and Hediger 2004; Mackenzie and Erickson 2004; Malandro and Killberg 1996; Palacin and Kanai 2004; Verrey 2003;Verrey et al. 2004). As stated in Section 2, metals can readily form complexes with proteins and amino acids, and several groups have provided evidence for cellular copper or zinc uptake as histidine complexes (Aiken et al. 1992; Harris 1993; Horn et al. 1995; Horn and Thomas 1996; Oakley et al. 2004). Our group has demonstrated transport of methylmercury as a cysteine complex (Ballatori 1994; Ballatori and Truong 1995b; Dutczak and Ballatori 1994; Kerper et al. 1992; Mokrzan et al. 1995). More recent studies have shown the involvement of specific amino acid transporters in metal transport. Of significance, Simmons-Willis and colleagues (2002) demonstrated that the methylmercury cysteine complex, which is structurally similar to methionine, is a substrate for the L-type large neutral amino acid transporters SLC7A5/LAT1 and SLC7A8/LAT2 when expressed in *Xenopus* oocytes. Inorganic mercury can also be transported via SLC7A9/$b^{0,+}$ as a complex with two molecules of cysteine or homocysteine (Bridges and Zalups 2004).

Peptide transporters may also play a large role in the transport and disposition of toxic metals. These proteins facilitate the transport of di- and tripeptides, and aid in the uptake and recycling of amino acids. To date, four peptide transporters have been identified (PEPT1, PEPT2, PHT1, PHT2: all are members of the SLC15 family, but have been characterized to different degrees). In addition, there is evidence for other as yet unidentified peptide transporters (Daniel and Kottra 2004; Smith et al. 2004; Terada and Inui 2004). PEPT1 was originally identified via expression cloning from a rabbit small intestine cDNA library, with human, rat, mouse, cow, and chicken homologues eventually being documented, along with the identification of a renal isoform, PEPT2 (Fei et al. 1994; Liu et al. 1995; Terada and Inui 2004). Human PEPT1 and PEPT2 can transport all 400 possible combinations of di- and 8000 different tripeptides and structurally related analogs, but not larger oligopeptides or free amino acids (Daniel and Kottra 2004). They both function as proton cotransporters, utilizing an extracellular proton gradient to drive substrate uptake. The low-affinity/high-capacity PEPT1 has been localized to the apical membrane in intestine and to a lesser extent in the kidney. PEPT2, a

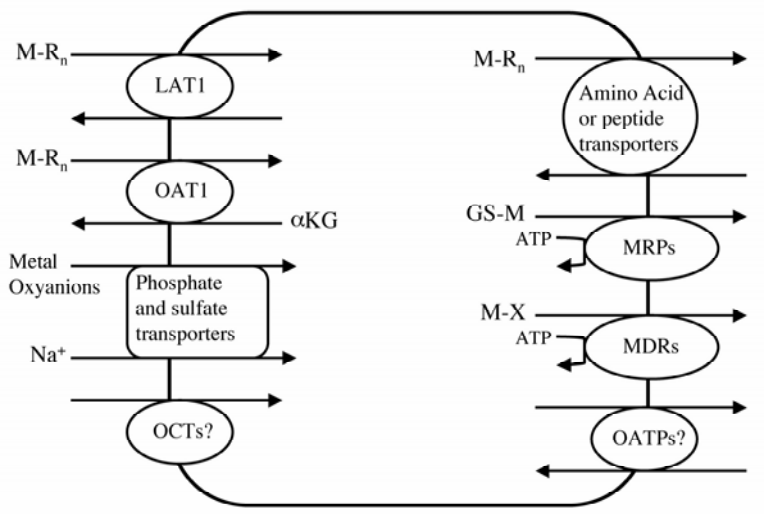


Fig. 3. Ion and organic solute transporters that may be involved in the uptake and export of metal complexes.

high-affinity/low-capacity transporter, is much more widely distributed. It is apically localized in the kidney, choroid plexus, lung and mammary gland (Daniel and Kottra 2004; Smith et al. 2004). Less information is known about the more recently discovered PHT1 and PHT2. They able to transport di- and tripeptides, but unlike PEPT1 and PEPT2, they can also accept free histidine as a substrate (Daniel and Kottra 2004).

4.2 Uptake on phosphate or sulfate transporters

One of the earliest metals transport mechanisms to be described was the transport of metal oxyanions on phosphate and sulfate carriers (Clarkson 1993; van Veen et al. 1994; Wetterhahn-Jennette 1981). Vanadate and arsenate are structurally similar to phosphate and can compete with phosphate for transport, as well as intracellular binding sites. Indeed, their toxicity is thought to be directly related to this competition. Similarly, chromate, selenate, and molybdate are structurally similar to sulfate, and are substrates for sulfate transporters.

Phosphate or sulfate transporters have been characterized functionally, and studies have identified some of the proteins at the molecular level (Markovich et al. 2005; Murer et al. 2004). In addition to these plasma membrane transporters, phosphate transporters are also present in intracellular organelles, including mitochondria (Pratt et al. 1991). Arsenate and vanadate most likely enter cells and their organelles on these phosphate transporters (Wetterhahn-Jennette 1981). By analogy, sulfate transporters probably mediates uptake of chromate, selenate, and molybdate.

4.3 MRP/ABCC-mediated excretion of GSH complexes and other organometallic complexes

Among the metal binding ligands, glutathione (GSH) is one of the most versatile and pervasive (Ballatori 1994). GSH is the most abundant non-protein sulfhydryl-containing compound within cells, at concentrations of 1-10 mM. The sulfhydryl group on the cysteine moiety has a high affinity for metals, forming thermodynamically stable but kinetically labile mercaptides with a number of metals including mercury, silver, cadmium, arsenic, lead, gold, zinc, and copper. There is now considerable evidence that GSH plays key roles in metal transport and metabolism (Ballatori 1994, 2002; Ballatori and Clarkson 1982, 1983). Of significance, cellular export of several metals is accomplished by transport of the corresponding GSH mercaptides on membrane proteins that normally transport either glutathione *S*-conjugates or GSH itself (Ballatori 1994; Ballatori and Truong 1995a; Ballatori et al. 1995; Dijkstra et al. 1995; Dutczak and Ballatori 1994).

Efflux of glutathione *S*-conjugates from mammalian cells is mediated by the multidrug resistance-associated proteins (MRP), of which nine have been identified in the human genome (MRP1 to MRP9). MRP1 is found on the plasma membrane of many cell types, whereas MRP2, which is also referred to as cMOAT (canalicular multispecific organic anion transporter) or cMRP (canalicular MRP), is selectively localized to the apical plasma membrane of specific transporting epithelia, including the hepatocyte canalicular membrane, and the apical membrane of kidney proximal tubules. Both MRP1 and MRP2 have a broad specificity for conjugates formed from GSH, glucuronides, or sulfate.

Several studies indicate that the MRP proteins also mediate cellular efflux of glutathione-metal complexes. First, cells overexpressing MRP1 are more resistant to arsenite, antimonite, and cisplatin, consistent with accelerated metal efflux, although resistance to the metals is relatively low (Borst et al. 1997; Chen et al. 1997; Deeley and Cole 1997). These metals can form divalent and trivalent complexes with GSH *in vitro*, and presumably are forming these GSH complexes *in vivo* as well. Zaman and coworkers (1995) demonstrated MRP-mediated extrusion of [^{35}S]cysteine-containing compounds after addition of arsenite, suggesting coupled efflux. Second, Ishikawa and coworkers (1996, 1997) demonstrated enhanced transport of a glutathione-platinum complex in membrane vesicles prepared from a tumor cell line overexpressing MRP. Third, mutant rats that lack mrp2 activity (TR$^-$, GY, or EHBR rats), exhibit impaired ability to transport several metals into bile, including zinc, excess copper, silver, cadmium, and methylmercury (Ballatori et al. 1995; Dijkstra et al. 1996, 1997; Houwen et al. 1990; Sugawara et al. 1997). In contrast, basal excretion of endogenous copper is unaffected in these mutant rats (Houwen et al. 1990). Fourth, the predicted MRP1 protein shows 43% amino acid identity with the YCF1 protein of *S. cerevisiae*, a protein which confers cadmium resistance by transporting and sequestering the glutathione-cadmium complex ($Cd(GS)_2$) in the yeast vacuole (Li et al. 1996, 1997; Szczypka et al. 1994; Wemmie et al. 1994). Of significance, human *MRP1* cDNA can complement the loss of cadmium resistance in *YCF1*-disrupted yeast cells (Tommasini et al. 1996). The predicted MRP1 protein sequence also shows 34% amino acid identity with

the *Leishmania* PgpA, ltpgpA, a transporter involved in resistance to arsenite and antimonite (Callahan and Beverley 1991; Dey et al. 1996). LtpgpA confers resistance to arsenite and trivalent antimonials, but not to pentavalent antimonials, zinc, cadmium or the typical MDR substrates vinblastine and puromycin (Callahan and Beverley 1991). Dey and coworkers (1996) demonstrated ATP-dependent transport of the As(III)-glutathione complex ($As(GS)_3$) in membrane vesicles of *Leishmania tarentolae*. In addition, a family of four MRP-related proteins has recently been identified in the nematode *C. elegans*, one of which (ceMRP1) confers resistance to cadmium and arsenite (Broeks et al. 1996). ceMRP1 is 47% identical to human MRP1 at the predicted amino acid level (Broeks et al. 1996). More recent studies have demonstrated that arsenic transport into bile depends on the MRP2 transporter and glutathione, and that arsenic triglutathione and methylarsenic diglutathione account for most of the arsenic in the bile (Kala et al. 2000, 2004). Likewise, MRP1 can transport arsenic-glutathione complexes (Leslie et al. 2004; Lorico et al. 2002).

Once exported from the cell, GSH, glutathione *S*-conjugates, as well as glutathione-complexes are degraded by the ectoenzymes γ-glutamyl transpeptidase and dipeptidase activities (Ballatori et al. 1986b; Hinchman and Ballatori 1994; Hinchman et al. 1991; Simmons et al. 1991), and the resulting products are partially reabsorbed by the cells (Ballatori et al. 1986a, 1988; Moseley et al. 1988; Hinchman et al. 1991; Simmons et al. 1992).

Studies in γ-glutamyl transpeptidase (GGT)-deficient mice provide direct evidence for a major role of this enzyme in regulating the metabolism, tissue distribution, and elimination of methylmercury and inorganic mercury (Ballatori et al. 1998a). To evaluate the role of GGT in the whole-body disposition of methylmercury, these studies compared the elimination of ^{203}Hg-methylmercury in GGT-deficient mice to that in wild type mice and mice heterozygous for this deficiency. The effects of *N*-acetylcysteine (NAC), a drug used to maintain the cysteine and GSH levels of GGT-deficient mice, were also examined. There were no differences in methylmercury excretion between the wild type and heterozygous mice; however, the GGT-deficient mice excreted methylmercury more rapidly at both dose levels (Ballatori et al. 1998a). Wild type and heterozygous mice excreted from 11-24% of the dose in the first 48 hours, whereas the GGT-deficient mice excreted 55-66% of the dose, with most of the methylmercury being excreted in urine. Urinary methylmercury excretion was further accelerated in mice that received NAC. In contrast to methylmercury, the whole body elimination of inorganic mercury was not affected by GGT deficiency, although the tissue distribution of inorganic mercury was markedly different in GGT-deficient male mice, with only 13% of the ^{203}Hg body burden in the kidneys of GGT-deficient mice versus ~50% in kidneys of wild type male mice. These findings provide direct evidence for a major role of GGT in regulating the tissue distribution and elimination of methylmercury and inorganic mercury (Ballatori et al. 1998a), and provide further support for the suggestion that NAC may be an excellent antidote in methylmercury poisoning (Ballatori et al. 1998b).

4.4 Organic solute carriers: OATP, OAT, OCT, and NTCP

As already mentioned, metals are normally present in biological fluids as complexes with endogenous molecules such as amino acids, peptides, or phospholipids, substances that are themselves transported across cell membranes by carrier proteins. These organic solute carriers are generally multispecific, that is, they accept substrates that differ considerably in their chemical structures, and most are probably unable to discriminate between substrates whose only modification is the presence of a metal ion. There is some evidence that some of these carriers can transport metal-substrate complexes (Aslamkhan et al. 2003; Ballatori 1994; Dutczak and Ballatori 1994; Kerper et al. 1992; Koh et al. 2002; Lungkaphin et al. 2004). This mode of transport may be particularly important for toxic metals, given the absence of selective transport systems for these metals.

4.5 Organic solute transport pumps: MDR1, MDR2, and BSEP

P-Glycoproteins are frequently responsible for the multidrug resistance (MDR) that is observed in tumor cells, but are also constitutively expressed in several epithelial and endothelial tissues, including the hepatocyte canalicular membrane. Bile canalicular membranes contain MDR1, which transports cationic drugs into bile, and MDR2, which transports phospholipids into bile (Smit et al. 1993). Sharma and coworkers (1996) demonstrated that cationic organometallic complexes are also substrates for MDR1. These investigators demonstrated that human epidermal carcinoma KB cells overexpressing MDR1 P-glycoprotein are more resistant to the cytotoxic activities of Al(III), Fe(III) and Ga(III)-complexes of (ethylenediamine)-*N,N'*-bis[propyl[(2-hydroxy-4,6-dimethoxybenzyl)-imino]].
Broeks and coworkers (1996) reported that the *C. elegans* equivalent MDR protein (cePGP) also contributes to heavy metal (cadmium and arsenite) resistance in this organism, but the mechanism involved is unknown. These observations raise the possibility that MDR proteins on the canalicular membrane may be involved in transport of metal complexes into bile.

An additional canalicular transporter that might be involved in biliary metal excretion is the ATP-dependent bile salt transporter, BSEP, although its role is probably minimal. Bile acid-metal complexes may form under certain conditions, and these would most likely be substrates for the multispecific BSEP protein.

5 Endocytosis and exocytosis

Endocytosis and exocytosis are complex and tightly controlled processes that are essential for maintaining cellular homoeostasis. Vesicle budding from the cell surface can be constitutive or ligand-induced, and can occur in a clathrin-dependent or -independent manner (Bonifacino and Glick 2004; Gundelfinger et al. 2003; Maxfield and McGraw 2004; Mousavi et al. 2004; Nichols 2003; Szymkiewicz et

al. 2004; Wu 2004). The constitutive pathway is involved mainly in the uptake of membrane proteins and other cargo that undergo continuous internalization and recycling, but is also involved in the ligand-independent internalization of signaling receptors. In ligand-induced endocytosis, specific molecules interact with cell surface receptors to stimulate endocytosis; however, the mechanisms of ligand-induced internalization are not yet known.

Of importance for metal transport, endocytosis is a very active process both in terms of the number of vesicles that are present at any one time on the cell surface, the rapidity with which they traffic to and from the cell membrane, and the volume that they internalize per unit time. It is estimated that coated pits cover up to 2% of the cell surface (Brown and Petersen 1999), and that the time course of endocytosis ranges from less than a second to hundreds of a second (Wu 2004). The volume of fluid taken up (and released) by vesicle trafficking is also quite large. For example, between 1-6 pl/cell.h are internalized in rat hepatocytes (Oka et al. 1989; Scharschmidt et al. 1986), or greater than 20% of the cell volume each hour. From this rate it can be estimated that between 5 and 50 times the hepatocytes' plasma membrane is endocytosed each hour (Blomhoff et al. 1989; Oka et al. 1989; Scharschmidt et al. 1986). This phenomenal rate of plasma membrane and fluid internalization has obvious implications for the transport of substances across the membrane, yet little information is available on its role in metal transport. The rapid rate of membrane and fluid transport may be particularly important in facilitating movement of metals that either have a high affinity for plasma membrane binding sites, or that are bound to ligands that are selectively cleared via membrane receptors. Because the process is bidirectional, membrane recycling functions to transport metals to and from blood plasma, and to and from apical compartments of polarized epithelial cells.

5.1 Endocytosis of transferrin and other metal complexes

Although the overall contribution of endocytosis to the transport of metals has probably been underestimated, the seminal importance of receptor-mediated endocytosis for the transport of iron and copper has long been recognized, and many of the individual steps in this process have been characterized at the molecular level (Aisen 1998).

Most investigators agree that the predominant mechanism of iron transport from blood plasma into hepatocytes is via the transferrin receptor (Morgan and Baker 1988), as illustrated in Figure 2. Nearly all of the iron in plasma (~99%) is normally associated with transferrin, a protein that binds iron in the Fe(III) oxidation state. Oxidation of Fe(II) to Fe(III) is catalyzed by the copper-containing enzyme ceruloplasmin (Fig. 2). The interaction of transferrin with its receptor promotes iron uptake by two mechanisms. First, receptor-mediated endocytosis leads to the internalization of diferric transferrin, followed by release of iron within acidic vesicles, and extrusion of iron-depleted transferrin (apotransferrin). Internalized endocytotic vesicles move rapidly (within minutes) to lysosomes and Golgi. The entrapped metal complexes may be metabolized, released to other in-

tracellular compartments, or transferred in coated and noncoated vesicles back across the plasma membrane. The mechanism by which the released iron is subsequently transferred from the endosome/lysosome to the cytosol is not clear, but is likely to be mediated by the DMT1 protein. A second and more speculative uptake mechanism involves the possibility that iron is released at the plasma membrane without internalization of the transferrin-receptor complex (Jordan and Kaplan 1994; Thorstensen and Romslo 1988; Thorstensen et al. 1995). In this model, transferrin-bound ferric iron is reduced to ferrous iron extracellularly, removed from the transferrin molecule, and transported into the cell, possibly by NRAMP2/DMT1.

In addition to transferrin receptors, ferritin receptors play a major role in hepatic iron uptake (Mack et al. 1983; Osterloh and Aisen 1989). Kupffer cells release a substantial fraction of the iron acquired by erythrophagocytosis in the form of ferritin, which is efficiently internalized by hepatocytes, via their ferritin receptors. Aisen (1998) suggests that ferritin may be more important than transferrin in mediating hepatic iron uptake at physiological concentrations of these ligands. This issue remains to be resolved.

A third receptor-mediated endocytotic mechanism involves the asialoglycoprotein receptors (Oka and Weigel 1983; Regoeczi et al. 1984; Stockert et al. 1980; Young et al. 1983). Galactose-specific asialoglycoprotein receptors are unique to hepatocytes, and have been identified in every mammalian species examined. One substrate for these receptors is asialotransferrin. Sinusoidal endothelial cells appear to be able to desialate transferrin (Tavassoli 1988); the iron carried by these sialic acid-depleted molecules can be rapidly cleared from the blood by receptor-mediated endocytosis. Hepatic iron uptake is faster from asialotransferrin than from fully sialylated transferrin (Rudolph et al. 1986).

Although the relative contributions of these auxiliary pathways of iron uptake are unknown, it is becoming quite clear that these pathways are important not only in iron-overload states, but work in parallel with the transferrin receptor to mediate normal hepatic iron uptake (Morley and Bezkorovainy 1985).

Interestingly, these iron binding proteins may also be involved in the transmembrane movement of other metal cations, including manganese, zinc, and vanadium. Most of the manganese in plasma is bound to transferrin, a finding that may explain the rapid hepatic clearance of Mn from plasma. Similarly, a substantial fraction of vanadium in rat plasma is associated with transferrin. Vanadium accumulates preferentially in tissues that are also abundant in iron (liver, spleen, and kidney). Chromatographic separation of vanadium in liver homogenates reveals that the metal coelutes with fractions corresponding to transferrin and ferritin. It has been speculated that ferritin may serve as a general metal detoxicant because of its ability to bind to a variety of metal cations, including Cd, Zn, Be, and Al (Joshi et al. 1989). Metals also bind to albumin, and this complex may also be transported by vesicular mechanisms (Tibaduiza and Bobilya 1996).

Vesicular exocytosis may also be involved in biliary excretion of iron and possibly other metals (LeSage et al. 1986; Ramm et al. 1994; Regoeczi and Chindemi 1995). Regoeczi and Chindemi (1995) measured the translocation of different

forms of transferrin from blood to bile in the rat, and found that only a small fraction of the metal-containing protein is excreted in bile.

6 Simple diffusion

There is increasing evidence that simple diffusion plays little if any role in the transport of most biologically active molecules, including metals, across cell membranes. For example, many compounds that were previously thought to be transported exclusively by simple diffusion, are now known to be transported largely by specific membrane channels, transporters, or pumps. These include proteins that mediate transport of small compounds such as water, urea, and ammonia (Khademi et al. 2004; King et al. 2004; Sands 2004; Shayakul and Hediger 2003), as well as proteins that mediate transport of relatively hydrophobic molecules such as fatty acids, prostaglandins, leukotrienes, cholesterol, and other sterols (Borst et al. 2000; Halestrap and Meredith 2004; Pighin et al. 2004; Schmitz et al. 2000; Shuster 2002; Stahl 2004; Tannert et al. 2003; Wang et al. 2001; Yu et al. 2004). As described in previous sections of this report, many metal transport proteins have now been identified for both the essential and many toxic metals. In contrast with simple diffusion, mediated transport can be regulated to adjust cellular concentrations, to respond to changing substrate availability and requirements, as well as to limit cellular concentrations of potentially toxic metals. Thus, it is clearly advantageous for transport to be mediated, and there is significant evolutionary pressure for such systems to continue to expand.

For metals, both their chemical properties and biological reactivities make it imperative that cellular concentrations be regulated. Moreover, most metals and metal-complexes are polar, hydrophilic, and quite often charged, properties that would exclude them from the hydrophobic interior of the plasma membrane. Simple diffusion through a membrane protein matrix or ion channel is also highly unlikely. Although most *in vitro* studies of metal transport conclude that a substantial fraction of transport is due to a "nonsaturable" component of transport (presumably diffusion) this component of transport is most likely an artifact of either the experimental conditions used to examine transport, or the cell or tissue isolation procedure. Kinetics consistent with simple diffusion may be obtained if: 1) the concentration of metal substrate is below the Km of the transport system. The effective metal substrate concentration in the incubation media is often artificially lowered by extensive binding to nonsubstrate ligands, 2) there is a large component of non-specific high affinity (or essentially irreversible) binding to cellular constituents, 3) transport occurs across a cell membrane damaged either during cell isolation or by increasing concentrations of the metal itself. For example, protease contaminants in the collagenase used for hepatocyte isolation can degrade transferrin receptors, thereby inhibiting the predominant iron uptake mechanism, and accelerating other nonspecific transport pathways (Morgan and Baker 1986). Finally, 4) uptake occurs by endocytosis. In this case, the metal would be transported nonspecifically along with the entrapped fluid and/or membrane vesicle

components. All of these mechanisms need to be considered before attributing a particular component of transport to "simple diffusion".

Studies of metal transport in isolated cell or vesicle systems are particularly susceptible to these artifacts. First, since a given metal normally binds to many endogenous ligands, some of which may be unidentified, it is difficult to reproduce *in vivo* conditions in a cell culture system. As discussed above, metals are normally complexed with amino acids, peptides, proteins, phospholipids, and other tissue constituents, and are distributed among all competing ligands on the basis of concentrations and relative affinities of available ligands. Because it is difficult to reproduce *in vivo* conditions in a culture dish, interpretation of transport studies performed *in vitro* should be performed with care.

Second, the propensity of metals to bind to things such as culture dishes, filters, components of the culture media, or more importantly cell membranes, creates both a practical and theoretical dilemma. This nonspecific attachment is difficult to eliminate and even more difficult to quantify. When metal salts are added to protein and amino acid-free culture media, the metal frequently associates rapidly with the cells. This rapid uptake is not due to the presence of an efficient "transport system" as often suggested, but may simply reflect the absence of competing ligands.

Although simple diffusion plays only a minor role in transport across cell membranes (i.e. transcellular transport), it may play a larger role in the paracellular movement of metals across epithelial barriers (Bronner 2003; Shachar-Hill and Hill 2002). Epithelial tissues with relatively leaky junctional complexes, such as the small intestine, exhibit substantial convective paracellular flow, which allow for the nonspecific movement of metals across tissue barriers. However, recent data indicate that even this relatively nonspecific permeation pathway may be regulated by specific membrane transport proteins (Shachar-Hill and Hill 2002). It has been proposed that paracellular flows may not be due to simple convection generated by osmotic flow through the junctions, but may be generated by active fluid transport within the junction, a mechano-osmotic process (Shachar-Hill and Hill 2002). In this model, the aquaporins are thought to function as osmocomparators within a feedback loop that regulates the paracellular fluid flow rate, resulting in an overall quasi-isotonic transport by the epithelium (Shachar-Hill and Hill 2002). However, this model has not yet been validated.

7 Summary

Although many metal transporter genes and metal permeation pathways have recently been identified, there are undoubtedly many others awaiting to be discovered and characterized. Indeed, of all of the genes in the human genome, less than half have been assigned a biological function, and for many of these genes their function is predicted from sequence identity rather than from direct experimental evidence. Thus, much exciting work remains to be done to identify and characterize the genes that are involved in metal transport, metabolism, and detoxification.

Acknowledgements

This work was supported in part by the National Institute of Health Grants ES06484 and DK48823, NIEHS Center Grants ES01247 and ES03828, and Toxicology Training Program Grant ES07026.

References

Abboud S, Haile DJ (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Biol Chem 275:19906-19912

Aiken SP, Horn NM, Saunders NR (1992) Effects of amino acids on zinc transport in rat erythrocytes. J Physiol 445:69-80

Aisen P (1988) Transferrin, the transferrin receptor, and the uptake of iron by cells. Met Ions Biol Syst 35:585-631

Andrews NC (2002) Metal transporters and disease. Curr Opin Chem Biol 6(2):81-86

Arredondo M, Munoz P, Mura CV, Nunez MT (2003) DMT1, a physiologically relevant apical Cu^{+1} transporter of intestinal cells. Am J Physiol Cell Physiol 284(6):C1525-C1530

Aslamkhan AG, Han YH, Yang XP, Zalups RK, Pritchard JB (2003) Human renal organic anion transporter 1-dependent uptake and toxicity of mercuric-thiol conjugates in Madin-Darby canine kidney cells. Mol Pharmacol 63(3):590-596

Atchison WD (2003) Effects of toxic environmental contaminants on voltage-gated calcium channel function: from past to present. J Bioenerg Biomembr 35:507-532

Atkinson PG, Blackwell JM, Barton CH (1997) Nramp1 locus encodes a 65 kDa interferon-gamma-inducible protein in murine macrophages. Biochem J 325:779-786

Baker TK, VanVooren HB, Smith WC, Carfagna MA (2003) Involvement of calcium channels in the sexual dimorphism of cadmium-induced hepatotoxicity. Toxicol Lett 137(3):185-192

Ballatori N (1994) Glutathione mercaptides as transport forms of metals. Adv Pharmacol 27:271-298

Ballatori N (2002) Transport of toxic metals by molecular mimicry. Environ Health Perspect 110:689-694

Ballatori N, Clarkson TW (1982) Developmental changes in the biliary excretion of methylmercury and glutathione. Science 216:61-63

Ballatori N, Clarkson TW (1983) Biliary transport of glutathione and methylmercury. Am J Physiol 244:G435-G441

Ballatori N, Truong AT (1989) Relation between biliary glutathione excretion and bile acid-independent bile flow. Am J Physiol 256:G22-G30

Ballatori N, Truong AT (1995a) Multiple canalicular transport mechanisms for glutathione *S*-conjugates. Transport on both ATP- and voltage-dependent carriers. J Biol Chem 270:3594-3601

Ballatori N, Truong AT (1995b) Mechanisms of hepatic methylmercury uptake. J Toxicol Environ Health 46:343-353

Ballatori N, Moseley RH, Boyer JL (1986a) Sodium gradient-dependent L-glutamate transport is localized to the canalicular domain of liver plasma membranes. J Biol Chem 261:6216-6221

Ballatori N, Jacob R, Boyer JL (1986b) Intrabiliary glutathione hydrolysis - a source of glutamate in bile. J Biol Chem 261:7860-7865

Ballatori N, Jacob R, Barrett C, Boyer JL (1988) Biliary catabolism of glutathione and differential reabsorption of its amino acid constituents. Am J Physiol 254:G1-G7

Ballatori N, Gatmaitan Z, Truong AT (1995) Impaired biliary excretion and whole body elimination of methylmercury in rats with congenital defect in biliary glutathione excretion. Hepatology 22:1469-1473

Ballatori N, Wang W, Lieberman MW (1998a) Accelerated methylmercury elimination in γ-glutamyl transpeptidase-deficient mice. Am J Pathol 152(4):1049-1055

Ballatori N, Lieberman MW, Wang W (1998b) N-Acetylcysteine as an antidote in methylmercury poisoning. Environ Health Perspect 106(5):267-271

Bannon DI, Portnoy ME, Olivi L, Lees PS, Culotta VC, Bressler JP (2002) Uptake of lead and iron by divalent metal transporter 1 in yeast and mammalian cells. Biochem Biophys Res Commun 295(4):978-984

Bannon DI, Abounader R, Lees PS, Bressler JP (2003) Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells. Am J Physiol Cell Physiol 284(1):C44-C50

Barbier O, Jacquillet G, Tauc M, Poujeol P, Cougnon M (2004) Acute study of interaction among cadmium, calcium, and zinc transport along the rat nephron *in vivo*. Am J Physiol Renal Physiol 287(5):F1067-F1075

Blazka ME, Shaikh ZA (1991) Differences in cadmium and mercury uptakes by hepatocytes: role of calcium channels. Toxicol Appl Pharmacol 110(2):355-63

Blitzer BL, Hostetler BR, Scott KA (1989) Hepatic adenosine triphosphate-dependent Ca2+ transport is mediated by distinct carriers on rat basolateral and canalicular membranes. J Clin Invest 83:1319-1325

Blomhoff R, Nenseter MS, Green MH, Berg T (1989) A multicompartmental model of fluid-phase endocytosis in rabbit liver parenchymal cells. Biochem J 262:605-610

Boll M, Daniel H, Gasnier B (2004) SLC36 family: proton-coupled transporters for the absorption of selected amino acids from extracellular and intracellular proteolysis. Pflugers Arch 447(5):776-779

Bonifacino JS, Glick BS (2004) The mechanisms of vesicle budding and fusion. Cell 116(2):53-66

Borst P, Kool M, Evers R (1997) Do CMOAT (MRP2), other MRP homologues, and LRP play a role in MDR. Semin Cancer Biol 8:205-213

Borst P, Zelcer N, van Helvoort A (2000) ABC transporters in lipid transport. Biochim Biophys Acta 486(1):28-44

Bressler JP, Olivi L, Cheong JH, Kim Y, Bannona D (2004) Divalent metal transporter 1 in lead and cadmium transport. Ann N Y Acad Sci 1012:42-52

Bridges CC, Zalups RK (2004) Homocysteine, system b0,+ and the renal epithelial transport and toxicity of inorganic mercury. Am J Pathol 165(4):1385-1394

Broeks A, Gerrard B, Allikmets R, Dean M, Plasterk RH (1996) Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. EMBO J 15:6132-6143

Bronner F (2003) Mechanisms of intestinal calcium absorption. J Cell Biochem 88(2):387-393

Brown CM, Petersen NO (1999) Free clathrin triskelions are required for the stability of clathrin-associated adaptor protein (AP-2) coated pit nucleation sites. Biochem Cell Biol 77(5):439-448

Bull PC, Cox DW (1994) Wilson disease and Menkes disease: new handles on heavy-metal transport. Trends Genet 10:246-252

Callahan HL, Beverley SM (1991) Heavy metal resistance: a new role for P-glycoprotein in *Leishmania*. J Biol Chem 266:18427-18430

Cellier M, Prive G, Belouchi A, Kwan T, Rodrigues V, Chia W, Gros P (1995) Nramp defines a family of membrane proteins. Proc Natl Acad Sci USA 92:10089-10093

Chen Z-S, Mutoh M, Sumizawa T, Furukawa T, Haraguchi M, Tani A, Akiyama S (1997) Reversal of heavy metal resistance in multidrug-resistant human KB carcinoma cells. Biochem Biophys Res Commun 236:586-590

Chung J, Wessling-Resnick M (2004) Lessons learned from genetic and nutritional iron deficiencies. Nutr Rev 62:212-215

Clarkson TW (1972) The pharmacology of mercury compounds. Annu Rev Pharmacol 12:375-406

Clarkson TW (1993) Molecular and ionic mimicry of toxic metals. Annu Rev Pharmacol Toxicol 33:545-571

Cox DW, Moore SD (2002) Copper transporting P-type ATPases and human disease. J Bioenerg Biomembr 34(5):333-338

Crofts JN, Barritt GJ (1990) The liver cell plasma membrane Ca2+ inflow systems exhibit a broad specificity for divalent metal ions. Biochem J 269(3):579-587

Daniel H, Kottra G (2004) The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. Pflugers Arch 477(5):610-618

Davis W, Chowrimootoo GF, Seymour CA (1996) Defective biliary copper excretion in Wilson's disease: the role of caeruloplasmin. Eur J Clin Invest 26:893-901

Deeley RG, Cole SPC (1997) Function, evolution and structure of multidrug resistance protein (MRP). Sem Cancer Biol 8:193-204

Dey S, Ouellette M, Lightbody J, Papadopoulou B, Rosen BP (1996) An ATP-dependent As(III)-glutathione transport system in membrane vesicles of *Leishmania tarentolae*. Proc Natl Acad Sci 93:2192-2197

Dijkstra M, In 't Veld G, van den Berg GJ, Muller M, Kuipers F, Vonk RJ (1995) Adenosine triphosphate-dependent copper transport in isolated rat liver plasma membranes. J Clin Invest 95:412-416

Dijkstra M, Havinga R, Vonk RJ, Kuipers F (1996) Bile secretion of cadmium, silver, zinc and copper in the rat. Involvement of various tranport systems. Life Sci 59:1237-1246

Dijkstra M, Kuipers F, van den Berg GJ, Havinga R, Vonk RJ (1997) Differences in hepatic processing of dietary and intravenously administered copper in rats. Hepatology 26:962-966

Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, Law TC, Brugnara C, Lux SE, Pinkus GS, Pinkus JL, Kingsley PD, Palis J, Fleming MD, Andrews NC, Zon LI (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature 403:776-781

Dutczak WJ, Ballatori N (1992) γ-Glutamyltransferase-dependent biliary-hepatic recycling of methyl mercury in the guinea pig. J Pharmacol Exp Ther 262:619-623

Eide DJ (2004) The SLC39 family of metal ion transporters. Pflugers Arch 447(5):796-800

Endo T, Kimura O, Sakata M (2002) Effects of P-glycoprotein inhibitors on cadmium accumulation in cultured renal epithelial cells, LLC-PK1, and OK. Toxicol Appl Pharmacol 185(3):166-171

Fei YJ, Kanai Y, Nussberger S, Ganapathy V, Leibach FH, Romero MF, Singh SK, Boron WF, Hediger MA (1994) Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature 368:563-566

Fleig A, Penner R (2004) The TRPM ion channel subfamily: molecular, biophysical and functional features. Trends Pharmacol Sci 25(12):633-639

Fleming MD, Trenor CC, Su MA, Foernzler D, Beier DR, Dietrich WF, Andrews NC (1997) Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat Genet 16:383-386

Gasnier B (2004) The SLC32 transporter, a key protein for the synaptic release of inhibitory amino acids. Pflugers Arch 447(5):756-759

Gitlin JD (2003) Wilson disease. Gastroenterology 125:1868-1877

Gundelfinger ED, Kessels MM, Qualmann B (2003) Temporal and spatial coordination of exocytosis and endocytosis. Nat Rev Mol Cell Biol 4:127-139

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388:482-488

Hahn P, Dentchev T, Qian Y, Rouault T, Harris ZL, Dunaief JL (2004) Immunolocalization and regulation of iron handling proteins ferritin and ferroportin in the retina. Mol Vis 10:598-607

Halestrap AP, Meredith D (2004) The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 447(5):619-628

Harris ED (1993) The transport of copper. Prog Clin Biol Res 380:163-179

Himeno S, Yanagiya T, Enomoto S, Kondo Y, Imura N (2002) Cellular cadmium uptake mediated by the transport system for manganese. Tohoku J Exp Med 196(1):43-50

Hinchman CA, Ballatori N (1994) Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. J Toxicol Environ Health 41:387-409

Hinchman CA, Matsumoto H, Simmons TW, Ballatori N (1991) Intrahepatic conversion of a glutathione conjugate to its mercapturic acid. J Biol Chem 266:22179-22185

Hinkle PM, Kinsella PA, Osterhoudt KC (1987) Cadmium uptake and toxicity via voltage-sensitive calcium channels. J Biol Chem 262(34):16333-16337

Holzer AK, Katano K, Klomp LW, Howell SB (2004) Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells. Clin Cancer Res 10:6744-6749

Horn NM, Thomas AL (1996) Interactions between the histidine stimulation of cadmium and zinc influx into human erythrocytes. J Physiol 496:711-718

Horn NM, Thomas AL, Tompkins JD (1995) The effect of histidine and cysteine on zinc influx into rat and human erythrocytes. J Physiol 489:73-80

Houwen R, Dijkstra M, Kuipers F, Smit EP, Havinga R, Vonk RJ (1990) Two pathways for biliary copper excretion in the rat. The role of glutathione. Biochem Pharmacol 39:1039-1044

Huang CL (2004) The transient receptor potential superfamily of ion channels. J Am Soc Nephrol 15(7):1690-1699

Hughes BP, Barritt GJ (1989) Inhibition of the liver cell receptor-activated Ca2+ inflow system by metal ion inhibitors of voltage-operated Ca2+ channels but not by other inhibitors of Ca2+ inflow. Biochim Biophys Acta 1013(3):197-205

Ishikawa T, Bao JJ, Yamane Y, Akimaru K, Frindrich K, Wright CD, Kuo MT (1996) Coordinated induction of MRP/GS-X pump and gamma-glutamylcysteine synthetase by heavy metals in human leukemia cells. J Biol Chem 271:14981-14988

Ishikawa T, Li ZS, Lu YP, Rea PA (1997) The GS-X pump in plant, yeast, and animal cells: structure, function, and gene expression. Biosci Rep 17:189-207

Jordan I, Kaplan J (1994) The mammalian transferrin-independent iron transport system may involve a surface ferrireductase activity. Biochem J 302:875-879

Joshi JG, Sczekan SR, Fleming JT (1989) Ferritin - a general metal detoxicant. Biol Trace Element Res 21:105-110

Jumarie C, Campbell PG, Berteloot A, Houde M, Denizeau F (1997) Caco-2 cell line used as an *in vitro* model to study cadmium accumulation in intestinal epithelial cells. J Membr Biol 158(1):31-48

Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW (2000) The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic. J Biol Chem 275(43):33404-33408

Kala SV, Kala G, Prater CI, Sartorelli AC, Lieberman MW (2004) Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione. Chem Res Toxicol 17(2):243-249

Kanai Y, Hediger MA (2004) The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. Pflugers Arch 447(5):469-479

Kerper LE, Ballatori N, Clarkson TW (1992) Methylmercury transport across the blood-brain barrier by an amino acid carrier. Am J Physiol 262:R761-R765

Khademi S, O'Connell J 3rd, Remis J, Robles-Colmenares Y, Miercke LJ, Stroud RM (2004) Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 A. Science 305(5690):1587-94

King LS, Kozono D, Agre P (2004) From structure to disease: the evolving tale of aquaporin biology. Nat Rev Mol Cell Biol 5(9):687-698

Koh AS, Simmons-Willis TA, Pritchard JB, Grassl SM, Ballatori N (2002) Identification of a mechanism by which the methylmercury antidotes N-acetylcysteine and dimercaptopropanesulfonate enhance urinary metal excretion: transport by the renal organic anion transporter-1, Oat1. Mol Pharmacol 62:921-926

Kwong WT, Friello P, Semba RD (2004) Interactions between iron deficiency and lead poisoning: epidemiology and pathogenesis. Sci Total Environ 330(1-3):21-37

LeSage GD, Kost LJ, Barham SS, LaRusso NF (1986) Biliary excretion of iron from hepatocyte lysosomes in the rat. A major excretory pathway in experimental iron overload. J Clin Invest 77:90-99

Leslie EM, Haimeur A, Waalkes MP (2004) Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. J Biol Chem 279(31):32700-32708

Li ZS, Szczypka M, Lu YP, Thiele DJ, Rea PA (1996) The yeast cadmium factor protein (YCF1) is a vacuolar glutathione *S*-conjugate pump. J Biol Chem 271:6509-6517

Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, Rea PA (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc Natl Acad Sci USA 94:42-47

Liu W, Liang R, Ramamoorthy S, Fei YJ, Ganapathy ME, Hediger MA, Ganapathy V, Liebach FH (1995) Molecular cloning of PEPT2, a new member of the H^+/peptide cotransporter family, from human kidney. Biochim Biophys Acta 1235:461-466

Liu Z, Carbrey JM, Agre P, Rosen BP (2004) Arsenic trioxide uptake by human and rat aquaglyceroporins. Biochem Biophys Res Commun 316(4):1178-1185

Liuzzi JP, Cousins RJ (2004) Mammalian zinc transporters. Annu Rev Nutr 24:151-172

Lorico A, Bertola A, Baum C, Fodstad O, Rappa G (2002) Role of the Multidrug Resistance Protein 1 in protection from heavy metal oxyanions: investigations *in vitro* and in MRP1-deficient mice. Biochem Biophys Res Commun 291(3):617-622

Lungkaphin A, Chatsudthipong V, Evans KK, Groves CE, Wright SH, Dantzler WH (2004) Interaction of the metal chelator DMPS with OAT1 and OAT3 in intact isolated rabbit renal proximal tubules. Am J Physiol Renal Physiol 286(1):F68-F76

Mackenzie B, Hediger MA (2004) SLC11 family of H+-coupled metal-ion transporters NRAMP1 and DMT1. Pflugers Arch 447(5):571-579

Mack U, Powell LW, Halliday JW (1983) Detection and isolation of a hepatic membrane receptor for ferritin. J Biol Chem 258:4672-4675

Mackenzie B, Erickson JD (2004) Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. Pflugers Arch 447(5):784-795

Malandro MS, Kilberg MS (1996) Molecular biology of mammalian amino acid transporters. Ann Rev Biochem 65:305-336

Markovich D, Regeer RR, Kunzelmann K, Dawson PA (2005) Functional characterization and genomic organization of the human Na(+)-sulfate cotransporter hNaS2 gene (SLC13A4). Biochem Biophys Res Commun 326:729-734

Maxfield FR, McGraw TE (2004) Endocytic recycling. Nat Rev Mol Cell Biol 5(2):121-132

McKie AT, Barlow DJ (2004) The SLC40 basolateral iron transporter family (IREG1/ferroportin/MTP1). Pflugers Arch 447(5):801-806

McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 5:299-309

McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F, Simpson RJ (2001) An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 291:1755-1759

Miyajima H (2002) Genetic disorders affecting proteins of iron and copper metabolism: clinical implications. Intern Med 41(10):762-769

Mokrzan EM, Kerper LE, Ballatori N, Clarkson TW (1995) Methylmercury-thiol uptake into cultured brain capillary endothelial cells on amino acid system L. J Pharmacol Exp Ther 272(3):1277-1284

Morgan EH, Baker E (1986) Iron uptake and metabolism by hepatocytes. Fed Proc 45:2810-2816

Morgan EH, Baker E (1988) Role of transferrin receptors and endocytosis in iron uptake by hepatic and erythroid cells. Ann N Y Acad Sci 526:65-82

Morley CG, Bezkorovainy A (1985) Cellular iron uptake from transferrin: is endocytosis the only mechanism? Int J Biochem 17:553-564

Moseley RH, Ballatori N, Murphy SM (1988) Na^+-Glycine cotransport in canalicular liver plasma membrane vesicles. Am J Physiol 255:G253-G259

Mousavi SA, Malerod L, Berg T, Kjeken R (2004) Clathrin-dependent endocytosis. Biochem J 377:1-16

Murer H, Forster I, Biber J (2004) The sodium phosphate cotransporter family SLC34. Pflugers Arch 447:763-767

Nichols B (2003) Caveosomes and endocytosis of lipid rafts. J Cell Sci 116(Pt 23):4707-4714

Nittis T, Gitlin JD (2002) The copper-iron connection: hereditary aceruloplasminemia. Semin Hematol 39(4):282-289

Oakley F, Horn NM, Thomas AL (2004) Histidine-stimulated divalent metal uptake in human erythrocytes and in the erythroleukaemic cell line HEL.92.1.7. J Physiol 561(Pt 2):525-534

Oka JA, Weigel PH (1983) Recycling of the asialoglycoprotein receptor in isolated rat hepatocytes. Dissociation of internalized ligand from receptor occurs in two kinetically and thermally distinguishable compartments. J Biol Chem 258:10253-10262

Oka JA, Christensen MD, Weigel PH (1989) Hyperosmolarity inhibits galactosyl receptor-mediated but not fluid phase endocytosis in isolated rat hepatocytes. J Biol Chem 264:12016-12024

Okubo M, Yamada K, Hosoyamada M, Shibasaki T, Endou H (2003) Cadmium transport by human Nramp 2 expressed in *Xenopus laevis* oocytes. Toxicol Appl Pharmacol 187(3):162-167

Olivi L, Sisk J, Bressler J (2001) Involvement of DMT1 in uptake of Cd in MDCK cells: role of protein kinase C. Am J Physiol Cell Physiol 281(3):C793-C800

Osterloh K, Aisen P (1989) Pathways in the binding and uptake of ferritin by hepatocytes. Biochim Biophys Acta 1011:40-45

Palacin M, Kanai Y (2004) The ancillary proteins of HATs: SLC3 family of amino acid transporters. Pflugers Arch 447:490-494

Palmiter RD, Huang L (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch 447(5):744-751

Park JD, Cherrington NJ, Klaassen CD (2002) Intestinal absorption of cadmium is associated with divalent metal transporter 1 in rats. Toxicol Sci 68(2):288-294

Pavoine C, Lotersztajn S, Mallat A, Pecker F (1987) The high affinity (Ca2+-Mg2+)-ATPase in liver plasma membranes is a Ca2+ pump. Reconstitution of the purified enzyme into phospholipid vesicles. J Biol Chem 262:5113-5117

Petris MJ (2004) The SLC31 (Ctr) copper transporter family. Pflugers Arch 447(5):752-755

Pham TN, Segui JA, Fortin C, Campbell PG, Denizeau F, Jumarie C (2004) Cadmium uptake in rat hepatocytes in relation to speciation and to complexation with metallothionein and albumin. J Cell Physiol 201(2):320-330

Picard V, Govoni G, Jabado N, Gros P (2000) Nramp 2 (DCT1/DMT1) expressed at the plasma membrane transports iron and other divalent cations into a calcein-accessible cytoplasmic pool. J Biol Chem 275(46):35738-35745

Pietrangelo A (2004) The ferroportin disease. Blood Cells Mol Dis 32(1):131-138

Pighin JA, Zheng H, Balakshin LJ, Goodman IP, Western TL, Jetter R, Kunst L, Samuels AL (2004) Plant cuticular lipid export requires an ABC transporter. Science 306(5696):702-704

Pratt RD, Ferreira GC, Pedersen PL (1991) Mitochondrial phosphate transport. Import of the H+/Pi symporter and role of the presequence. J Biol Chem 266:1276-1280

Ramm GA, Powell LW, Halliday JW (1994) Pathways of intracellular trafficking and release of ferritin by the liver *in vivo*: the effect of chloroquine and cytochalasin D. Hepatology 19:504-513

Regoeczi E, Chindemi PA (1995) Translocation of different forms of transferrin from blood to bile in the rat. Hepatology 21:1063-1069

Regoeczi E, Chindemi PA, Debanne MT (1984) Partial resialylation of human asialotransferrin types 1 and 2 in the rat. Can J Biochem & Cell Biol 62:853-858

Reuter H (1983) Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 301:569-574

Rolfs A, Hediger MA (2001) Intestinal metal ion absorption: an update. Curr Opin Gastroenterol 17(2):177-183

Rudolph JR, Regoeczi E, Chindemi PA, Debanne MT (1986) Preferential hepatic uptake of iron from rat asialotransferrin: possible engagement of two receptors. Am J Physiol 251:G398-G404

Ryu DY, Lee SJ, Park DW, Choi BS, Klaassen CD, Park JD (2004) Dietary iron regulates intestinal cadmium absorption through iron transporters in rats. Toxicol Lett 152(1):19-25

Sacher A, Cohen A, Nelson N (2001) Properties of the mammalian and yeast metal-ion transporters DCT1 and Smf1p expressed in *Xenopus laevis* oocytes. J Exp Biol 204(Pt 6):1053-1061

Safaei R, Howell SB (2005) Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs. Crit Rev Oncol Hematol 53:13-23

Sands JM (2004) Renal urea transporters. Curr Opin Nephrol Hypertens 13(5):525-532

Schaefer M, Gitlin JD (1999) Genetic disorders of membrane transport. IV. Wilson's disease and Menkes disease. Am J Physiol 276(2 Pt 1):G311-G314

Schmitz G, Kaminski WE, Orso E (2000) ABC transporters in cellular lipid trafficking. Curr Opin Lipidol 11(5):493-501

Schuster VL (2002) Prostaglandin transport. Prostaglandins Other Lipid Mediat 68-69:633-647

Scharschmidt BF, Lake JR, Renner EL, Licko V, Van Dyke RW (1986) Fluid phase endocytosis by cultured rat hepatocytes and perfused rat liver: implications for plasma membrane turnover and vesicular trafficking of fluid phase markers. Proc Natl Acad Sci USA 83:9488-9492

Shachar-Hill B, Hill AE (2002) Paracellular fluid transport by epithelia. Int Rev Cytol 215:319-350

Sharma V, Crankshaw CL, Piwnica-Worms D (1996) Effects of multidrug resistance (MDR1)P-glycoprotein expression levels and coordination metal on the cytotoxic potency of multidentate (N4O2) (ethylenediamine)bis[propyl(R-benzylimino)]metal(III) cations. J Med Chem 39:3483-3490

Shayakul C, Hediger MA (2004) The SLC14 gene family of urea transporters. Pflugers Arch 447(5):603-609

Simons TJ, Pocock G (1987) Lead enters bovine adrenal medullary cells through calcium channels. J Neurochem 48:383-389

Simmons TW, Hinchman CA, Ballatori N (1991) Polarity of hepatic glutathione and glutathione *S*-conjugate efflux, and intraorgan mercapturic acid formation in the skate. Biochem Pharmacol 42:2221-2228

Simmons TW, Anders MW, Ballatori N (1992) Cysteine and *S*-(1,2-dichlorovinyl)-L-cysteine transport in rat liver canalicular membrane vesicles: Potential reabsorption

mechanisms for biliary metabolites of glutathione and its *S*-conjugates. J Pharmacol Exp Ther 262:1182-1188

Simmons-Willis TA, Koh AS, Clarkson TW, Ballatori N (2002) Transport of a neurotoxicant by molecular mimicry: the methylmercury-L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2. Biochem J 367(Pt 1):239-246

Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lugt NMT, van Roon MA, van der Valk MA, Berns AJM, Borst P (1993) Homogeneous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 75:451-462

Smith DE, Johanson CE, Keep RF (2004) Peptide and peptide analog transport systems at the blood-CSF barrier. Adv Drug Deliv Rev 56(12):1765-1791

Stahl A (2004) A current review of fatty acid transport proteins (SLC27). Pflugers Arch 447(5):722-727

Stockert RJ, Haimes HB, Morell AG, Novikoff PM, Novikoff AB, Quintana N, Sternlieb I (1980) Endocytosis of asialoglycoprotein-enzyme conjugates by hepatocytes. Lab Invest 43:556-563

Sugawara N, Lai YR, Arizono K, Kitajima T, Inoue H (1997) Lack of biliary excretion of Cd linked to an inherent defect of the canalicular isoform of multidrug resistance protein (cMrp) does not abnormally stimulate accumulation of Cd in the *Eisai hyperbilirubinemic* (EHB) rat liver. Archives Toxicol 71:336-339

Supek F, Supekova L, Nelson H, Nelson N (1996) A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. Pro Natl Acad Sci USA 93:5105-5110

Supek F, Supekova L, Nelson H, Nelson N (1997) Function of metal-ion homeostasis in the cell division cycle, mitochondrial protein processing, sensitivity to mycobacterial infection and brain function. J Exp Biol 200:321-30

Suzuki T, Kawakita M (1993) Uncoupling of ATP splitting from Ca(2+)-transport in Ca(2+)-transporting ATPase of the sarcoplasmic reticulum as a result of modification by N-(3-pyrene)maleimide: activation of a channel with a specificity for alkaline earth metal ions. J Biochem 114:203-209

Szczypka MS, Wemmie JA, Moye-Rowley WS, Thiele DJ (1994) A yeast metal resistance protein similar to human cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance-associated protein. J Biol Chem 269:22853-22857

Szymkiewicz I, Shupliakov O, Dikic I (2004) Cargo- and compartment-selective endocytic scaffold proteins. Biochem J 383(Pt 1):1-11

Tallkvist J, Bowlus CL, Lonnerdal B (2001) DMT1 gene expression and cadmium absorption in human absorptive enterocytes. Toxicol Lett 122(2):171-177

Tallkvist J, Bowlus CL, Lonnerdal B (2003) Effect of iron treatment on nickel absorption and gene expression of the divalent metal transporter (DMT1) by human intestinal Caco-2 cells. Pharmacol Toxicol 92(3):121-124

Tannert A, Pohl A, Pomorski T, Herrmann A (2003) Protein-mediated transbilayer movement of lipids in eukaryotes and prokaryotes: the relevance of ABC transporters. Int J Antimicrob Agents 22(3):177-187

Tavassoli M (1988) The role of liver endothelium in the transfer of iron from transferrin to the hepatocyte. Annals NY Acad Sci 526:83-92

Templeton DM (1990) Cadmium uptake by cells of renal origin. J Biol Chem 265(35):21764-21770

Terada T, Inui K (2004) Peptide transporters: structure, function, regulation and application for drug delivery. Curr Drug Metab 5(1):85-94

Thorstensen K, Romslo I (1988) Uptake of iron from transferrin by isolated rat hepatocytes. A redox-mediated plasma membrane process? J Biol Chem 263, 8844-8850

Thorstensen K, Trinder D, Zak O, Aisen P (1995) Uptake of iron from N-terminal half-transferrin by isolated rat hepatocytes. Evidence of transferrin-receptor-independent iron uptake. Eur J Biochem 232:129-133

Tibaduiza EC, Bobilya DJ (1996) Zinc transport across an endothelium includes vesicular cotransport with albumin. J Cellular Physiol 167:539-547

Tommasini R, Evers R, Vogt E, Mornet C, Zaman GJ, Schinkel AH, Borst P, Martinoia E (1996) The human multidrug resistance-associated protein functionally complements the yeast cadmium resistance factor 1. Pro Natl Acad Sci USA 93:6743-6748

van Veen HW, Abee T, Kortstee GJ, Konings WN, Zehnder AJ (1994) Translocation of metal phosphate via the phosphate inorganic transport system of *Escherichia coli*. Biochem 33:1766-1770

Verry F (2004) System L: heteromeric exchangers of large, neutral amino acids involved in directional transport. Pflugers Arch 445(5):529-533

Verry F, Closs EI, Wagner CA, Palacin M, Endou H, Kanai Y (2004) CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch 447(5):532-542

Voskoboinik I, Camakaris J (2002) Menkes copper-translocating P-type ATPase (ATP7A): biochemical and cell biology properties, and role in Menkes disease. J Bioenerg Biomembr 34:363-371

Vulpe CD, Packman S (1995) Cellular copper transport. Ann Rev Nutr 15:293-322

Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat Genet 3:7-13

Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat Genet 21:195-199

Wemmie JA, Szczypka MS, Thiele DJ, Moye-Rowley WS (1994) Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. J Biol Chem 269:32592-32597

Wetterhahn-Jennette K (1981) The role of metals in carcinogenesis: Biochemistry and metabolism. Environ Health Perspect 40:233-252

Wang W, Seward DJ, Li L, Boyer JL, Ballatori N (2001) Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate. Proc Natl Acad Sci USA 98:9431-9436

Wijmenga C, Klomp LW (2004) Molecular regulation of copper excretion in the liver. Proc Nutr Soc 63:31-39

Wu LG (2004) Kinetic regulation of vesicle endocytosis at synapses. Trends Neurosci 27:548-554

Wu LJ, Leenders AG, Cooperman S, Meyron-Holtz E, Smith S, Land W, Tsai RY, Berger UV, Sheng ZH, Rouault TA (2004) Expression of the iron transporter ferroportin in synaptic vesicles and the blood-brain barrier. Brain Res 1001(1-2):108-117

Yamaguchi Y, Heiny ME, Suzuki M, Gitlin JD (1996) Biochemical characterization and intracellular localization of the Menkes disease protein. Proc Natl Acad Sci USA 93:14030-14035

Young SP, Bomford AB, Williams R (1983) Dual pathways for the uptake of rat asialotransferrin by rat hepatocytes. J Biol Chem 258:4972-4976

Yu L, Gupta S, Xu F, Liverman AD, Moschetta A, Mangelsdorf DJ, Repa JJ, Hobbs HH, Cohen JC (2004) Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. J Biol Chem (in press)

Zaman GJR, Lankelma J, van Tellingen O, Beijnen J, Bekker H, Paulusma C, Oude Elferink RPJ, Baas F, Borst P (1995) Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. Proc Natl Acad Sci USA 92:7690-7694

Zhou B, Gitschier J (1997) hCTR1: a human gene for copper uptake identified by complementation in yeast. Proc Natl Acad Sci USA 94:7481-7486

Ballatori, Nazzareno
Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine, 575 Elmwood Avenue, Rochester, NY 14642
Ned_Ballatori@urmc.rochester.edu

Madejczyk, Michael S.
Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine, 575 Elmwood Avenue, Rochester, NY 14642

Metals in biology: past, present, and future

Barry P. Rosen

"For now we see through a glass, darkly; but then face to face: now I know in part; but then shall I know even as also I am known."
I Cor. 13:12.

Abstract

This chapter reviews basic concepts in metal biology and suggests a vision for the future of metals in medicine. Important developments in the field include the discovery of metallochaperones that prevent free metals from reeking havoc inside of cells. These intracellular metal ion carriers may work in conjunction with scaffold proteins or may deliver their cargo directly to metalloenzymes or metal transport proteins. Another area reviewed is the mechanism of metalloid uptake and detoxification. This leads into the future of metals in medicine, using examples from past and recent history.

1 Introduction

This chapter will review recent concepts and developments in the field of metal biology. With scientific knowledge accumulating at an accelerating pace, we have learned so much over the past decade that only the highlights can be presented here. Fortunately, the details of many of these concepts have been recounted in previous chapters, allowing this chapter to focus on a limited number of important developments.

2 Concepts and developments

2.1 Chaperones and scaffolds

Over the last decade, it has become clear that most transition and heavy metal ions do not exist as free ions in the cytosol but are instead sequestered by a variety of proteins variously called metal ion chaperones, scaffolds, or intracellular carriers (Field et al. 2002). There may be fine distinctions between these terms, but, for the purposes of this review, they will be considered in the group of metal chaperones.

Topics in Current Genetics, Vol. 14
M.J. Tamás, E. Martinoia (Eds.): Molecular Biology of Metal Homeostasis and Detoxification
DOI 10.1007/4735_94 / Published online: 26 July 2005

2.1.1 Copper chaperones

The biology and chemistry of these metal chaperones have been described in detail in Chapters 2 and 5. The first copper chaperone, *Saccharomyces cerevisiae* Atx1p (for antioxidant protein), was identified nearly a decade ago (Culotta et al. 1995). Although the 73-residue Atx1p was originally identified as a suppressor of SOD1 (superoxide dismutase) mutants, its physiological role is as an intracellular Cu(I) chaperone for Ccc2p, a golgi-related Cu(I)-translocating ATPase. Ccc2p delivers copper to the multicopper oxidase Fet3p, which is involved in high affinity iron uptake (Lin et al. 1997). As a result, mutations in Atx1p have an iron deficiency phenotype that can be rescued by increased medium copper. Similarly, its human homologue ATOX1 (HAH1) is a chaperone for the Menkes protein (ATP7A), which is an intestinal copper uptake pump, and for the Wilson protein (ATP7B), which is a liver copper pump for extrusion of excess copper into bile.

A second copper chaperone, the 69-residue yeast protein Cox17p (63-residue COX17 in humans) was identified as the copper delivery protein for mitochondrial cytochrome oxidase (Glerum et al. 1996). A third copper chaperone for superoxide dismutase was subsequently identified as the *LYS7* gene product, the 249-residue Ccs1p (Culotta et al. 1997). The *lys7Δ* strain cannot insert copper into SOD, but otherwise the phenotype is relatively mild, with a requirement for lysine and methionine during aerobic growth. Thus, the copper chaperone is not essential for aerobic growth of yeast. In humans, genetic defects in SOD result in some forms of familial amyotrophic lateral sclerosis (ALS), so the physiological role of the mammalian homologue, CCS, is of considerable interest. A mouse knockout is more sensitive to paraquat and has reduced levels of active SOD (Wong et al. 2000), but the involvement of CCS in ALS is unclear and controversial.

One concept that comes out of these studies is that copper chaperones are ubiquitous, but another concept is that they are not essential for growth, especially for unicellular organisms. In higher organisms such as humans, their biological roles may become apparent during development or later in life. A third concept, and perhaps the most important, is the physical complementarity between the copper chaperones and their partner proteins. This is best illustrated by the interaction of Ccs1p and Sod1p. The structures of Ccs1p and the interacting domain of Sod1p are so similar that they interact with each other as if they were two subunits of a homodimer (Lamb et al. 1999). Atx1p is likewise a structural homologue of the N-terminal metal binding domain of its partner, the Ccc2p copper pump (Rosenzweig et al. 1999). This metal binding domain is widespread and has evolved the ability to bind other metals such as mercury and nickel, as described below.

2.1.2 MerP and MerA, mercury binding proteins

Mercury resistance (*mer*) operons are widespread (Barkay et al. 2003). MerP is a 72-residue periplasmic protein of uncertain function. The structure of MerP has been determined and has the βαββαβ fold characteristic of copper chaperones but binds Hg(II) rather than Cu(I) (Serre et al. 2004). Central to resistance is MerA, an

NADPH-linked mercuric reductase that reduces Hg(II) to volatile Hg(0) (Schiering et al. 1991). The core of MerA is homologous to members of the pyridine nucleotide disulfide oxidoreductase family, including enzymes such as glutathione reductase. The N-terminal residues is homologous to MerP and to copper chaperones but, like MerP, binds Hg(II) rather than Cu(I) (Steele and Opella 1997). Thus, while neither MerP nor MerA are metal chaperones, they are evolutionarily related to each other and to other metal binding proteins. This illustrates that concept that metal binding domains can spread through genomes, using the same protein ligands to evolve different specificity and consequently new functions.

2.1.3 UreE, a nickel chaperone

Urease is a nickel-containing enzyme that converts urea to nitrogen and carbonate. It is present in a number of bacterial species including *Klebsiella aerogenes*. Urease has a binuclear metal center with two Ni(II) bridged by a carbamylated lysine residue (Park and Hausinger 1995). UreE is a Ni(II) chaperone or scaffold protein that aids in the assembly of the metal center in urease. The 1.5 Å structure of a C-terminally deleted UreE from *K. aerogenes* has been reported (Song et al. 2001). UreE is a two-domain protein. The N-terminal domain is similar to Hsp-70, while the C-terminal domain is related to the copper chaperone Atx1p. However, UreE does not contain the cysteine rich copper binding motif of Atx1p and appears to bind metal with a histidine-rich motif. This extends the concept that proteins can modify their metal binding sites to change selectivity. While MerP modified the use of the same protein ligands as a copper site to bind a different metal, the homologous domain in UreE has evolved an entirely new binding site for nickel using different ligands.

2.1.4 NarJ, a molybdenum chaperone

Nitrate reductase from *E. coli* uses a cofactor molybdopterin guanine dinucleotide (Mo-(bis-MGD)) (Blasco et al. 1998). NarJ is a 236-residue Mo chaperon or scaffold protein that is required for assembly of the Mo cofactor in nitrate reductase (Vergnes et al. 2004). An unrelated protein, TorD, has been proposed to be a molybdenum chaperone for trimethylamine N-oxide reductase enzyme (TorA) in *E. coli* (Pommier et al. 1998).

2.1.5 ArsD, an arsenic chaperone

Resistance to arsenic and antimony is widely spread in both gram-positive and gram-negative bacteria (Rosen 2002). The best-characterized system encoded by plasmid R773 in *E. coli* confers resistance to arsenite [As(III)], arsenate [As(V)], and antimonite [Sb(III)]. The *arsRDABC* operon of R773 codes for five proteins. ArsR is an As(III)-responsive transcriptional regulator. Resistance correlates with active extrusion of arsenite from the cell by a primary pump, the ArsAB As(III)-translocating ATPase, as discussed in more detail below. Arsenate resistance re-

quires expression of a third structural gene for ArsC, which reduces arsenate to the substrate of the ArsAB pump (Mukhopadhyay and Rosen 2002).

We have shown that the fifth protein, the 120-residue ArsD, has weak transcriptional regulatory properties (Chen and Rosen 1997). Our most recent data suggest that ArsD is a metalloid chaperone that delivers As(III) to the ArsAB As(III)-translocating ATPase (YF Lin, AR Walmsley, and BP Rosen, manuscript in preparation). The initial motivation for this conjecture was a genomics analysis of *ars* operons that contain an *arsD* gene (Rosen 1999). As mentioned, every sequenced bacterial genome contains an *ars* operon. The overwhelming majority are three-gene *arsRBC* operons. The *arsD* gene is found in only a small number of operons, fourteen to date. Strikingly, every *arsD* gene precedes an *arsA* gene. Most are five-gene *arsRDABC* operons, but, in some, *arsDA* follows the *arsRBC* genes, and in the archaea *H. halobium*, *arsDA* is transcribed divergently from *arsRC*. We infer 1) ArsD and ArsA co-evolved, 2) the *arsDA* genes moved laterally into operons as a unit, and 3) ArsD has a biochemical function specifically related to ArsA. Four lines of evidence support this hypothesis. First, by yeast two-hybrid analysis, ArsD and ArsA interact *in vivo*. Second, purified ArsD and ArsA can be chemically crosslinked in a 1:1 complex through their metal binding sites. Third, the rate of dissociation of metalloid from ArsD is enhanced 10^4-fold by ArsA, consistent with transfer of As(III) from the chaperone to the ATPase. Fourth, and most important, ArsD increases the affinity for As(III) of ArsA but does not change the V_{max}, which makes the enzyme more effective at low concentrations of metalloid, a property expected for a metallochaperone. We propose that ArsD and ArsB both bind to the same site on ArsA sequentially in a cycle of metal transfer from ArsD to ArsA to ArsB concomitant with ATP binding and then hydrolysis by ArsA.

2.1.6 Frataxin and Isu, iron chaperone and scaffold

Assembly of iron-sulfur clusters requires a series of proteins that serve as chaperones, scaffolds, and sulfur donors (Mansy and Cowan 2004). In bacteria, iron-sulfur cluster (ISC) assembly takes place in the cytosol. The related pathway in eukaryotes takes place in the mitochondrion, although the proteins are nuclear-encoded. The yeast mitochondrial system is the best characterized (Balk and Lill 2004). Frataxin is 174-residue a mitochondrial protein that functions as a chaperone to insert Fe into the Isu scaffold protein (Muhlenhoff et al. 2002; Mansy and Cowan 2004). Mutation in human frataxin results in Friedreich's ataxia, a human autosomal recessive neurodegenerative disease (Alper and Narayanan 2003). There are two *ISU* genes in yeast, encoding Isu1p and Isu2p, both of which are homologues of bacterial IscU. The Isu scaffold is a dimer of two Isu polypeptides (Gerber et al. 2004). It can be either a homodimer of either or a heterodimer composed of Isu1p and Isu2p. Consequently, deletion of either *ISU* gene has little effect on iron-sulfur center assembly, but the double deletion is lethal. To complete ISC assembly, inorganic sulfur is donated by the cysteine desulfurase Nfs1p (IscS in bacteria) (Strain et al. 1998). Interestingly, this makes ISC assembly the only essential role for the mitochondrion in yeast.

2.2 Transporters for assembled metal complexes

2.2.1 The ABC7 transporter

How do proteins with assembled metal complexes reach the compartments in which they function? Although many metal ion transporters are known, only a few systems for the transport of assembled complexes have been identified. One such transporter is Atm1p (ABC7 in humans), which encodes a mitochondrial ABC ATPase (Kispal et al. 1999). An *ATM1* deletion strain exhibits an oxidative stress-related phenotype and a 30-fold increase in free mitochondrial iron levels (Kispal et al. 1997). Mutations in human ABC7 result in X-linked sideroblastic anemia and ataxia, which are also associated with increased mitochondrial accumulation of iron (Csere et al. 1998; Allikmets et al. 1999). Atm1p is localized in the inner mitochondrial membrane with the nucleotide binding domains on the matrix side, suggesting that it pumps its substrate from the matrix to the cytosol. Atm1p was shown to be required for the assembly of cytosolic iron-sulfur cluster proteins. Most proteins with iron-sulfur clusters are mitochondrial, but there are some cytosolic ISC proteins. For example, aconitase, an enzyme of the citric acid cycle that converts citrate to isocitrate, is an ISC protein. Mammalian cytosolic aconitase is a bifunctional protein that can be converted to the iron regulated transcription factor IRP1 by disassembly of the ICS, signaling a need for iron. IRP1 binds to and stabilizes the mRNA of iron proteins such as the iron storage protein ferritin and the iron delivery protein transferritin (Eisenstein 2000).

The 690-residue Atm1p is a half-sized ABC transporter, with one nucleotide binding domain and one set of membrane spanning segments. It most likely functions as a homodimer, but isolation of the functional complex has not been reported. The C-terminal soluble portion of the protein containing the nucleotide binding domain has been expressed and shown to exhibit ATPase activity (Chen and Cowan 2003). When expressed in high levels, another mitochondrial ABC transporter, Mdl1p, can suppress deletion of *ATM1*, which may explain why the *ATM1* deletion is not lethal (Chloupkova et al. 2003). Mdl1p transports mitochondrial peptides generated by AAA proteases, so it is tempting to suggest that it can transport the FeS-containing substrate of Atm1p.

What does Atm1p do? It must be moving assembled irons-sulfur clusters from the mitochondrial matrix to the cytosol. It is clearly an ABC pump, and members of this family are capable of transporting a wide variety of substrates, from small molecules such as ions, amino acids and sugars, to peptides and large proteins (Locher 2004). However, ABC transporters are not known to transport folded proteins, so the possibility that it moves a protein with an assembled iron sulfur cluster is remote. Could it transport a free ISC in analogy to the BtuCD ABC pump, which transports cobalamin, the 1350 Da vitamin B_{12}? However, the cobalt-containing, heme-like corrin ring system is stable, whereas an ISC would be labile in the absence of a scaffold. Perhaps a relatively small scaffold such as a cysteine-rich peptide might serve as the transport substrate of members of the ABC7 family.

2.2.2 The TAT system

How do proteins with assemble metal centers get from their site of synthesis to their site of function, which usually requires movement across a membrane? Many exported and integral membrane proteins are translocated across membranes in an unfolded state. The SecA ATPase motor and SecYEG pore of bacteria and the analogous complexes in archaea and in the eukaryotic endoplasmic reticulum recognize an N-terminal signal sequence, which is the starting point for threading the polypeptide through the translocase pore (Veenendaal et al. 2004). The signal sequence is subsequently cleaved, and the protein is refolded. The pore has a diameter of only 5-8 Å, which is sufficient for translocating the polypeptide chain, and perhaps even an α-helix, but not a folded protein with an assembled metal center (Clemons et al. 2004).

A second major pathway for protein translocation in bacteria and the thylocoid membrane of chloroplasts is the TAT (twin-arginine translocase) system (Berks et al. 2003). TAT refers to the twin-arginine motif (S<u>RR</u>XFLK) usually found in the C-terminal region of the translocated substrate. This translocase is quite different from the SecAYEG system in that it can transport entire folded proteins, including those with assembled cofactors such as iron-sulfur centers and molybdopterin cofactors. It is energized by the electrochemical proton gradient, with no ATP hydrolysis involved. There are four polypeptides in the complex, TatA, B, C, and E. TatA is the major component in terms of mass, forming a ring of α-helices. TatE is homologous to TatA and has an overlapping function, although there is 50-100 fold more TatA than TatE. TatB is also related to TatA and TatE but has a separate function. The ratio of TatA to TatB is 20:1. Each TatA, B and E polypeptide has a single membrane-spanning α-helix. TatC is a polytopic membrane protein with four to six α-helices and unrelated to the other three Tat proteins. It may be the initial binding site for Tat substrates. Some bacteria have TatD, which is a soluble nuclease not obligatory to Tat function. The structure of the complex is not known, but, since TatA is present in many more copies than the other proteins, it must be the principal pore component. The pore has been visualized by negative stain electron microscopy (Sargent et al. 2001). It has an inner diameter of 70 Å, which is just large enough to allow diffusion of folded proteins. The mechanism of translocation is as yet unknown, but obviously the pore must be gated to prevent the contents of the cell from leaking out and must have a means to discriminate against non-Tat substrates.

2.3 Pathways of metalloid uptake and detoxification

Another important biological concept centers on the relationship of metal uptake to sensitivity and efflux to detoxification. Of particular relevance are the types and mechanisms of transporters of ions of the heavy metals, transition metals, and metalloids such as Cu(I), Ag(I), Zn(II), Cd(II), Pb(II), Co(II), As(III), and Sb(III) (Wong et al. 2004). This includes both uptake and efflux of metals that are essential, toxic, or both. This section will focus on transport systems that transport the

metalloids arsenic and antimony, toxic elements with no known biological function.

Arsenic and antimony straddle the metallic series. They are semi-metals or metalloids, with two biologically important oxidation states, pentavalent (V), and trivalent (III). In their metallic forms, they are chemically soft Lewis acids, as opposed to the hard Lewis acids of Groups I and II elements such as Na^{+} and Ca^{2+}. Hard Lewis acids bind to proteins through relatively weak ionic interactions with hard Lewis bases such as the carboxyl oxygens of glutamate or aspartate residues. In contrast, soft Lewis acids (or simply soft metal ions) form strong bonds with soft Lewis bases such as the thiolates of cysteine residues and the imidazolium nitrogens of histidine residues. These nearly covalent interactions with cysteines and histidines in proteins account for much of the biological properties and toxicity of the trivalent metalloids. There apparently is no single target for As(III), which has been shown to inactivate up to 200 enzymes, including mitochondrial energy generating pathways and enzymes involved in DNA synthesis and repair (Ratnaike 2003).

Maintaining suitable intracellular concentrations of essential metals while excluding toxic metals such as arsenic and antimony was one of the earliest challenges of the first cells. This ancient environmental challenge could have been the driving force for the evolution of mechanisms for metal ion homeostasis and detoxification. The presence of arsenic resistance (*ars*) genes in the genome of every living organism sequenced to date illustrates first that *ars* genes must be ancient and second that arsenic must still be ubiquitous in the environment, providing the selective pressure that maintains them in present-day organisms. Today arsenic enters the ecosphere through both geochemical and anthropogenic sources. For example, according to the United States Geological Survey of arsenic in ground water of the United States (http://co.water.usgs.gov/trace/arsenic/), parts of the Midwest and Northeast have arsenic concentrations that exceed 10 µg/L, the World Health Organization's (WHO) provisional guideline arsenic in drinking water. In many places the concentration of arsenic in water supplies exceeds 50 µg/L, the present United States Environmental Protection Agency (EPA) maximum allowable limit (MCL). After considerable political (but not scientific) debate, the EPA will decrease the MCL to 10 µg/L in 2006. In other parts of the world, arsenic levels in the water supplies have reached crisis levels, but nowhere is it more severe than West Bengal and Bangladesh, where 46 million people drink arsenic-contaminated well water that, in some cases, contains 1000-fold more arsenic than the WHO limit (Alam et al. 2002). WHO initiated the use of deep tube wells in West Bengal and Bangladesh to prevent the spread of infectious diseases such as cholera, which contaminated the surface sources of drinking water. Thus, the distinction between natural and anthropogenic exposure to arsenic is blurred by human intervention in environmental crises. Another example of an ecosystem contaminated with concentrations of arsenic as high as 380 µg/ml is the River Tinto (Huelva, Southwestern Spain), which stems from the effluent of an ancient Roman mine in the Iberian Pyritic Belt (Lopez-Archilla et al. 2001). Despite these extreme conditions, the River Tinto supports a high degree of microbial diversity,

including an *Aspergillus* species that is highly resistant to arsenic (Canovas et al. 2003).

There are numerous modern anthropogenic sources of arsenic. Arsenic is the sole active ingredient of a number of herbicides such as Ortho Crabgrass Killer Formula II (8.4% methylarsonate) and Atochem Desiccant L-10 (used for harvesting cotton – 75% arsenic acid). In addition, organic arsenicals are used as feed supplements for turkeys and pigs. Chromated copper arsenate (CCA) is a major source of arsenic around homes and schools, since most wooden decks and playground equipment are soaked in this wood preservative. A Brake fern (*Pteris vittata*) that hyper-accumulates arsenic was isolated from a CCA-contaminated site in central Florida (Ma et al. 2001).

2.3.1 Trivalent metalloid efflux systems

With the ubiquitous presence of arsenic in both soil and water, it is little wonder that in every organism examined there are transport systems that detoxify As(III) and Sb(III) by catalyzing removal from the cytosol (Rosen 2002). The best-characterized prokaryotic system is the ArsAB ATP-coupled As(III)/Sb(III) efflux pump. The 583-residue ArsA ATPase is a member of a family of ATPases that probably arose from GTPases. ArsA binds to ArsB (Dey et al. 1994), which is the ion-conducting subunit of the pump. ArsB is a 45-kDa integral membrane protein that spans the inner membrane 12 times (Wu et al. 1992). ArsB has a novel dual mode of energy coupling depending on its association with ArsA (Dey and Rosen 1995). Arsenic efflux in bacteria can be catalyzed by either ArsB alone functioning as a secondary transporter or by the ArsAB complex, functioning as a transport ATPase. In fact, *E. coli* can utilize either mode physiologically. First, it has a chromosomal *arsRBC* operon that allows it to catalyze translocation of trivalent metalloids as a secondary carrier protein (Carlin et al. 1995). Second, there are a variety of plasmids such as R773 and R46 that carry *arsRDABC* operons, allowing *E. coli* to use ATP for arsenite extrusion (Rosen 1999). This illustrates an important concept: the difference between the two modes of energy coupling is efficiency. A secondary carrier in which transport of an ion is coupled to a membrane potential of –180 mV can form a maximum concentration gradient of 10^3. Thus, if the concentration of As(III) outside the cells is 1 mM, the intracellular concentration cannot be reduced to less than 1 μM. In contrast, an ATP-coupled pump is more efficient, capable of concentration gradients as high as 10^6, equivalent to a concentration of 1 nM intracellular As(III) at 1 mM external metalloid concentration. This may explain why cells expressing the plasmid *arsRDABC* operon are considerably more metalloid resistant than cells expressing only the chromosomal *arsRBC* operon (Dey and Rosen 1995). A second concept that comes out of these studies is that primary pumps such as the ArsAB ATPase, the F_0F_1 ATPase, and ABC ATPases most likely evolved from the union of soluble ATPases and secondary transporters as a matter of efficiency (Rosen et al. 1992).

In contrast to ArsA, where activation results from the reaction of the soft metal As(III) with cysteine thiolates, ArsB contains only a single cysteine residue that can be altered without effect on resistance or transport (Chen et al. 1996). Thus, its

catalytic mechanism cannot involve thiol chemistry, and ArsB is most likely a true carrier protein. ArsB is an antiporter that catalyzes exchange of a neutral trivalent metalloid species for a proton (Meng et al. 2004). The true ArsB substrate is likely a six-membered ring composed of an oxo-bridged trimer of arsenic trioxide, as discussed in more detail below. A third concept from these studies is that a transport system such as the Ars pump can simultaneously use two distinct types of metalloid chemistry: 1) soft metal binding of the trivalent metalloid to the ArsA subunit and 2) transport of a neutral nonmetallic species through the membrane catalyzed by the ArsB subunit.

In eukaryotes, arsenite resistance is conferred by two types of efflux proteins. Acr3p is a secondary carrier that extrudes As(III) and Sb(III) from cells of *Saccharomyces cerevisiae* (Wysocki et al. 1997; Ghosh et al. 1999). The *ACR3* gene is part of a three-gene cluster (Bobrowicz et al. 1997), along with *ACR1*, which encodes an As(III)-responsive transcription factor (Bobrowicz and Ulaszewski 1998) (also called *YAP8* (Wysocki et al. 2004)) and *ACR2*, which encodes an arsenate reductase (Mukhopadhyay and Rosen 1998; Mukhopadhyay et al. 2000). The *ACR* genes are not widespread and are not found in higher eukaryotes. Rather, the closest Acr3p homologues are found in archaea and prokaryotes, for example, the SKIN element arsenite carrier protein that confers arsenite resistance in *B. subtilis* (Sato and Kobayashi 1998). In most eukaryotes, As(III)/Sb(III) efflux is catalyzed by members of the MRP (multidrug resistance-associated protein) group of the ABC superfamily of transport ATPases (Haimeur et al. 2004). In *S. cerevisiae*, the MRP homologue Ycf1p catalyzes ATP-dependent pumping of glutathione conjugates of heavy metal ions and metalloids, including Cd(II) (Li et al. 1996), As(III) and Sb(III) (Ghosh et al. 1999) into the vacuole. In the eukaryotic protozoan *Leishmania*, the MRP family member PgpA transports $As(GS)_3$ into intracellular vesicles (Legare et al. 2001). In human liver, a major route of arsenic detoxification is MRP2-catalyzed extrusion of arsenic-glutathione complexes into bile (Kala et al. 2000).

2.3.2 Trivalent metalloid uptake systems

Arsenic efflux systems evolved for detoxification purposes. In contrast, it is unlikely that organisms evolved transport systems for the uptake of toxic metalloids since they have no biological function. Instead, metalloids enter cells adventitiously through existing nutrient uptake systems. For over 25 years, it has been recognized that arsenate, the pentavalent arsenic oxyanion, is taken up by phosphate transport systems in bacteria (Rosenberg et al. 1977). More recently yeast phosphate transporters have been shown to take up arsenate (Bun-ya et al. 1996), and similar phosphate uptake pathways are likely to be responsible for arsenate uptake in most other organisms.

A question of considerable interest is the routes of metalloid uptake. The uptake systems for As(III) and Sb(III) were not identified until recently, when we made the unexpected observations that these inorganic oxyacids are brought into cells by transporters for trioses (Sanders et al. 1997) and hexoses (Liu et al. 2004).

Aquaglyceroporins: A century ago Paul Ehrlich, who developed his magic bullet, the organic arsenical Salvarsan, for the treatment of syphilis and sleeping sickness, said in his 1908 Nobel Prize address, "*If a substance is able to kill, this can happen only because it accumulates in cells [because] the arsenoceptor of the cell is able to take up the arsenic radicle"*. Ehrlich found that cells rapidly became resistant to arsenic, and he predicted *"the arsenoceptor had suffered a reduction in its avidity"*. Guided by Ehrlich's words and thoughts, we reasoned that a mutant of *E. coli* that could not take up As(III) or Sb(III) would exhibit resistance. We isolated a Sb(III) resistant mutant and mapped the mutation to the *glpF* gene, which encodes the glycerol facilitator, GlpF (Sanders et al. 1997). The *glpF* mutant lost 90% of its ^{73}As(III) uptake, demonstrating that GlpF is the major route of As(III) uptake in *E. coli* (Meng et al. 2004).

GlpF was one of the first members of the aquaporin superfamily to be identified, nearly a quarter of a century ago (Heller et al. 1980). About a decade later, Peter Agre identified the gene for human AQP1, which he showed was a water channel (Preston et al. 1992). For this accomplishment, Agre was awarded the Nobel Prize in Chemistry in 2003. The superfamily has two major branches (Borgnia et al. 1999). Members of the aquaporin clade are primarily water channels because their pore diameter is too small for larger molecules (Walz et al. 1997). Members of the aquaglyceroporins clade such as GlpF, which has a larger pore diameter than AQP1 (Fu et al. 2000; Stroud et al. 2003), are channels that facilitate downhill movement of water, glycerol, and other small neutral molecules (Agre and Kozono 2003). It should be pointed out that channels such as aquaporins, which move uncharged molecules, are not concentrative; they only facilitate movement, equilibrating solutes inside and outside. Thus, a channel for a neutral solute can increase the rate but not the extent of uptake. Any apparent increase in amount inside the cell probably reflects binding or sequestration of the substrate.

How can a channel for the triose glycerol facilitate transmembrane movement of inorganic As(III) and Sb(III)? Physiologically, the trivalent metalloid salts are frequently depicted as the monovalent oxyanions arsenite and antimonite. With a pK_a of 9.2, arsenous acid ($HAsO_2$) would be predominately undissociated at physiological pH. Indeed, the pK_a of antimonious acid is 11.8, which means that there is essentially no antimonite at neutral pH. Thus, GlpF must be transporting the undissociated oxyacids of the trivalent metalloids, but in what form? GlpF is a polyol transporter, so we predicted that the metalloids would form the inorganic equivalent of a polyol in solution. Arsenous acid is prepared from anhydrous arsenic trioxide (As_2O_3). We predicted from quantum chemical studies that arsenic trioxide would be hydrated in solution, and we showed by extended X-ray absorption fine structure that, at neutral pH, As(III) has a nearest-neighbor coordination geometry of approximately 3 As-O bonds at an average bond length of 1.77 Å (Ramirez-Solis et al. 2004). Thus the dominant form of arsenic trioxide at physiological pH is $As(OH)_3$. This answers the question: GlpF recognizes $As(OH)_3$ as a molecular mimic of glycerol.

S. cerevisiae has a GlpF homologue, Fps1p (Tamas et al. 1999). Wysocki and coworkers (Wysocki et al. 2001) showed that deletion of *FPS1* produced sensitiv-

ity to As(III) and that uptake is mediated by Fps1p, demonstrating that yeast, like *E. coli*, take up As(III) by an aquaglyceroporin. This observation was central to our identification of mammalian As(III) uptake systems by functional complementation in yeast (Liu et al. 2002). A strain lacking both *ACR3* and *YCF1* is hypersensitive to metalloids. We disrupted *FPS1* in the *acr3Δ ycf1Δ* background. This triple deletion is resistant to $As(OH)_3$ and does not take up $^{73}As(OH)_3$ and $^{125}Sb(OH)_3$. This yeast strain was used for complementation with mammalian genes. There are four mammalian aquaglyceroporins, AQP3, AQP7, AQP9, and AQP10. We examined the ability of cloned rat AQP9 and mouse AQP7 to complement the yeast deletion. The rat AQP9 is able to functionally complement the $As(OH)_3$ resistant phenotype in the yeast mutant, while the mouse AQP7 does not. In fact, the AQP9-complemented strain is more sensitive to metalloids than the parent with a wild type *FPS1* gene. The complemented mutant takes up $^{73}As(OH)_3$ and $^{125}Sb(OH)_3$ at a faster rate than its parent, suggesting that AQP9 facilitates uptake of trivalent metalloids better than Fps1p, although differences in expression levels are also possible.

In addition to functional complementation in yeast, *Xenopus* oocytes were injected with the cRNAs for rat AQP9 and mouse AQP7. With either gene, the oocytes facilitate $^{73}As(OH)_3$ uptake, but AQP9 is considerably more effective (Liu et al. 2002). We next cloned all of the human aquaglyceroporins, AQP3, AQP7, AQP9, and AQP10 and expressed them in oocytes (Liu et al. 2004). Human AQP9 and AQP7 (but not AQP3 or AQP10) are effective $As(OH)_3$ transporters. Again, AQP9 is considerably better than AQP7. We believe that these results are of potential significance to human health and disease. Health effects associated with arsenic exposure include cardiovascular and peripheral vascular disease, neurological disorders, diabetes mellitus and various cancers, including liver, bladder, kidney, and skin (Abernathy et al. 2003). In countries such as Bangladesh, the effects are exacerbated by malnutrition. We hypothesize that arsenic uptake is linked to nutrition. In rats, AQP9 expression is elevated 20-fold by starvation (Carbrey et al. 2003). If the same is true in human, then poor nutrition could result in so much arsenic accumulation in liver that it would overwhelm our intrinsic detoxification mechanisms. Conversely, improving the nutritional status of affected individuals could ameliorate the effects of arsenic in drinking water.

Hexose permeases: We recently discovered that the major pathway for arsenite uptake in yeast is catalyzed by hexose permeases (Liu et al. 2004). When glucose is omitted from the uptake assay, the cells take up much more $^{73}As(III)$ than in the presence of glucose. In the absence of glucose, only about 25% of the As(III) enters the cells by Fps1p. This apparently paradoxical result suggested to us that glucose might be inhibiting uptake of arsenite. We found that glucose, galactose, mannose or fructose nearly completely inhibits As(III) uptake in a strain with an *FPS1* disruption. Pentoses and disaccharides do not inhibit. A yeast strain lacking *FPS1*, *ACR3* and all 20 genes for hexose permeases exhibits less than 10% of wild type arsenite transport. When *HXT1, HXT3, HXT4, HXT5, HXT7*, or *HXT9* are individually expressed in that strain, hexose-inhibitable As(III) uptake is restored. These results demonstrate that hexose permeases catalyze the majority of transport of the trivalent metalloid arsenic trioxide.

How can hexose permeases facilitate arsenite transport? Even though we have shown that the predominate species in solution is $As(OH)_3$, another minor form is predicted to be a trimer of arsenic trioxide, a six-membered oxo-bridged ring $(AsO)_3(OH)_3$ (Hamson and Stosick 1938). Theoretical considerations suggest that this form would be stable in solution (Tossell 1997). There are 109 oxo-bridged arsenicals entries in the Cambridge Structural Database, and nearly all are cyclic, including 10 with hexose-like six-membered $(AsO)_3$ rings, so our proposed structure is not unique. Note that polymerization of $(AsO)_n$ can from only even-numbered rings, with six-membered rings the most common in the Cambridge Structural Database. Pentose-like five-membered rings cannot be formed, which may explain why the metalloids are transported by hexose but not pentose transporters. We propose that this six-membered ring is an inorganic molecular mimic of the chair form of the glucose molecule and is the form of arsenic taken up by yeast and mammalian hexose permeases.

3 Visions for the future

There are many possible visions for the future. There may be new discoveries on the basic science of metals in biology; after all, who would have predicted metallochaperones? There may be new applications for metals in the environment. For example, fertilizing the oceans with iron is predicted to enhance fixation of carbon dioxide by plankton and reverse global warming (Coale et al. 2004). This now appears to be unlikely (Buesseler et al. 2004), illustrating that predictions are often not as fruitful as serendipity, which may yield the unexpected but also point to new directions and paradigms. Albert Szent-Györgyi (Nobel Prize in Physiology or Medicine, 1937) said of serendipity "*a discovery is an accident meeting a prepared mind*". In the area of metals in biology, one of the best examples of serendipity was the discovery of the antitumor agent cisplatin. Rosenberg and colleagues noted that an electrolysis product from a platinum electrode inhibited growth of *E. coli* (Rosenberg et al. 1965). Possessing prepared minds, they predicted that the platinum compound cis-diammintetrachloroplatinum(IV) tetrarchloride, which they called cisplatin, would inhibit the growth of cancer cells (Rosenberg et al. 1969), inspiring a new field of metallo-drug design.

3.1 Metals and medicine

One of the more likely predictions we can make here is that metals will play an ever more important role in medicine and the treatment of clinical disease. The strong interactions of soft metal with oxygen, nitrogen and sulfur ligands in proteins, and nucleic acids make them good poisons. As Paracelsus (1493-1541), the father of modern pharmacology, declared, "*The dose makes the poison*". At an appropriate dose, toxic metals are effective pharmacological agents. Although metals have been used for thousands of years to treat human ailments, it was Paul Ehrlich

who first designed organometallic compounds as antimicrobial agents. His organoarsenical arsphenamine (Salvarsan) was the first effective cure for syphilis and was used to treat such famous historical figures as Vladimir Lenin and the Baroness Karen Christence Dinesen Blixen-Finecke.

While Salvarsan is no longer in clinical use, it spurred the development of newer organometallic drugs. Even though the current success story, cisplatin, was discovered by accident, there are many new metal-containing drugs currently in clinical trials that are the result of rational drug design. These include compounds with transition metals such as platinum, ruthenium, technetium, gold, iron, manganese and copper. For rational design, it is crucial to understand the mechanism of action of each drug, as well as the transporters that catalyze their entry into and out of cells and organelles. These new drugs have recently been reviewed by Zhang and Lippard (Zhang and Lippard 2003), and so will not be elaborated further here.

3.2 Metalloid chemotherapy

3.2.1 Arsenic and trypansomiasis

While Ehrlich developed his magic bullet Salvarsan for syphilis, a bacterial disease, the origins of his interest in chemotherapy stemmed from the use of atoxyl, aminophenyarsenate, which was used for the treatment of sleeping sickness, caused by the eukaryotic parasite *Trypanosoma brucei*. This drug had been used as an antitrypanosomal drug since 1905, but the side effects of atoxyl were too severe, which led to Ehrlich's search for less noxious alternatives. It has also been used as a feed additive to treat enteric infections in pigs and poultry but causes blindness and kidney toxicity in animals. The less toxic arsenical drug melarsoprol (Melarsen) is still in use today for treatment of tryposomomal diseases. Even though this drug has little similarity to adenosine, surprisingly, it is taken up into the parasite by the P2 adenosine transport, and loss of this transporter leads to resistance due to loss of uptake (Carter and Fairlamb 1993). This is another example of Ehrlich's premise that many drugs are taken up by transport systems, and that mutation of the transporter leads to resistance.

3.2.2 Antimony and leishmaniasis

We have been interested in determining the mechanisms of drug uptake and resistance in the protozoan parasite *Leishmania*. This organism is a trypanosomid and belongs to the same group of flagellates as trypanosomes. *Leishmania* has a complex life cycle involving two hosts. In the sand fly, the organism exists in the flagellated promastigote form. When the fly bites a vertebrate host, it develops into the obligate intracellular amastigote form inside of the host macrophage. The most common forms of leishmaniais are cutaneous, which causes skin sores, and the more serious visceral form (or Kala-azar), which affects spleen, liver, and bone marrow. Leishmaniasis affects 12 million people worldwide. The first line of drug

for the treatment of this disease is the pentavalent antimonial Pentostam. In endemic regions, resistance to this class of drugs is a major impediment to treatment. Drug resistance correlates with amplification of the gene for PgpA, a member of the ABC superfamily (Papadopoulou et al. 1994). PgpA is located in intracellular compartments and catalyzes the sequestration of As-thiol complexes (Legare et al. 2001). Resistance also correlates with expression of a second efflux system for As-thiol and Sb(III)-thiol complexes (Dey et al. 1996). In *Leishmania* the major thiol is trypanothioine (TSH), a glutathione-spermidine conjugate (Fairlamb et al. 1985), and the metalloid-trypanothione complex is a substrate of the ABC transporters (Mukhopadhyay et al. 1996). Once the rate of the efflux systems are increased, resistance is limited by the availability of the thiols to which As(III) or Sb(III) conjugates (Grondin et al. 1997; Haimeur et al. 1999). Step-wise selection for resistant mutants results in isolation of strains in which the various steps in the thiol biosynthetic pathways are overexpressed. Synthesis of TSH is normally rate-limited by the availability of GSH, in particular the amount of the enzyme γ-glutamylcysteine synthetase (γGCS). When the *GSH1* gene for γGCS is amplified, cells become resistant to both $As(OH)_3$ and $Sb(OH)_3$. The rate-limiting step in TSH biosynthesis then becomes the enzyme ornithine decarboxylase (ODCase), which is on the pathway of spermidine biosynthesis. Selection for higher-level resistance results in increased production of ODCase. Thus, an enlightening concept is that selection for drug resistance can result in amplification of sequential steps in inter-related pathways. At each step another enzyme or transporter becomes rate-limiting, and its gene is the target for the next mutation or amplification.

Those resistance mechanisms involve efflux or sequestration of the trivalent metalloids. We next considered the possibility that *Leishmania* become resistant to Pentostam by mutation or downregulation of the uptake system. We cloned an AQP gene from *L. major* (*LmAQP1*) and *L. tarentolae* (*LtAQP1*), respectively (Gourbal et al. 2004). Transfection of *LmAQP1* into three different species of *Leishmania*, *L. tarentolae*, *L. infantum*, and *L. major* produces hypersensitivity to both $As(OH)_3$ and $Sb(OH)_3$ in all three species. More importantly, expression of *LmAQP1* reverses drug resistance in every type of drug resistant mutant, no matter the immediate cause of resistance. Increased rates of uptake of $As(OH)_3$ or $Sb(OH)_3$ correlates with metalloid sensitivity of the wild type and drug resistant transfectants. The question arises whether uptake of trivalent metalloids by this aquaglyceroporin is relevant to action of the drug, which contains Sb(V), not Sb(III). However, transfection of *LmAQP1* in a Pentostam resistant field isolate also sensitized the parasite in the macrophage-associated amastigote form. This indicates that at least a portion of the Sb(V) is reduced to Sb(III) in the macrophage, and the intracellular amastigote takes up the Sb(III) product, which is the active form of the drug. A portion of the Sb(V) also enters the amastigote directly, where it is reduced to Sb(III) by LmACR2, a parasite arsenate reductase (Zhou et al. 2004). This is the first report of uptake of a metalloid drug by an AQP in *Leishmania* and suggests a strategy to reverse resistance in the field.

3.2.3 Arsenic and cancer chemotherapy

Trivalent arsenic is also used as chemotherapeutic drugs for the treatment of acute promyelocytic leukemia (APL). In particular, arsenic trioxide is the active ingredient of the antileukemia drug Trisenox (Bentley and Chasteen 2002). Arsenic therapy had long been used in China, where it was shown to be an effective treatment for APL (Chen et al. 1996). Those studies led to its trial in the United States (Soignet et al. 1998) and to its approval by the U.S. Food and Drug Administration in September 2000 as the drug Trisenox ^{TM} for the treatment of relapsed or refractory APL. The mechanism of Trisenox action is obscure, and, until recently, its route of uptake into leukemia cells was likewise unknown. Since drug action usually requires uptake of the drug, we considered it important to determine the transport system responsible for Trisenox uptake and so investigated the role of aquaglyceroporins. We showed that AQP9 expression modulates the drug sensitivity of leukemic cells. AQP9 was transfected into several; leukemia cell lines, and the transfectants became hypersensitive to Trisenox and Sb(III). Trisenox hypersensitivity correlated with increased expression of AQP9 and increased drug uptake. A promyelocytic leukemia cell line also became hypersensitive to Trisenox when treated with vitamin D, which increases expression of AQP9. The possibility of using pharmacological agents to increase expression of uptake genes holds out the promise of new therapies for the treatment of leukemia and perhaps other forms of cancer.

4 Conclusion

In conclusion, the development of new metallopharmaceuticals will come to pass in part through applied research in the rational design of drugs, as Paul Ehrlich foresaw a century ago. But basic research using of model systems will play a large part as well. Many of the metallochaperones were all discovered through the use of microbial genetics. The serendipitous discovery of cisplatin came through a study of *E. coli* physiology. From our identification of the *E. coli* GlpF as the route of uptake of As(III) in 1997 to the prospect of improved treatment from leukemia demonstrates the power of prokaryotic model systems and illustrates the importance of investigation into the mechanisms of metal uptake and detoxification. Jacques Monod (Nobel Prize in Physiology or Medicine, 1965) said it best: "*Anything that is true of E. coli must be true of elephants, except more so*".

Acknowledgements

The work from our laboratory described in this review was supported by grants GM55425, GM52216, AI45428, and ES10344 from the National Institutes of Health and a Wellcome Trust Biomedical Research Collaborative Grant.

References

Abernathy CO, Thomas DJ, Calderon RL (2003) Health effects and risk assessment of arsenic. J Nutr 133:1536S-1538S

Agre P, Kozono D (2003) Aquaporin water channels: molecular mechanisms for human diseases. FEBS Lett 555:72-78

Alam MG, Allinson G, Stagnitti F, Tanaka A, Westbrooke M (2002) Arsenic contamination in Bangladesh groundwater: a major environmental and social disaster. Int J Environ Health Res 12:235-253

Allikmets R, Raskind WH, Hutchinson A, Schueck ND, Dean M, Koeller DM (1999) Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). Hum Mol Genet 8:743-749

Alper G, Narayanan V (2003) Friedreich's ataxia. Pediatr Neurol 28:335-341

Balk J, Lill R (2004) The cell's cookbook for iron--sulfur clusters: recipes for fool's gold? Chembiochem 5:1044-1049

Barkay T, Miller SM, Summers AO (2003) Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol Rev 27:355-384

Bentley R, Chasteen TG (2002) Arsenic curiosa and humanity. Chem Educator 7:51-60

Berks BC, Palmer T, Sargent F (2003) The Tat protein translocation pathway and its role in microbial physiology. Adv Microb Physiol 47:187-254

Blasco F, Dos Santos JP, Magalon A, Frixon C, Guigliarelli B, Santini CL, Giordano G (1998) NarJ is a specific chaperone required for molybdenum cofactor assembly in nitrate reductase A of *Escherichia coli*. Mol Microbiol 28:435-447

Bobrowicz P, Ulaszewski S (1998) Arsenical-induced transcriptional activation of the yeast *Saccharomyces cerevisiae ACR2* and *ACR3* genes requires the presence of the *ACR1* gene product. Cell Mol Biol Lett 3:13-20

Bobrowicz P, Wysocki R, Owsianik G, Goffeau A, Ulaszewski S (1997) Isolation of three contiguous genes, *ACR1*, *ACR2* and *ACR3*, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. Yeast 13:819-828

Borgnia M, Nielsen S, Engel A, Agre P (1999) Cellular and molecular biology of the aquaporin water channels. Annu Rev Biochem 68:425-458

Buesseler KO, Andrews JE, Pike SM, Charette MA (2004) The effects of iron fertilization on carbon sequestration in the Southern Ocean. Science 304:414-417

Bun-ya M, Shikata K, Nakade S, Yompakdee C, Harashima S, Oshima Y (1996) Two new genes, *PHO86* and *PHO87*, involved in inorganic phosphate uptake in *Saccharomyces cerevisiae*. Curr Genet 29:344-351

Canovas D, Mukhopadhyay R, Rosen BP, de Lorenzo V (2003) Arsenate transport and reduction in the hyper-tolerant fungus Aspergillus sp. P37. Environ Microbiol 5:1087-1093

Carbrey JM, Gorelick-Feldman DA, Kozono D, Praetorius J, Nielsen S, Agre P (2003) Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. Proc Natl Acad Sci USA 100:2945-2950

Carlin A, Shi W, Dey S, Rosen BP (1995) The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. J Bacteriol 177:981-986

Carter NS, Fairlamb AH (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature 361:173-176

Chen CA, Cowan JA (2003) Characterization of the soluble domain of the ABC7 type transporter Atm1. J Biol Chem 278:52681-52688

Chen GQ, Zhu J, Shi XG, Ni JH, Zhong HJ, Si GY, Jin XL, Tang W, Li XS, Xong SM, Shen ZX, Sun GL, Ma J, Zhang P, Zhang TD, Gazin C, Naoe T, Chen SJ, Wang ZY, Chen Z (1996) *In vitro* studies on cellular and molecular mechanisms of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia: As_2O_3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. Blood 88:1052-1061

Chen Y, Dey S, Rosen BP (1996) Soft metal thiol chemistry is not involved in the transport of arsenite by the Ars pump. J Bacteriol 178:911-913

Chen Y, Rosen BP (1997) Metalloregulatory properties of the ArsD repressor. J Biol Chem 272:14257-14262

Chloupkova M, LeBard LS, Koeller DM (2003) *MDL1* is a high copy suppressor of *ATM1*: evidence for a role in resistance to oxidative stress. J Mol Biol 331:155-165

Clemons WM Jr, Menetret JF, Akey CW, Rapoport TA (2004) Structural insight into the protein translocation channel. Curr Opin Struct Biol 14:390-396

Coale KH, Johnson KS, Chavez FP, Buesseler KO, Barber RT, Brzezinski MA, Cochlan WP, Millero FJ, Falkowski PG, Bauer JE, Wanninkhof RH, Kudela RM, Altabet MA, Hales BE, Takahashi T, Landry MR, Bidigare RR, Wang X, Chase Z, Strutton PG, Friederich GE, Gorbunov MY, Lance VP, Hilting AK, Hiscock MR, Demarest M, Hiscock WT, Sullivan KF, Tanner SJ, Gordon RM, Hunter CN, Elrod VA, Fitzwater SE, Jones JL, Tozzi S, Koblizek M, Roberts AE, Herndon J, Brewster J, Ladizinsky N, Smith G, Cooper D, Timothy D, Brown SL, Selph KE, Sheridan CC, Twining BS, Johnson ZI (2004) Southern Ocean iron enrichment experiment: carbon cycling in high- and low-Si waters. Science 304:408-414

Csere P, Lill R, Kispal G (1998) Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast Atm1p. FEBS Lett 441:266-270

Culotta VC, Joh HD, Lin SJ, Slekar KH, Strain J (1995) A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. J Biol Chem 270:29991-29997

Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD (1997) The copper chaperone for superoxide dismutase. J Biol Chem 272:23469-23472

Dey S, Dou D, Tisa LS, Rosen BP (1994) Interaction of the catalytic and the membrane subunits of an oxyanion- translocating ATPase. Arch Biochem Biophys 311:418-424

Dey S, Ouellette M, Lightbody J, Papadopoulou B, Rosen BP (1996) An ATP-dependent As(III)-glutathione transport system in membrane vesicles of *Leishmania tarentolae*. Proc Natl Acad Sci USA 93:2192-2197

Dey S, Rosen BP (1995) Dual mode of energy coupling by the oxyanion-translocating ArsB protein. J Bacteriol 177:385-389

Eisenstein RS (2000) Iron regulatory proteins and the molecular control of mammalian iron metabolism. Annu Rev Nutr 20:627-662

Fairlamb AH, Blackburn P, Ulrich P, Chait B, Cerami A (1985) Trypanothione: a novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. Science 227:1485-1487

Field LS, Luk E, Culotta VC (2002) Copper chaperones: personal escorts for metal ions. J Bioenerg Biomembr 34:373-379

Fu D, Libson A, Miercke LJ, Weitzman C, Nollert P, Krucinski J, Stroud RM (2000) Structure of a glycerol-conducting channel and the basis for its selectivity. Science 290:481-486

Gerber J, Neumann K, Prohl C, Muhlenhoff U, Lill R (2004) The yeast scaffold proteins Isu1p and Isu2p are required inside mitochondria for maturation of cytosolic Fe/S proteins. Mol Cell Biol 24:4848-4857

Ghosh M, Shen J, Rosen BP (1999) Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 96:5001-5006

Glerum DM, Shtanko A, Tzagoloff A (1996) Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J Biol Chem 271:14504-14509

Gourbal B, Sonuc N, Bhattacharjee H, Legare D, Sundar S, Ouellette M, Rosen BP, Mukhopadhyay R (2004) Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. J Biol Chem 279:31010-31017

Grondin K, Haimeur A, Mukhopadhyay R, Rosen BP, Ouellette M (1997) Co-amplification of the gamma-glutamylcysteine synthetase gene *gsh1* and of the ABC transporter gene *pgpA* in arsenite-resistant *Leishmania tarentolae*. Embo J 16:3057-3065

Haimeur A, Conseil G, Deeley RG, Cole SP (2004) The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Metab 5:21-53

Haimeur A, Guimond C, Pilote S, Mukhopadhyay R, Rosen BP, Poulin R, Ouellette M (1999) Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite- resistant Leishmania. Mol Microbiol 34:726-735

Hamson GC, Stosick AJ (1938) The molecular structure of arsenious oxide, As_4O_6, phosphorus trioxide, P_4O_6, phosphorus pentoxide, P_40_{10}, and hexamethylenetetramine, $(CH_6),N_4$, by electron diffraction. J Am Chem Soc 60:1814-1822

Heller KB, Lin EC, Wilson TH (1980) Substrate specificity and transport properties of the glycerol facilitator of *Escherichia coli*. J Bacteriol 144:274-278

Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW (2000) The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic. J Biol Chem 275:33404-33408

Kispal G, Csere P, Guiard B, Lill R (1997) The ABC transporter Atm1p is required for mitochondrial iron homeostasis. FEBS Lett 418:346-350

Kispal G, Csere P, Prohl C, Lill R (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. Embo J 18:3981-3989

Lamb AL, Wernimont AK, Pufahl RA, Culotta VC, O'Halloran TV, Rosenzweig AC (1999) Crystal structure of the copper chaperone for superoxide dismutase. Nat Struct Biol 6:724-729

Legare D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulou B, Ouellette M (2001) The *Leishmania* ATP-binding cassette protein *PGPA* is an intracellular metal-thiol transporter ATPase. J Biol Chem 276:26301-26307

Li ZS, Szczypka M, Lu YP, Thiele DJ, Rea PA (1996) The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. J Biol Chem 271:6509-6517

Lin SJ, Pufahl RA, Dancis A, O'Halloran TV, Culotta VC (1997) A role for the *Saccharomyces cerevisiae ATX1* gene in copper trafficking and iron transport. J Biol Chem 272:9215-9220

Liu Z, Boles E, Rosen BP (2004) Arsenic trioxide uptake by hexose permeases in *Saccharomyces cerevisiae*. J Biol Chem 279:17312-17318

Liu Z, Carbrey JM, Agre P, Rosen BP (2004) Arsenic trioxide uptake by human and rat aquaglyceroporins. Biochem Biophys Res Commun 316:1178-1185

Liu Z, Shen J, Carbrey JM, Mukhopadhyay R, Agre P, Rosen BP (2002) Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci USA 99:6053-6058

Locher KP (2004) Structure and mechanism of ABC transporters. Curr Opin Struct Biol 14:426-431

Lopez-Archilla AI, Marin I, Amils R (2001) Microbial community composition and ecology of an acidic aquatic environment: the Tinto River, Spain. Microb Ecol 41:20-35

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED (2001) A fern that hyperaccumulates arsenic. Nature 409:579

Mansy SS, Cowan JA (2004) Iron-sulfur cluster biosynthesis: toward an understanding of cellular machinery and molecular mechanism. Acc Chem Res 37:719-725

Meng YL, Liu Z, Rosen BP (2004) As(III) and Sb(III) uptake by GlpF and efflux by ArsB in *Escherichia coli*. J Biol Chem 279:18334-18341

Muhlenhoff U, Richhardt N, Ristow M, Kispal G, Lill R (2002) The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins. Hum Mol Genet 11:2025-2036

Mukhopadhyay R, Dey S, Xu N, Gage D, Lightbody J, Ouellette M, Rosen BP (1996) Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. Proc Natl Acad Sci USA 93:10383-10387

Mukhopadhyay R, Rosen BP (1998) The *Saccharomyces cerevisiae ACR2* gene encodes an arsenate reductase. FEMS Microbiol Lett 168:127-136

Mukhopadhyay R, Rosen BP (2002) Arsenate reductases in prokaryotes and eukaryotes. Environ Health Perspect 110 Suppl 5:745-748

Mukhopadhyay R, Shi J, Rosen BP (2000) Purification and characterization of Acr2p, the *Saccharomyces cerevisiae* arsenate reductase. J Biol Chem 275:21149-21157

Papadopoulou B, Roy G, Dey S, Rosen BP, Ouellette M (1994) Contribution of the *Leishmania* P-glycoprotein-related gene *ltpgpA* to oxyanion resistance. J Biol Chem 269:11980-11986

Park IS, Hausinger RP (1995) Requirement of carbon dioxide for *in vitro* assembly of the urease nickel metallocenter. Science 267:1156-1158

Pommier J, Mejean V, Giordano G, Iobbi-Nivol C (1998) TorD, a cytoplasmic chaperone that interacts with the unfolded trimethylamine N-oxide reductase enzyme (TorA) in *Escherichia coli*. J Biol Chem 273:16615-16620

Preston GM, Carroll TP, Guggino WB, Agre P (1992) Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. Science 256:385-387

Ramirez-Solis A, Mukopadhyay R, Rosen BP, Stemmler TL (2004) Experimental and theoretical characterization of arsenite in water: insights into the coordination environment of As-O. Inorg Chem 43:2954-2959

Ratnaike RN (2003) Acute and chronic arsenic toxicity. Postgrad Med J 79:391-396

Rosen BP (1999) Families of arsenic transporters. Trends Microbiol 7:207-212

Rosen BP (2002) Biochemistry of arsenic detoxification. FEBS Lett 529:86-92

Rosen BP, Dey S, Dou D, Ji G, Kaur P, Ksenzenko M, Silver S, Wu J (1992) Evolution of an ion-translocating ATPase. Ann N Y Acad Sci 671:257-272

Rosenberg B, Vancamp L, Krigas T (1965) Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature 205:698-699

Rosenberg B, VanCamp L, Trosko JE, Mansour VH (1969) Platinum compounds: a new class of potent antitumour agents. Nature 222:385-386

Rosenberg H, Gerdes RG, Chegwidden K (1977) Two systems for the uptake of phosphate in *Escherichia coli*. J Bacteriol 131:505-511

Rosenzweig AC, Huffman DL, Hou MY, Wernimont AK, Pufahl RA, O'Halloran TV (1999) Crystal structure of the Atx1 metallochaperone protein at 1.02 Å resolution. Structure Fold Des 7:605-617

Sanders OI, Rensing C, Kuroda M, Mitra B, Rosen BP (1997) Antimonite is accumulated by the glycerol facilitator GlpF in *Escherichia coli*. J Bacteriol 179:3365-3367

Sargent F, Gohlke U, De Leeuw E, Stanley NR, Palmer T, Saibil HR, Berks BC (2001) Purified components of the Escherichia coli Tat protein transport system form a double-layered ring structure. Eur J Biochem 268:3361-3367

Sato T, Kobayashi Y (1998) The *ars* operon in the skin element of *Bacillus subtilis* confers resistance to arsenate and arsenite. J Bacteriol 180:1655-1661

Schiering N, Kabsch W, Moore MJ, Distefano MD, Walsh CT, Pai EF (1991) Structure of the detoxification catalyst mercuric ion reductase from Bacillus sp. strain RC607. Nature 352:168-172

Serre L, Rossy E, Pebay-Peyroula E, Cohen-Addad C, Coves J (2004) Crystal structure of the oxidized form of the periplasmic mercury-binding protein MerP from *Ralstonia metallidurans* CH34. J Mol Biol 339:161-171

Soignet SL, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti LJ, Corso D, DeBlasio A, Gabrilove J, Scheinberg DA, Pandolfi PP, Warrell RPJ Jr (1998) Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N Engl J Med 339:1341-1348

Song HK, Mulrooney SB, Huber R, Hausinger RP (2001) Crystal structure of *Klebsiella aerogenes* UreE, a nickel-binding metallochaperone for urease activation. J Biol Chem 276:49359-49364

Steele RA, Opella SJ (1997) Structures of the reduced and mercury-bound forms of MerP, the periplasmic protein from the bacterial mercury detoxification system. Biochemistry 36:6885-6895

Strain J, Lorenz CR, Bode J, Garland S, Smolen GA, Ta DT, Vickery LE, Culotta VC (1998) Suppressors of superoxide dismutase (*SOD1*) deficiency in *Saccharomyces cerevisiae*. Identification of proteins predicted to mediate iron-sulfur cluster assembly. J Biol Chem 273:31138-31144

Stroud RM, Nollert P, Miercke L (2003) The glycerol facilitator GlpF its aquaporin family of channels, and their selectivity. Adv Protein Chem 63:291-316

Tamas MJ, Luyten K, Sutherland FC, Hernandez A, Albertyn J, Valadi H, Li H, Prior BA, Kilian SG, Ramos J, Gustafsson L, Thevelein JM, Hohmann S (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol Microbiol 31:1087-1104

Tossell JA (1997) Epidemiologic evidence of diabetogenic effect of arsenic. Geochim Cosmochim Acta 61:1613-1623

Veenendaal AK, van der Does C, Driessen AJ (2004) The protein-conducting channel SecYEG. Biochim Biophys Acta 1694:81-95

Vergnes A, Gouffi-Belhabich K, Blasco F, Giordano G, Magalon A (2004) Involvement of the molybdenum cofactor biosynthetic machinery in the maturation of the *Escherichia coli* nitrate reductase A. J Biol Chem 279:41398-41403

Walz T, Hirai T, Murata K, Heymann JB, Mitsuoka K, Fujiyoshi Y, Smith BL, Agre P, Engel A (1997) The three-dimensional structure of aquaporin-1. Nature 387:624-627

Wong MD, Fan B, Rosen BP (2004) Bacterial transport ATPases for monovalent, divalent and trivalent soft metal ions. In: Futai M, Wada Y, Kaplan JH (eds) Handbook of ATPases. Wiley-VCH, Weinheim, pp 159-178

Wong PC, Waggoner D, Subramaniam JR, Tessarollo L, Bartnikas TB, Culotta VC, Price DL, Rothstein J, Gitlin JD (2000) Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. Proc Natl Acad Sci USA 97:2886-2891

Wu J, Tisa LS, Rosen BP (1992) Membrane topology of the ArsB protein, the membrane subunit of an anion-translocating ATPase. J Biol Chem 267:12570-12576

Wysocki R, Bobrowicz P, Ulaszewski S (1997) The *Saccharomyces cerevisiae ACR3* gene encodes a putative membrane protein involved in arsenite transport. J Biol Chem 272:30061-30066

Wysocki R, Chery CC, Wawrzycka D, Van Hulle M, Cornelis R, Thevelein JM, Tamás MJ (2001) The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. Mol Microbiol 40:1391-1401

Wysocki R, Fortier PK, Maciaszczyk E, Thorsen M, Leduc A, Odhagen A, Owsianik G, Ulaszewski S, Ramotar D, Tamas MJ (2004) Transcriptional activation of metalloid tolerance genes in *Saccharomyces cerevisiae* requires the AP-1-like proteins Yap1p and Yap8p. Mol Biol Cell 15:2049-2060

Zhang CX, Lippard SJ (2003) New metal complexes as potential therapeutics. Curr Opin Chem Biol 7:481-489

Zhou Y, Messier N, Ouellette M, Rosen BP, Mukhopadhyay R (2004) *Leishmania major* LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug Pentostam. J Biol Chem 279:37445-37451

Rosen, Barry P.
Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, Detroit, MI 48201 USA
brosen@med.wayne.edu

Index

abscissic acid, 205
acidification, 217, 229, 306
acrodermatitis enteropathica, 41, 132
acute promyelocytic leukaemia, 396, 499
Alzheimer's disease, 119
amino acid transporters, 464
amyloplasts, 202
amyotrophic lateral sclerosis, 19, 119, 486
anemias, 171
AP-1-like transcription factor, 410, 428
apoplasmic space, 194, 284
apoptosis, 107, 132, 139, 396
aquaglyceroporin, 405, 463, 494
aquaporin, 405, 494
arsenate reductase, 359, 415, 493
arsenic chaperone, 487
arsenic resistance, 410, 428
arsenic trioxide, 396, 493
ascorbate, 204, 227
ataxia, 73, 109, 176, 489
ATP-binding cassette, 20, 176, 249, 352, 410

bacteroids, 197
bZIP transcription factor, 400, 420

cadmium resistance, 17, 401
cadmium toxicity, 139, 399
cadmium uptake, 407, 460
cation diffusion facilitator, 247, 321, 413
cell cycle arrest, 89, 370, 431
cell cycle regulation, 24, 107, 431
chelation, 18, 68, 217, 273, 306, 409
chemotherapy, 396, 497
chlorophyll, 205, 225, 248
chloroplast, 200, 221, 319
cisplatin, 5, 496
citrate, 198, 241, 275, 318
copper binding, 65, 107, 227, 367, 460, 487
copper chaperone, 8, 50, 65, 106, 462, 486
copper deficiency, 103
copper homeostasis, 1, 50, 101, 235
copper oxidase, 65, 196, 227, 486
copper toxicity, 101, 113
copper uptake, 103, 247, 486
copper-dependent enzymes, 107
copper-dependent transcription factor, 83
Cu-responsive element (CuRE), 245
cytochrome c oxidase, 1, 84, 105, 226

disulphide bond, 426
divalent metal ion transporter, 70, 155
DNA binding, 22, 38, 81, 246, 348, 429
DNA repair, 397

ecosystems, 303, 317
electron energy loss spectroscopy (EELS), 285
electron transport chain, 13, 73, 217
embryo, 105, 136, 162, 200, 283
ethylene, 227
etioplasts, 202

Fenton chemistry, 101, 193
ferric chelate reductases, 196
ferritin, 67, 155, 200, 404, 456, 489
frataxin, 75, 488
Friedrich's ataxia, 73

GATA-like transcription factor, 62
germination, 204, 228
glutathione (GSH), 237, 279, 306, 403, 466
glutathione biosynthesis, 313, 418
glutathione conjugate, 401, 493
glutathione reductase, 207, 306, 402, 487

H^+/metal-ion cotransport, 459
helix-loop-helix, 196, 243, 360
heme biosynthesis, 73, 156, 352
hereditary hemochromatosis, 159
horizontal gene transfer, 411
hydrogen peroxide, 352, 399, 404
hydroxyl radical, 19, 101, 249, 399
hyperaccumulator plants, 299

indicator species, 305
iron chaperone, 67, 488
iron deficiency, 86, 155, 193, 275, 486
iron export, 65, 161, 460
iron homeostasis, 59, 158, 194, 460
iron overload, 159, 200
iron regulatory proteins (IRP), 157
iron response element, 166
iron storage, 84, 157, 201
iron uptake, 59, 157, 194, 407, 469
iron-sulfur cluster, 70, 488
ischemia, 132

leghemoglobin, 197
leishmaniasis, 397, 497
Lethal milk syndrome, 132
lipid peroxidation, 397

MAP kinase, 143
Menkes disease, 9, 108, 247
metal binding, 4, 41, 114, 217, 282, 349, 486
metal chelator, 197, 230, 273, 301, 374
metal immobilization, 273
metal remobilization, 233
metal sequestration, 253, 283, 411
metal tolerance, 395
metal-dependent gene regulation, 347
metalloid efflux, 492
metalloid uptake, 493
metalloprotease, 40, 139, 216
metallothionein, 18, 52, 104, 249, 282, 341, 414, 456
methyl cycle, 400, 418
mitochondrial iron, 62, 176, 489
molybdenum chaperone, 487
mRNA processing, 178
mRNA stability, 343
mRNA turnover, 84
mugineic acid, 197, 236, 275
Multidrug Associated and Toxin Efflux (MATE) family, 206
multidrug resistance, 20, 349, 468, 493

natural resistance associated macrophage protein, 201, 356, 407, 458
neuronal cell death, 132
nickel chaperone, 487
nicotianamine, 197, 231, 289, 320
nitrogenase, 197
nodules, 197
nonessential metals, 395, 455

oncogenesis, 107
operon, 347, 395, 487
organic acids, 197, 232, 278
organic anion transporter, 466
oxidative phosphorylation, 3, 141, 398
oxidative stress, 19, 73, 207, 226, 306, 343, 397, 489

Parkinson's disease, 119
phloem, 198, 233, 283
photosynthesis, 193, 225
phytochelatin, 249, 279, 308, 341, 409
phytochelatin synthase, 249, 279, 402, 414
phytoextraction, 301
phytoremediation, 281, 299, 318, 396
phytorestoration, 304
phytosiderophores, 195, 236, 275
phytostabilization, 303
phytovolatization, 302
prokaryotic transcription factor, 343, 362
proteasome, 222, 434
P-type ATPase, 9, 107, 237, 347, 401, 462

receptor-mediated endocytosis, 155, 469
redox potential, 216
redox-inactive metals, 402
rhizofiltration, 303
rhizosphere degradation, 302

simple diffusion, 471
sulphate assimilation, 418
sulphur sparing, 419
superoxide dismutase, 3, 50, 225, 423, 486

thylakoid membrane, 202, 223
transition metals, 216, 497
trypanothione, 412, 498
trypansomiasis, 497

ubiquitin, 44, 221, 432
ubiquitin ligase, 433

vacuolar sequestration, 253, 321, 412

Wilson disease, 110

X-linked sideroblastic anemia, 73, 174, 489
X-ray absorption spectroscopy (XAS), 281
xylem, 198, 232, 284, 300

zinc deficiency, 41, 131
zinc finger, 39, 134, 366, 402
zinc homeostasis, 37, 133
zinc pools, 134
Zinc Response Elements (ZREs), 38
zinc sensing receptor, 143
zinc signaling, 142
zinc transport, 40, 135
ZIP family, 41, 138, 235, 325, 407
ZnT family, 135