Toshiyuki Fukada · Taiho Kambe *Editors*

Zinc Signals in Cellular Functions and Disorders

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Foreword

Our understanding of the roles of the essential metal zinc in health and disease processes is advancing rapidly, as is evident from the remarkable studies presented in this book on zinc signaling mechanisms. Certainly many excellent scientists have contributed to the foundations of this field, and I trust that they will not be offended if I cannot mention them all in this limited space. However, I find the pioneering studies of several clinicians and molecular biologists particularly noteworthy of mention. The clinicians Ananda Prasad and Harold Sandstead traveled to Iran in the late 1950s and 1960s and found young adults who had failed to thrive and mature and then deduced that this was due to dietary zinc deficiency (Prasad 1984). By the early 1970s, Edward Moynahan identified acrodermatitis enteropathica as a genetic disease of zinc deficiency in humans (Moynahan 1974). These findings led the American National Academy of Sciences in the 1970s to realize that humans could, in fact, become zinc deficient and that zinc deficiency could cause disease.

The molecular biologist Richard Palmiter first described the mammalian metallothionein genes and demonstrated their dramatic transcriptional induction by zinc and then created mouse models which over-express or lack metallothioneins (MT-I and II) (Palmiter 1987; Masters et al. 1994). Subsequently Walter Schaffner identified and cloned the transcription factor MTF-1, which regulates metallothionein gene transcription in response to zinc (Radtke et al. 1993). These pivotal studies paved the way for thousands of subsequent studies. Although my group provided compelling data that the unique zinc-finger domain of MTF-1 functions as a zinc sensor (Laity and Andrews 2007), the structural basis for that mechanism remains to be resolved in detail. Nonetheless the concept that substantial changes in "available" zinc in higher eukaryotic cells and organisms are sensed by the cell was fundamental to our understanding of zinc biology and zinc homeostasis mechanisms. We now understand that zinc fluxes modify kinase signal transduction cascades and control the localization and stability of several zinc transporters. Using the MT over-expressing or knockout mice created by Richard Palmiter, we presented some of the first evidence that the mouse metallothioneins provide a biologically important labile pool of zinc (Dalton et al. 1996; Andrews and Geiser 1999). These proteins are now considered to function as zinc buffers. Richard Palmiter's contribution did not end with the metallothioneins.

He subsequently cloned the first mammalian zinc efflux transporter (ZnT1; Slc30a1), described the ZnT gene family, and created mouse models that lacked ZnTs (Palmiter and Findley 1995; Palmiter and Huang 2004). His ZnT3 knockout mouse model has been and continues to be employed in hundreds of neurobiology studies (Cole et al. 1999).

Another fundamental advance in the field was the identification of the first ZIP family member IRT-1 in Mary Lou Guerinot's laboratory (Eide et al. 1996). In collaboration with David Eide they showed that *Saccharomyces* ZRT zinc transporters (Zhao and Eide 1996) and *Arabidopsis* IRT1 iron transporters belong to a structurally related family of metal ion transporters, thus the acronym **Z**rt-**I**rt-like **P**roteins (Guerinot 2000). The ZIP proteins are found in all eukaryotes, and orthologues are found in bacteria. Since the identification of this family of metal ion transporters, there have been hundreds of publications on their structure, regulation, and functions. Pioneering studies by Jane Gitschier (Wang et al. 2002) and Sebastien Kury (Kury et al. 2002) identified *Zip4* mutations in patients with acrodermatitis enteropathica about 30 years after the description of this devastating zinc deficiency disease by Moynahan (1974). Among the 14 known ZIP family members, we now have mouse knockout models of over half of these genes. My group created mouse knockout models of *Zip1* through *Zip5* which includes mouse models of acrodermatitis enteropathica (Kambe et al. 2008; Dufner-Beattie et al. 2007; Geiser et al. 2012, 2013). Our studies revealed that expression of the *Zip4* gene in intestinal enterocytes and embryonic visceral endoderm in mice is essential for viability and that the loss of function of this gene causes a rapid shift from anabolic to catabolic metabolism in the animal accompanied by a devastating loss of intestinal integrity and impaired stem cell differentiation.

As you will see when you read this book, the field of zinc biology has matured rapidly in the past decade. The current availability of zinc-sensing fluorescent probes, zinc-transporter genes, and expression vectors, antibodies (still a weak point), and genetic mouse models allows investigators to probe mechanistic aspects of zinc metabolism in great depth. Evidence for functions of zinc and specific zinc transporters in several diseases has emerged, including functions in cancer as well as in normal growth and development. Studies of structure–function relationships in zinc transport proteins are rapidly progressing, and an active field of investigation involves understanding the biophysics of zinc–protein interactions in regulatory proteins and the multiple mechanism of cellular and organismal zinc sensing. We can look forward to many exciting and novel findings in this field over the next few years.

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References

Andrews GK, Geiser J (1999) Expression of metallothionein-I and -II genes provides a reproductive advantage during maternal dietary zinc deficiency. J Nutr 129:1643–1648

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 U S A 96:1716–1721

Dalton TP, Fu K, Palmiter RD, Andrews GK (1996) Transgenic mice that over-express metallothionein-I resist dietary zinc deficiency. J Nutr 126:825–833

Dufner-Beattie J, Weaver BP, Geiser J, Bilgen M, Larson M, Xu W et al. (2007) The mouse acrodermatitis gene Slc39a4 (*ZIP4*) is essential for development and heterozygosity causes hypersensitivity to zinc deficiency. Hum Mol Genet 16:1391–1399

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 U S A 93:5624–5628

Geiser J, Venken KJ, De Lisle RC, Andrews GK (2012) A mouse model of acrodermatitis enteropathica: loss of intestine zinc transporter ZIP4 (Slc39a4) disrupts the stem cell niche and intestine integrity. PLoS Genet 8:e1002766

Geiser J, De Lisle RC, Andrews GK (2013) The zinc transporter Zip5 (Slc39a5) regulates intestinal zinc excretion and protects the pancreas against zinc toxicity. PLoS One 10: e0082149

Guerinot ML (2000) The ZIP family of metal transporters. Biochim Biophys Acta 1465:190–198

Kambe T, Geiser J, Lahner B, Salt DE, Andrews GK (2008) Slc39a1 to 3 (subfamily II) Zip genes in mice have unique cell-specific functions during adaptation to zinc deficiency. Am J Physiol Regul Integr Comp Physiol 294:R1474–R1481

Kury S, Dreno B, Bezieau S, Giraudet S, Kharfi M, Kamoun R et al. (2002) Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. Nat Genet 31:239–240

Laity JH, Andrews GK (2007) Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor (MTF-1). Arch Biochem Biophys 463:201–210

Masters BA, Kelly EJ, Quaife CJ, Brinster RL, Palmiter RD (1994) Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. Proc Nat Acad Sci U S A 91:584–588

Moynahan EJ (1974) Letter: *Acrodermatitis enteropathica*: a lethal inherited human zinc- deficiency disorder. Lancet 2:399–400

Palmiter RD (1987) Molecular biology of metallothionein gene expression. EXS 52:63–80

Palmiter RD, Findley SD (1995) Cloning 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

Prasad AS (1984) Discovery and importance of zinc in human nutrition. Fed Proc 43:2829–2834

Radtke F, Heuchel R, Georgiev O, Hergersberg M, Gariglio M, Dembic Z et al. (1993) Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. EMBO J 12:1355–1362

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

Zhao H, Eide D (1996) The yeast *ZRT1* gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc Nat Acad Sci U S A 93:2454–2458

Preface

More than five decades ago, Dr. Ananda S. Prasad discovered zinc (Zn) as the essential trace element for human life. Zn deficiency was the first discovery in Zn imbalance-related abnormality that causes growth retardation, immunodeficiency, hypogonadism, and neuronal and sensory dysfunctions. Human diseases including cancer, diabetes, osteoporosis, dermatitis, and auto-immune and neurodegenerative diseases have been shown to be associated with abnormal Zn status. Investigations of the biological roles of Zn, however, had been challenging because Zn compounds are normally colorless, and the natural status of Zn is stable as a divalent cation, unlike other bioactive metals such as iron and copper.

Until now, there have been at least four issues that advanced our knowledge about the significant roles of Zn in physiology and diseases. First: bioinformatics, which revealed that approximately 10 % of all proteins in humans may bind with Zn. Second: genetic approaches using animal models and human genetics, which contributed to demonstrating the physiological roles of Zn in cells, tissues, and the whole body. Third: investigation of Zn transporters and metallothioneins in vitro and in vivo, which provided a variety of information on the importance of Zn transportation within and between cells, which led us to the fourth issue: Zn indeed acts as a signaling factor like calcium, called "Zn signaling". Because this is a quite new field, we were motivated to introduce the current status of the study of Zn signaling and to review the whole scheme of this area to date.

The present book overviews up-to-date information on the study of Zn signaling, describing not only the essence of Zn signaling including its history, the molecular analysis of the structures and functions of Zn transporters and metallothioneins, and detection techniques for Zn signals, but also the involvement of Zn signaling in physiology and disease status as in brain function, immunity, inflammation, skeletogenesis, diabetes, and cancer. Besides the introduction of new insights in the study of Zn signaling, this book aims to address the many unsolved problems in the field. For this reason, we made a great effort to furnish educational contexts that will provide great introductions for students, young scientists, and clinical personnel. These contexts can also be valuable references for the pioneers and aficionados among researchers involved with Zn. So that all these goals would mesh, we as editors invited contributions from investigators who are world leaders in this field.

We believe the publication of this book is timely for reviewing the nature of Zn signaling, in which there is growing evidence that Zn signals regulate intra- and extracellular events leading to biological homeostasis, as all the authors will discuss. Also, we are confident that readers will find the book valuable for teaching, lecturing, and other outreach activities that can help make known to the public the importance of Zn itself. Finally, we express our heartfelt thanks to the splendid contributions of all authors, which will lead us to our goal.

Yokohama, Kanagawa, Japan — Toshiyuki Fukada, Ph.D.
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Contents

Chapter 1
Introduction: "Zinc Signaling"–The Blossoming Field of Zinc Biology

Taiho Kambe

Abstract Zinc plays an indispensable role in life. It regulates a great number of protein functions including transcription factors, enzymes, adapters, receptors, and growth factors as a structural or catalytic factor. Recently, a further function of zinc has received extensive interest and attention because of its potential importance as a signaling mediator. Zinc plays a dynamic role as an extracellular and intracellular signaling factor, which enables communication between cells in an autocrine, paracrine, or endocrine fashion, conversion of extracellular stimuli to intracellular responses, and regulation of various intracellular signaling pathways. These zinc functions are recognized as "zinc signaling," which has critical roles in physiology, and thus their imbalance can cause a variety of problems with regard to human health. This book extensively reviews the field from the basic aspects to the crucial roles of zinc signaling in biological processes, discussing the future directions and questions at both the molecular and the physiological level.

Keywords Zinc • Zinc homeostasis • Zinc signaling

1.1 Introduction

There are two types of zinc ions in the body. One is protein-binding zinc, which functions as a structural component and enzyme cofactor to stabilize and functionalize proteins. Proteome analyses estimate that approximately 10 % of proteins encoded in the human genome have potential zinc-binding motifs (Andreini et al. 2006). The other is labile zinc, which means "free" zinc ions. The amount of free zinc ions in the cytosol maintained is extremely low and is estimated to be less than nanomolar concentrations, while it is high in some kind of zinc containing vesicles such as insulin granules and synaptic vesicles. (Kambe et al. 2004; Maret 2011). Recent studies have revealed exciting information about the dynamic roles of free zinc ions in a great number of biological processes (Frederickson et al. 2005; Hirano et al. 2008; Sensi et al. 2009; Haase and Rink 2009, 2014; Fukada et al. 2011), which has highlighted the signaling functions of zinc, generally called "zinc signaling" (Fig. 1.1).

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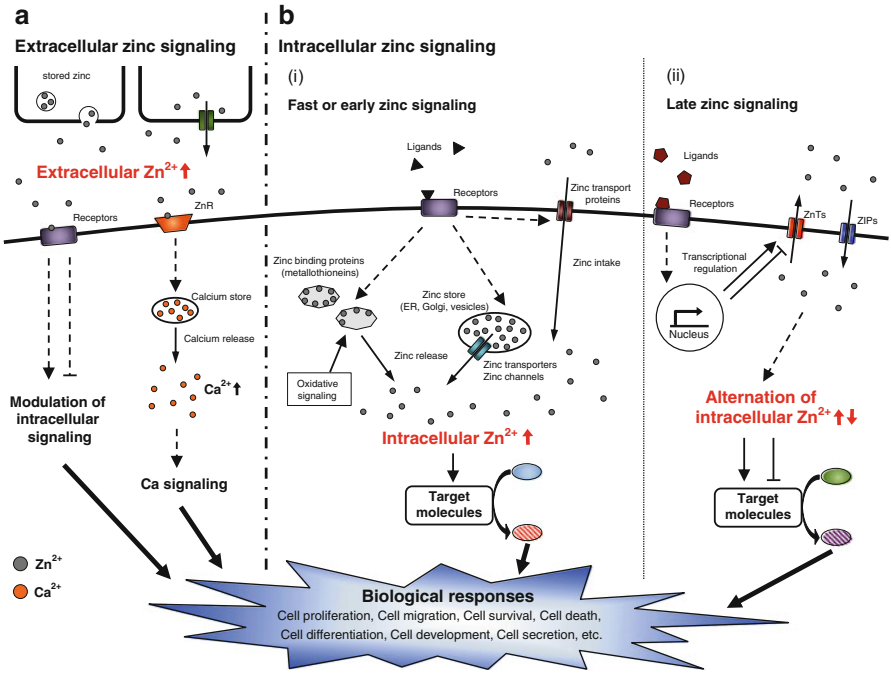

Fig. 1.1 Schematic diagrams of zinc signaling. Zinc signaling is caused by alternation in zinc concentrations in both the extracellular and intracellular milieu and can be divided into several types. **a** "Extracellular zinc signaling" takes place in extracellular milieu such as the synaptic cleft. In extracellular zinc signaling, zinc, which is released from cells via exocytosis of vesicular zinc or zinc efflux across the plasma membrane by zinc transport proteins, acts as a ligand of receptors expressed on the cellular membrane of target cells. One example is the ZnR (zinc receptor) signaling that activates calcium (Ca) signaling in different types of cells, and another example is α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) receptor signaling, which can modulate intracellular signaling in postsynaptic cells. **b** "Intracellular zinc signaling" is the result of extracellular stimuli via their receptors, which generally can be divided into two groups depending on the timescale in which it operates. *i* "Fast or early zinc signaling" occurs within seconds or a minute after the extracellular stimulus. In fast or early zinc signaling, intracellular free zinc is influxed from the extracellular space or released from intracellular zinc stores, such as the endoplasmic reticulum (ER), Golgi, and some vesicles, by zinc transport proteins, including ZIPs (Zrt, Irt-like proteins), AMPAR, and L-type calcium channels. Furthermore, zinc is released from cytosolic zinc-binding proteins such as metallothionein (MT), which can be triggered by oxidative stimuli. The increased intracellular free zinc functions as an intracellular second messenger as does Ca. *ii* "Late zinc signaling" occurs in the timescale of hours. In late zinc signaling, extracellular stimuli induce transcriptional regulation of zinc transport proteins including ZIPs and ZnTs (Zn transporters). This regulation can contribute to the alteration in intracellular zinc concentrations and zinc distribution across the cellular membrane, leading to regulation of downstream signaling pathways. All types of zinc signaling modulate a wide variety of biological responses such as cell proliferation, cell migration, cell survival, cell death, cell differentiation, cell development, cell secretion

Zinc signaling occurs via alterations in zinc concentrations in both extracellular and intracellular sites. It contributes to multifarious physiological and pathological processes, and the mechanisms underlying zinc signaling events have begun to be elucidated at the molecular level. Thus, it is very timely to review the extensive functions of zinc signaling. This book has 15 chapters that give a comprehensive understanding of the current knowledge of zinc signaling in physiology and pathogenesis. As an introduction to the book, zinc signaling is briefly overviewed from several different perspectives.

1.2 "Extracellular" Versus "Intracellular"

Zinc signaling can fall into two classes from the perspective of the location where it occurs: one is extracellular zinc signaling, and the other is intracellular zinc signaling (Fig. 1.1). The former has been extensively investigated in synaptic transmission in the central nervous system (CNS) (see Chaps. 4, 8, and 9), where zinc released in synaptic clefts from presynaptic neurons can modulate neurotransmitter functions via a number of receptor channels and transporters on postsynaptic neurons through its zinc-binding sites (Frederickson et al. 2005; Sensi et al. 2009). Besides synaptic transmission, zinc released with insulin from pancreatic β cells reduces insulin secretion from the β cells and suppresses insulin clearance in the liver (see Chap. 13). Thus, extracellular zinc signaling mediates communication between cells in an autocrine, paracrine, or endocrine fashion. For sensing extracellularly released zinc, zinc receptor (ZnR), which is identical to G protein-coupled receptor 39 (GPR39), is important (see Chap. 6).

Intracellular zinc signaling, which plays a role in a great number of cell types, has been extensively investigated in immune cells (see Chaps. 10 and 11). In the cytosol, zinc functions as a second messenger of extracellular signals similarly to Ca. The "zinc wave," which has been identified as zinc release from the perinuclear area including the endoplasmic reticulum (ER) in antigen-stimulated mast cells (Yamasaki et al. 2007), is well known (see Chap. 5). Release of zinc from proteins with zinc-thiol/disulfide coordination such as metallothionein (MT) is thought to contribute to intracellular zinc signaling (see Chaps. 2 and 4). In intracellular zinc signaling, a number of molecular targets have been identified, including protein tyrosine phosphatases (PTPs), phosphodiesterases (PDEs), caspases, and kinases (Huber and Hardy 2012; Wilson et al. 2012). In oocytes, zinc is taken up during the final stage of maturation, and then released into the extracellular milieu on fertilization, which is named the "zinc spark." Zinc spark decreases intracellular zinc content, thereby contributing to regulation of cell signaling (see Chap. 15).

1.3 "Membrane Transport Protein-Mediated" Versus "Cytosolic Protein-Mediated"

Alteration of zinc concentrations initiates zinc signaling; therefore, zinc transport proteins have critical roles in this process (see Chap. 3). ZIP (Zrt, Irt-like protein) and ZnT (Zn transporter) transporters are the major two zinc transport proteins and thus have crucial roles in zinc signaling (see Chaps. 12 and 14). Both transporters are localized to the plasma membrane and membranes of the intracellular compartments, which indicates that zinc is mobilized between the extracellular space or the lumen of intracellular compartments and the cytosol across cellular membranes. In addition to ZIPs and ZnTs, it has been shown that other membrane transport proteins are involved in triggering zinc signaling (see Chaps. 3 and 5).

An alternative mechanism of zinc signaling is by zinc release from proteins with oxidation-sensitive zinc-binding sites in the cytosol, which is triggered via oxidative signaling (see Chap. 2). However, zinc released by oxidative stress can be involved in deleterious signaling pathway (see Chap. 4). Most likely, cooperative regulation of zinc signaling by zinc transport proteins and cytosolic proteins such as MT is required for crucial roles in physiology.

1.4 "Fast" and "Early" Versus "Late"

Extracellular stimuli can increase or decrease the cytosolic concentration of labile zinc within minutes or several hours (Fig. 1.1). Thus, intracellular zinc signaling can also be classified into several classes according to the timescale in which it acts (see Chap. 10). Zinc signaling occurring within minutes is termed "fast" or "early" zinc signaling. Fast or early zinc signaling does not need transcription of proteins (reviewed by Hirano et al. 2008; Haase and Rink 2009, 2014; Fukada et al. 2011). Fast zinc signaling occurs within seconds to a minute, and early zinc signaling, for example, a "zinc wave," occurs within minutes after triggering; in both cases, zinc ions serve as a type of second messenger (see Chaps. 5 and 10). The other type of zinc signaling, which requires transcription of zinc transport proteins including ZIP and ZnT transporters, occurs on a timescale of hours. This type of zinc signaling is termed "late" zinc signaling. In late zinc signaling, local zinc homeostasis is changed in zinc concentrations and distribution by changes of expression levels of zinc transport proteins in response to various stimuli such as cytokines and lipopolysaccharide (Kambe 2013). Thus, late zinc signaling is important as homeostatic control mechanisms.

1.5 Conclusions

The increasing body of evidence clearly reveals that zinc signaling is essential for life, but it is also clear that recent findings represent the very tip of the iceberg. Zinc signaling is essential for human physiology, and its dysfunction likely causes health deterioration and diseases including cancer, inflammatory diseases, diabetes, and neurodegenerative diseases, as shown in this book. There are still many questions and ongoing discussions in the field of zinc signaling. However, novel techniques, such as genetically encoded fluorescent sensors (see Chap. 7), will contribute to clarifying those questions and discussions, similarly to the case of small fluorescent probes, which have greatly contributed to detecting and monitoring both extracellular and intracellular zinc signaling (Nolan and Lippard 2009).

This book is the first one to extensively review zinc signaling. Readers will enjoy each chapter with interest and find that zinc signaling definitely goes mainstream!

References

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196–201

Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6:449–462

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signaling in health and diseases: zinc signaling. J Biol Inorg Chem 16:1123–1134

Haase H, Rink L (2009) Functional significance of zinc-related signaling pathways in immune cells. Annu Rev Nutr 29:133–152

Haase H, Rink L (2014) Zinc signals and immune function. Biofactors 40:27–40

Hirano T, Murakami M, Fukada T, Nishida K, Yamasaki S, Suzuki T (2008) Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. Adv Immunol 97:149–176

Huber KL, Hardy JA (2012) Mechanism of zinc-mediated inhibition of caspase-9. Protein Sci 21:1056–1065

Kambe T (2013) Regulation of zinc transport. In: Culotta V, Scott RS (eds) Encyclopedia of inorganic and bioinorganic chemistry: metals in cells. Wiley, Chichester, pp 301–309

Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M (2004) Overview of mammalian zinc transporters. Cell Mol Life Sci 61:49–68

Maret W (2011) Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. Biometals 24:411–418

Nolan EM, Lippard SJ (2009) Small-molecule fluorescent sensors for investigating zinc metalloneurochemistry. Acc Chem Res 42:193–203

Sensi SL, Paoletti P, Bush AI, Sekler I (2009) Zinc in the physiology and pathology of the CNS. Nat Rev Neurosci 10:780–791

Wilson M, Hogstrand C, Maret W (2012) Picomolar concentrations of free zinc(II) ions regulate receptor protein-tyrosine phosphatase beta activity. J Biol Chem 287:9322–9326

Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E et al (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645

Chapter 2
Molecular Aspects of Zinc Signals

Wolfgang Maret

Abstract Zinc ions (Zn^{2+}) have chemical properties that make them ideally suited to carry biological information in intracellular and intercellular communication. Such zinc signaling has much in common with calcium signaling in terms of fast binding in coordination environments of proteins, but there are also important differences between the two metal ions. Biological control with zinc occurs at much lower metal ion concentrations. Zinc ions bind with higher affinity and hence dissociation rates are slower, resulting in longer-lasting biological effects. Selectivity of coordination environments is different as zinc employs oxygen, nitrogen, and sulfur donors from ligands whereas calcium binds almost exclusively to oxygen donors. Zinc and calcium ions are redox inert but sulfur donors in zinc/thiolate coordination environments confer redox activity, thereby linking zinc metabolism and redox metabolism. In humans, 24 zinc transporters and more than 12 metallothioneins exert precise control over cellular zinc homeostasis, cellular redistribution, and transients of zinc ions that are used for biological regulation. Zinc ions are stored in subcellular compartments and released by different stimuli. Rises in cytosolic Zn^{2+} concentrations target proteins and affect a variety of cellular processes, such as phosphorylation signaling and gene expression. Zinc signaling complements and interacts with calcium signaling and redox signaling and is an integral part of the cellular signal transduction network. It has fundamental importance for health and disease.

Keywords Zinc • Zinc buffering • Zinc homeostasis • Zinc muffling • Zinc signaling

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2.1 Historic Perspective: The Emerging Role of Zinc Ions in Cellular Control

Through its catalytic and structural functions in about 3,000 human proteins, zinc is associated with about 10 % of all proteins and affects most aspects of cellular biology (Andreini et al. 2006; Rink 2011). The concept that zinc has regulatory functions in multicellular organisms enhances its importance even further. The concept is recent but rooted in rather early reports. One relates to the transcription factor MTF-1 (metal regulatory transcription factor-1), which senses increased cellular zinc concentrations and mediates zinc-dependent gene expression (Günther et al. 2012). The other is the discovery of autoradiographically detectable zinc in certain parts of the brain (hippocampus), which was found to be *zinc ions* (Zn^{2+}) stored in a yet to be defined chemical form in synaptic vesicles of specific zinc-rich neurons and secreted into the synaptic cleft when these neurons are stimulated. Acting like a classical neurotransmitter in these specialized neurons, the zinc ions affect presynaptic and postsynaptic physiology (Frederickson et al. 2005; Sensi et al. 2009; Takeda et al. 2013). These discoveries expanded into fields other than neurobiology when zinc ions were detected autoradiographically in other organs and tissues (Danscher and Stoltenberg 2005). It is now known that cells sequester zinc ions in cellular vesicles ("zincosomes") and other organelles and that this is a way for the cell to store zinc and to make it available for specific processes. This aspect is different from iron metabolism, where ferrous ions are oxidized to ferric ions and stored in the protein ferritin. In addition to the specialized neurons, a variety of cells secrete zinc ions by vesicular exocytosis (Box 2.1). This process differs from merely exporting zinc, which is an activity of zinc transporters in every cell. The presence of subcellular pools of zinc ions was verified and thus became a much more general issue. Investigations then began to address the functional significance of the zinc ions that are not bound to proteins.

Box 2.1 Cells Secreting Zinc Ions

- Pancreatic β cells and acinar cells
- Prostate epithelial cells (tubuloacinar cells)
- Mammary gland epithelial cells
- Epididymal epithelial cells
- Paneth cells (crypts of Lieberkühn)
- Somatotrophic cells (pituitary gland)
- Osteoblasts
- Platelets
- Mast cells
- Granulocytes, neutrophils
- Fertilized oocytes

Numerous experiments demonstrated that changing zinc in cells by either adding zinc ions extracellularly or by removing them with chelating agents affects multiple signaling pathways (Beyersmann and Haase 2001). These observations gain new significance as they mimic the events that occur when transients of zinc ions are induced intracellularly. There has been an initial focus on immune cells (Hirano et al. 2008; Haase and Rink 2014), but the field is rapidly moving into investigations of other cell types (Fukada et al. 2011; Taylor et al. 2012a). In particular, with the advent of fluorescent chelating agents ("probes") for detecting cellular zinc ions and their fluctuations it became known that zinc ions are intracrine, paracrine, and perhaps even endocrine signals. With this abbreviated account of the origins of the field, three principles are now recognized for the ways cellular zinc ions are made available for signaling functions:

1. Vesicular exocytosis
2. Opening of gated channels on vesicular/organellar stores and the plasma membrane
3. Modification of zinc/thiolate coordination environments in proteins

The first two principles involve zinc in vesicles whereas the third involves zinc in proteins.

In this chapter, I discuss the molecular framework that makes signaling with zinc ions possible. To understand zinc signaling, it is critical to address and define the meaning of the terms "zinc ions" and "zinc signals." These terms are used with ambiguity in the literature. Biologists tend to refer to "zinc," but what they mean is chemically the zinc ion. Zinc(II) (Zn^{2+}) is the only valence state of zinc in biology. Implied in signaling are free zinc ions that are not bound to proteins. Zinc in proteins is also in the form of zinc ions but tightly bound and hence not free. This distinction is the only basis for the term "free," and the term does not refer to an absence of coordinating ligands. There is presently no method to examine the chemical speciation of zinc at the low concentration of free zinc ions in the cytosol or in organelles, and therefore it is not a foregone conclusion that the main species is the hydrated ion $Zn(H_2O)_6^{2+}$. Spectroscopic analysis of zinc in cellular vesicles, where the free zinc ion concentrations are considerably higher, demonstrated interactions with ligands other than water (Wellenreuther et al. 2009). In vesicular exocytosis, zinc ions with their ligands move to an environment with a different pH value. Whether ligand exchange occurs after exocytosis is not known. Free zinc ions may have different ligands and concentrations in the cell ("i", intracellular), outside the cell ("e", extracellular) and in organelles ("o", organellar), and therefore the concentrations of zinc ions in the different pools are referred to as $[Zn^{2+}]_{i,e,o}$. An alternative to low molecular weight complexes of zinc ions being diffusible signals would be that zinc signaling occurs via protein-mediated zinc transfer without the ion ever being free (Fig. 2.1). Such interprotein associative zinc transfer has been observed in vitro (Maret et al. 1997). However, aside from metallothioneins, metallochaperones for such zinc delivery to another protein are not known. Given the large number of structurally diverse zinc proteins, associative zinc transfer is unlikely to be the general case for zinc redistribution as it would require too many

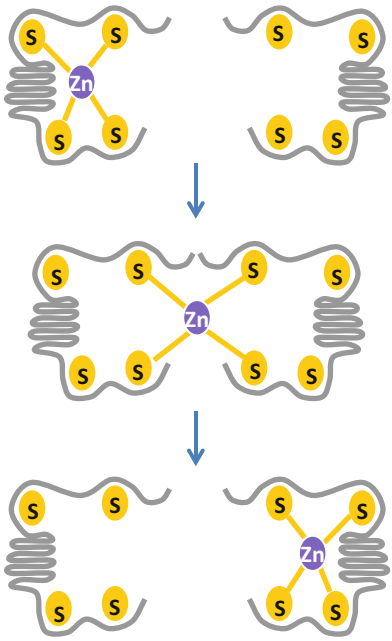

Fig. 2.1 Zinc/thiolate coordination environments as conduits for zinc transfer. Zinc ions can be transferred between two proteins by swapping ligands (associative mechanism). Zinc ions are never free in this reaction. Sulfur donors are particularly well suited for such reactions

chaperones for recognition of all the different zinc proteins, in contrast to copper, the most competitive ion in the Irving–Williams series (see following), which is controlled by chaperones. The fact that free zinc ion concentrations and fluctuations can be measured and that they have functions at rather low concentrations makes them not negligible and supports the role of dissociative mechanisms in zinc signaling. Such mechanisms of redistribution do not exclude the participation of other, low molecular weight ligands as such ligands have been shown to accelerate the rate of zinc removal from proteins (Jacob et al. 1998; Chong and Auld 2007).

Under which conditions zinc ions become "zinc signals" also needs further consideration as the term zinc signaling is being used quite broadly. I suggest that one uses the analogy with the classic signaling metal ion Ca^{2+} to define zinc signaling (Table 2.1) and then focuses on the differences between the two ions, namely, characteristics of zinc such as its binding to sulfur and its higher affinity for proteins and other ligands. As for calcium signaling, the term zinc signaling should be used for fast processes, induced transients of zinc ions, and subsequent binding to an effector.

$$\text{Physiological stimulus} \rightarrow Zn^{2+} \rightarrow \text{effector}$$

This sequence does not exclude MTF-1 from participating in zinc signaling, but it is the rapid activation of MTF-1 as the direct result of a zinc signal rather than the slower consequence of this activation resulting in changes in mRNA and protein abundance.

Table 2.1 Comparison of calcium and zinc signals

Ca^{2+}		Zn^{2+}
Dissimilar:	*Similar*: Subcellular stores involved in release Buffers and mufflers Plasma membrane receptor	*Dissimilar*:
Outward gradient		Inward gradient
O coordination environments		O, N, and S coordination environments
e.g., calmodulin (O chemistry)		e.g., metallothionein (S chemistry)
		Chemical mechanism of release
Short duration of effects		Long duration of effects
Transients >100 nM		Transients >100 pM
Mostly activation of processes		Mostly inhibition of processes

One finds terms such as labile zinc, mobile zinc, or rapidly exchanging zinc in the literature. These terms imply different characteristics of zinc or lack a chemical definition altogether. In fact, they may prevent understanding the molecular basis of zinc ion signals. I believe it is best practice to use one operational definition such as free zinc to refer to zinc ions that are not bound to proteins, albeit with the notion that the coordination environment of the zinc ions is not defined. How strongly proteins bind zinc and how zinc is buffered determine the concentrations of these free zinc ions.

2.2 Control of Zinc Ions

2.2.1 Zinc Buffering

The concepts of free zinc and zinc signaling are intricately linked to metal buffering. Metal buffering is treated as is pH buffering. In the same way that one defines a pH value (hydrogen potential, $pH = -\log [H^+]$), one defines a pZn value (zinc potential, $pZn = -\log [Zn^{2+}]$). pH is determined by the pK_a of the buffering acid–base pair (1), and pZn is determined by the pK_d of the buffering ligand(s) (2):

(1) $pH = pK_a + \log ([A]/[AH])$
(2) $pZn = pK_d + \log ([L]/[ZnL])$

For the most part, the zinc buffering ligands in biology are the coordinating side chains of proteins (Asp, Glu, His, Cys). The dissociation constants (pK_d) for *cytosolic* zinc proteins are picomolar, $pZn = <9$ and >12 (Maret 2004a; Krężel and Maret 2008), although some may be lower, as discussed later. Thus, on the

basis of equilibrium constants, the concentrations of free zinc ions should also be picomolar. One could argue that biomolecules other than proteins contribute to zinc buffering. Many biomolecules bind zinc (free amino acids, carboxylic acids, peptides such as glutathione, ATP, etc.). However, these biomolecules do not bind zinc as tightly as proteins and hence it is unlikely that they contribute significantly to zinc buffering, at least not in binary or 1:1 complexes. To examine this point, the analogy to pH buffering is again helpful. A buffer with a different pK_a value contributes to a buffer at a given pK_a only if higher concentrations compensate for the differences in pK_a values. As we are concerned with logarithmic scales, one pK_a unit would need to be compensated by one order of magnitude in concentrations. Thus, ligands with different pK_d values would contribute to zinc buffering only at much higher concentrations. For zinc-binding biomolecules with lower pK_d values, such as those just mentioned, high concentrations would be required to contribute to physiological zinc buffering. An equally important consideration is how strongly zinc is buffered: this is *not* an issue of the pZn value but an issue of the *buffering capacity at the particular pZn where zinc is buffered*.

2.2.2 Zinc-Buffering Capacity

There is considerable uncertainty in the literature regarding cellular zinc-buffering capacity because it is often assumed to be high as a result of the many ligands that *potentially* bind zinc. It is not the buffering capacity that keeps the free zinc ion concentrations low but the affinity of the ligands. The buffering capacity determines how resistant the pZn is to change.

It is often remarked that "zinc is tightly/strongly buffered" when investigators mean that there is very little free zinc. The low free zinc ion concentrations are a consequence of the high-affinity binding sites in proteins, not the buffering capacity. Tight and weak buffering refers primarily to the buffering capacity, *which is not high as it allows pZn fluctuations to occur*. We determined the cellular buffering capacity at the physiological pZn to be only about 10 % of the total zinc ion concentrations of a few hundred micromolar (Krężel and Maret 2006). This finding has important consequences as it means that some coordination sites with high affinity for zinc are not occupied at physiological pZn and can bind zinc when free zinc ion concentrations increase. These sites determine physiological zinc buffering. Overall, the zinc-buffering capacity of all the ligands is high, but it is not relevant. Zinc is (and must be) buffered at a particular physiological pZn, and only the buffering capacity of the ligands that control this pZn is important. If the buffering capacity were high at physiological pZn, zinc signaling could not occur as any zinc signal would be quenched immediately. Changes in pZn are necessary for zinc signaling to occur. Thus, the cytosolic zinc-buffering capacity and the physiological pZn are major determinants for the role of zinc ions in signaling.

2.2.3 The Role of Zinc Transporters: Muffling

Transporters bring zinc ions into the cell and into cellular compartments or export them from the cell and from these compartments. Transport processes also contribute to biological metal ion buffering and have been referred to as muffling in the calcium field (Thomas et al. 1991). Muffling zinc ions increases the cellular zinc-buffering capacity because it removes an excess of cytosolic zinc ions (Colvin et al. 2010). Thus, what we see as buffering in a cell is a combination of (thermodynamic) buffering by ligands and (kinetic) muffling by transporters. The contribution of transporters in muffling depends on their transport characteristics and the capacity of intracellular stores. Very little quantitative information is available to define these parameters that determine the timescale, frequency, and amplitudes of zinc signals.

Computational approaches in combination with experimental data have been used to model muffling and zinc transients in neurons (Colvin et al. 2008). It became evident that a steady-state buffer model is not sufficient to explain how cells handle extracellular zinc loads, which can mimic cellular transients of zinc ions. A cellular protein that binds zinc and delivers it to a store was required. One molecule that satisfies the required characteristics is metallothionein (MT). A dual role in buffering and muffling zinc, that is, transporting zinc for the delivery to a store, resolves the issue that the concentrations of MTs are generally not high enough to account for the overall cellular zinc-buffering capacity.

This discussion explains why changing the cellular zinc muffling and buffering capacities and hence the pZn but not the total zinc concentration allows zinc redistribution.

2.2.4 Quantitative Measurements

A quantitative approach is needed to define signaling with zinc ions. Total cellular zinc concentrations are rather high, that is, a few hundred micromolar. However, the free zinc ion concentrations (pZn) are picomolar, about six orders of magnitude lower. Free zinc ion concentrations have been estimated and reported in two classic articles. One considers that zinc inhibits muscle phosphoglucomutase, a magnesium enzyme, with high affinity for zinc in vitro (Peck and Ray 1971). For the enzyme not to be zinc inhibited in muscle, the free zinc ion concentration must be less than 32 pM (Ray 1969). It is noteworthy that this consideration merely sets a limit on the enzyme being fully active but it leaves the possibility that free zinc ion concentrations are indeed higher than 32 pM in muscle tissue and modulate the activity of this enzyme. In the other article, free zinc ion concentrations of 24 pM in erythrocytes have been determined (Simons 1991). One expects pZn values in this range on the basis of the affinity of zinc proteins for zinc. With the advent of new molecular probes for zinc ions, that is, chelating agents that become highly fluorescent when binding zinc, picomolar values of free zinc were confirmed in different cell lines,

although the reported values vary between tens and hundreds of picomolar (Krężel and Maret 2006; Bozym et al. 2006; Vinkenborg et al. 2009; Qin et al. 2011). Zinc sensors based on the zinc enzyme carbonic anhydrase measure 5–10 pM, but other protein sensors as well as low molecular weight probes measure hundreds of pM (see table 1 in Maret 2013a). Any probe or sensor that is brought into the cell to measure free zinc contributes to zinc buffering and *lowers free zinc ion concentrations*. We have measured this effect as a function of different probe concentrations and applied an extrapolation to a zero probe concentration to correct for the additional buffering of the probe, and indeed found higher free zinc ion concentrations in the absence of the probe (Krężel and Maret 2006). Some investigators refer to the zinc ions that the probes measure as labile zinc. When extrapolation is applied, the zinc measured cannot be labile because there is no probe to remove zinc from other ligands. The term labile zinc should not indicate that zinc bound weakly to proteins coexists with tightly bond zinc in the same cellular compartment. The free zinc ion concentration determined in the absence of a probe is simply the result of existing equilibria. Tightly bound zinc can be kinetically labile, however. For example, MT binds zinc tightly but the sulfur ligands allow fast exchange with other zinc/thiolate coordination environments (Maret et al. 1997). Measured cellular pZn values indicate that predictions based on zinc-binding affinities of proteins are valid and that there is no buffering increment from other biomolecules that would lower the free zinc ion concentration further.

Although consensus is building regarding cellular (cytosolic) pZn values and zinc-buffering capacity, pZn values in different subcellular compartments are just beginning to be defined. Estimates are 0.9 pM for the endoplasmic reticulum (ER), 0.2 pM for the Golgi (Qin et al. 2011), 0.2 pM for the mitochondrial matrix (McCranor et al. 2012), and 0.14 pM for mitochondria (Park et al. 2012). However, there is a fundamental issue with these concentrations as well as those given for small structures such as erythrocytes. Taking the proton as an example, calculations demonstrate that there are limits to volumes and concentrations where there is essentially no longer a single free ion (Bal et al. 2012a). In other words, given the same concentrations for a larger volume such as the cell and for a much smaller volume such as an organelle leads to a seemingly unrealistically small number of ions in the latter.

Few data exist on extracellular free zinc ion concentrations. Experimental estimates are about 200 pM for horse blood plasma (Magneson et al. 1987), suggesting there is virtually no gradient between extracellular and intracellular free zinc. With higher total intracellular (>100 μM) than extracellular (<10 μM) zinc concentrations, one wonders about the source of energy to transport zinc into the cell against this gradient. However, recent examination of human blood plasma revealed free zinc concentrations of about 500 nM (Maarten Merkx, personal communication). This finding has important implications as it allows zinc binding to serum albumin, which has an apparent zinc-binding constant of about 100 nM (Ohyoshi et al. 1999; Bal et al. 2012b). Such zinc ion concentrations are also in the range for free zinc to be considered as the substrate of zinc transporters. For example, zebrafish ZIP1 has a K_m value for zinc of less than 500 nM (Qiu et al. 2005).

The necessity of buffering zinc in cells with resulting picomolar free zinc ion concentrations becomes evident when considering the properties and the control of

other essential metal ions. A fundamental principle in inorganic chemistry, the Irving–Williams series (Irving and Williams 1948), describes relative binding affinities of divalent transition metal ions and the free metal ion concentrations resulting from binding equilibria. For the cell to keep the biochemistry of metal ions separated from each other, every metal ion needs to be controlled in a specific range of pM. Without such control, the more competitive ions such as zinc and copper would bind to sites that need to be occupied by the less competitive ions. There is no free ranging of metal ions over concentrations needed for the control of other metal ions. According to the Irving–Williams series, the affinity of the biologically relevant divalent metals ions to the same ligands is $Mn < Fe < Co < Ni < Cu > Zn$; that is, zinc is the most strongly bound divalent ion after copper. Thus, for the binding equilibrium

$$P + Zn^{2+} \rightarrow ZnP + 2H^{+}$$

with picomolar affinities of the proteins (P), picomolar free zinc ion concentrations result. There is a scarcity of data about how greatly the affinities of zinc proteins for zinc differ. If there were sites with more loosely bound zinc, there would be considerable dissociation of zinc, loss of function, and interference with the functions of other metal ions. There is no evidence for a hierarchy in such a way that some proteins yield their zinc under zinc deficiency to preserve the functions of other, potentially more essential zinc proteins. Some proteins bind zinc more strongly, with femtomolar affinities for zinc (Sikorska et al. 2012). The reason for this could be that at very low protein concentrations, such as for low abundance zinc proteins, zinc would dissociate if the binding were not strong enough, thereby compromising the function of the protein.

First principles also relate free zinc ion concentrations to biological events. It has been estimated that it would take about 14 h for a zinc enzyme to obtain its zinc at a concentrations of 1 pM free zinc and with a comparatively fast on-rate (Heinz et al. 2005). Clearly, if free zinc concentrations were so low—as they appear to be in some cellular compartments (see earlier)—they could not serve as a source of zinc for enzymes unless low molecular substances enhance the transfer rates (see foregoing) (Heinz et al. 2005). Measured free zinc ion concentrations in the range of a few hundred picomolar, however, are sufficient for association rates on a biologically relevant timescale, in the range of seconds. Of course, this argument also applies to zinc signaling: Zinc ion concentrations need to be at least a few hundred picomolar to affect proteins sufficiently rapidly.

Zinc sites of most zinc proteins seem to be fully occupied with zinc. It would be a significant waste of energy to synthesize a large protein and then not have the zinc available for function. How the synthesis of a zinc protein and the supply of zinc are coordinated and how, when, and where zinc proteins acquire their zinc is not known. There is no experimental evidence that mononuclear sites in zinc proteins are regulated by zinc ion fluxes. The evidence is less clear for enzymes with a second, co-catalytic zinc. These enzymes could be regulated through zinc ion fluctuations. Zinc regulation must occur above the free zinc ion concentrations

that keep zinc proteins saturated. Regulation also requires mechanisms that restore the steady-state free zinc ion concentrations after transients have occurred.

Several investigators attempted to quantify *global* fluctuations of intracellular free zinc ion concentrations. The fluctuations are a few hundred picomolar above the steady-state concentrations of free zinc ions and may reach about one nanomolar (Table 2 in Maret 2013a). For example, when cells are deprived of extracellular zinc, they have a way of mobilizing zinc ions intracellularly (Li and Maret 2009). Also, synchronized rat pheochromocytoma (PC12) cells increase their free zinc ion concentrations at two stages during the cell cycle (Li and Maret 2009). Generally, fluctuations have been measured in different states of a cell and rarely in real time. Hence, the time period over which changes develop and persist is largely unknown.

However, *local* free zinc ion concentrations are expected to be significantly higher. They could establish microdomains akin to those in calcium signaling. Induced local zinc ion transients seem to be fast acting not to act pleiotropically or change gene expression. Although the transients are likely short lived, the effects are expected to be long lasting. Again, first principles support this prediction. With high affinity for zinc and fast on-rates, the corresponding off-rates must be slow. For example, the half-life for zinc dissociation from carbonic anhydrase with a pK_d of 11.4 (pH 7.0) is about 250 days (Ippolito et al. 1995). Of course, carbonic anhydrase is not thought to be regulated by zinc. Because proteins that are targets of fluctuating zinc ions have affinities that are not significantly lower than those of genuine zinc proteins (see following), the off-rates, and hence the duration of zinc signals, are still of the order of many hours or even days. Thus, zinc signaling is expected to elicit long-lasting effects, which is in contrast to calcium signaling. Overall, this interpretation is consistent with zinc ions being inhibitory for cellular functions (Williams 1984) and being involved in long-term adjustments of the state of a cell, such as growth, differentiation, and survival.

Genuine zinc ion signals are to be distinguished from global changes to a different steady-state concentration of free zinc ions (pZn). For example, growth-arrested, proliferating, differentiating, and apoptotic intestinal epithelial cancer cells (HT-29) all have different pZn values (Krężel and Maret 2006), which seem to be the consequence of adjustments in buffering rather than changes in total zinc concentrations. If changes of total zinc were responsible for such adjustments, unreasonable large fluctuations in the range of hundreds of micromolar zinc would be required. Global adjustments of buffering can be brought about by changes of redox potentials that may affect specific redox pairs coupled to zinc binding and release. About 30 % of the zinc-buffering capacity at physiological pH depends on sulfur (thiol) donors and therefore is redox sensitive (Krężel et al. 2007). Changing the expression of zinc homeostatic proteins such as ZnT1 and MT, which are controlled by MTF-1, also changes zinc buffering. The dynamics of the MT pool is an example of how zinc buffering and pZn are interrelated. In different states of HT-29 cells, concentrations of total zinc, free zinc, metallothionein, buffering capacity, and the redox state are correlated (Krężel and Maret 2006). Changed buffering increases or decreases free zinc ion concentrations, and this is a cause for long-term adjustments. Indeed transcriptomics, and

more recently proteomics, investigations have shown extensive changes in protein expression profiles in response to zinc added to growth media or zinc removed by chelating agents.

2.3 Molecular Mechanisms of Proteins Involved in Cellular Zinc Homeostasis

In contrast to proteins that use zinc as a permanent cofactor, the proteins involved in regulating zinc (zinc transporters, metallothioneins, and MTF-1) and in being regulated by zinc have mechanisms for moving zinc and binding it reversibly in sites with coordination dynamics (Maret and Li 2009; Maret 2011a, 2012). MTF-1 has six zinc fingers (C_2H_2 coordination of zinc: C = Cys, H = His) for DNA recognition, but it is neither entirely clear how they are involved together with a metal-reponsive activation domain in zinc sensing nor whether free zinc ions are sensed in the cytosol before MTF-1 translocates to the nucleus (Laity and Andrews 2007; Günther et al. 2012). Three-dimensional (3D) structures of MTF-1 or any of the 24 mammalian zinc transporters [10 members of the ZnT family (SLC30A) and 14 members of the Zip family (SLC39A)] have not been reported and therefore there is virtually no insight into the transport and sensing mechanisms. However, detailed information about the sequences, biological regulation, and genetics of these transporters is available (Fukada and Kambe 2011). The number of zinc transporters is remarkable as homeostatic mechanisms for other metals ions such as iron and copper rely on only a few transporters. None of the mammalian zinc transporters uses ATP as a source of energy. The *Escherichia coli* Yiip protein, which belongs to the ZnT family, functions as a Zn^{2+}/H^+ antiporter and has served as the only model for human ZnTs (Lu and Fu 2007). It has three different zinc-binding sites, all of which use oxygen (Asp/Glu) and nitrogen (His) donors; one is located between the transmembrane helices and one at the interface between the transmembrane domain and the cytoplasmic domain. The third site is binuclear and located between the dimer interface of the cytoplasmic domains. It is thought to be a sensor site of cytoplasmic zinc ion concentrations. Once zinc is bound at these sites, a conformational change of the protein occurs and triggers zinc transport. At present, it is not known whether transporters contribute directly to maintaining physiological pZn. If they do, they would need to have K_m values in the picomolar range, many orders of magnitude lower than their experimentally observed values. Alternatively, the substrates could be proteins that deliver zinc to the transporters by an associative mechanism. Which zinc complexes are the substrates for these transporters and which ligands receive zinc on the opposite side of the membrane is not known.

About a dozen human MT genes are expressed and participate in the control of cellular zinc homeostasis (Li and Maret 2008). Their zinc coordination involves exclusively zinc/sulfur (thiolate) interactions. They have been thought to store zinc, and there is an extensive literature about their possible functions as cellular antioxidants. At least two chemical properties of MTs and the molecular biology

of MT gene expression support a dynamic role in zinc metabolism. One is that MTs have different binding constants for the seven zinc ions. The zinc affinities of human MT-2 are in the range where zinc regulation takes place, allowing MTs to transport, accept, and donate zinc ions dependent on cellular conditions (Maret 2011b). Although four zinc ions are bound with affinities similar to those of other zinc proteins, two zinc ions bind less tightly and one zinc ion only with nanomolar affinity, making MT a protein that is not saturated with zinc at physiological pZn (Krężel and Maret 2007). These properties would allow MT to buffer zinc exactly in the range where such buffering is required. The second property is that MTs are redox-active zinc proteins. Zinc itself is redox inert and remains Zn^{2+}, and hence zinc proteins were generally not considered to be redox proteins. However, the oxidation of the sulfur donors of the cysteine ligands of zinc causes zinc dissociation whereas the reduction of cystines (disulfides) to cysteines (thiols) generates zinc-binding capacity (Maret and Vallee 1998). This property establishes a cellular redox cycle that links redox changes and the availability of zinc from MT and other proteins with zinc/thiolate sites (Chen and Maret 2001; Maret 2009). Because of the redox activity of the sulfurs, MTs are indeed "antioxidants"; however, any discussion of an antioxidant function needs to consider that MT is oxidized when it reacts with oxidants and releases zinc ions that are potent effectors at very low concentrations, and that the released zinc itself can have pro-antioxidant and pro-oxidant functions depending on its concentrations (Maret 2008a).

2.4 Stimulation of Zinc(II) Ion Fluctuations

This section focuses only on the three basic mechanisms of zinc release. The signaling pathways and proteins triggering or controlling zinc release are treated in considerable depth in other chapters of this book.

2.4.1 Vesicular Exocytosis and Paracrine Signals

Zinc transporters ZnT3, ZnT8, and ZnT2 load cellular vesicles with zinc in the brain, pancreatic β cells, and mammary epithelial cells, respectively, for exocytosis of zinc ions. Neither the concentration of zinc in the vesicles (millimolar?) nor the local concentration of released zinc (submicromolar?) is exactly known. Exocytotic vesicles have slightly acidic pH favoring free zinc ions, but once the zinc is exocytosed it is in an environment of higher pH, which favors zinc binding to proteins. Zinc ions secreted from neurons diffuse over a distance of 100 μm, and the signal is eliminated within tens of seconds (Ueno et al. 2002). This investigation demonstrates the significance of the spatiotemporal characteristics of extracellular zinc signals and makes it an issue over which space and time measured zinc ion concentrations are averaged. Owing to their high affinity for proteins, free zinc ions

are "sticky" and, depending on the environment, only fleetingly existent, a phenomenon that has been referred to as a zinc "veneer" forming on the surface of cells (Nydegger et al. 2012). Zinc ions secreted from specific neurons are paracrine signals affecting postsynaptic events. Zinc secreted together with insulin from pancreatic β cells is discussed as an effector of pancreatic α cells and, in addition, has been suggested to be an endocrine signal affecting blood insulin clearance by the liver (Tamaki et al. 2013). Potentially, there are many more functions for the zinc ions secreted from different cells (Box 2.1).

The release of zinc ions from fertilized mouse oocytes into the extracellular space follows calcium ion oscillations and has been referred to as "zinc sparks." Whether this process involves vesicular exocytosis is not known. The role of zinc release is thought to be that the embryo needs to resume progression through the cell cycle upon fertilization; this happens after the oocyte has taken up significant amounts of zinc at the latest stage of maturation and became growth arrested after the first meiotic division (Kim et al. 2011).

2.4.2 *Release from Intracellular Stores, Influx from the Plasma Membrane, and Intracrine Signals*

Stimulation of the macrophage immunoglobulin E FcεRI receptor and ERK/IP_3 signaling triggers first Ca^{2+} and then Zn^{2+} release from a vesicular store that ZnT2 loads with zinc (Yamasaki et al. 2007). The release has been referred to as a "zinc wave." Intracellular zinc waves occur in minutes, whereas extracellular zinc sparks occur in seconds, demonstrating different dynamics of zinc signals. In neurons, the zinc store is also IP_3 sensitive (Stork and Li 2010). Growth factor stimulation of breast cancer cells leads to casein kinase 2-dependent phosphorylation of ZIP7, which opens an endoplasmic reticulum store for zinc release (Taylor et al. 2012b). Similarly, lysosomal zinc release in interleukin 2-stimulated T cells is a required signal for proliferation (Kaltenberg et al. 2010). Several ZIP transporters of the LIV-1 family also seem to trigger zinc signals from their location at the plasma membrane. For instance, N-terminal proteolytic cleavage of ZIP6 leads to translocation to the plasma membrane and zinc influx (Hogstrand et al. 2013). Given the high inward gradient of total zinc, it is unclear how the extracellular zinc is provided and what transport mechanism is involved.

2.4.3 *Redox Mechanisms*

An alternative way to releasing zinc by opening a subcellular store is oxidative signaling in the cytosol. The basis for this zinc release mechanism is the bonding of zinc to sulfur donors in thiolate coordination environments and sulfur

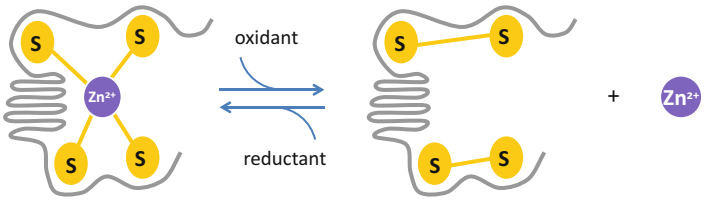

Fig. 2.2 Zinc/thiolate redox switches. Zinc/thiolate (Cys) coordination environments can react with oxidants resulting in the oxidation of sulfur (to disulfides for example, as shown here) and the release of zinc ions. Reduction of the disulfide regenerates zinc-binding capacity. In this way, redox signaling or changes of specific redox pairs can modulate free zinc ion concentrations and the function of proteins that lose their zinc and others which are targets of the released zinc. These reactions are not restricted to zinc in tetrathiolate sites

ligand-centered redox activity (Maret 2004a, 2006) (Fig. 2.2). The chemistry is specific for sulfur and does not occur in calcium coordination environments. It involves different oxidation states of sulfur, not only thiol–disulfide equilibria, in reactions with a host of physiologically significant reactive oxygen, nitrogen, and sulfur species, reactive carbonyls, and reducible selenium compounds (Chen and Maret 2001; Hao and Maret 2006).

Zinc/thiolate sites, such as those in MTs, are molecular switches operating under many pathophysiological conditions where reactive species release zinc ions that become cytotoxic, for example, for neurons (Aizenman et al. 2000). These switches are also indicated under physiological conditions where a redox signal is transduced into a zinc signal. Particular emphasis has been given to nitric oxide (NO) signaling. Metallothionein has been shown to be the source of intracellularly released zinc ions in endothelial cells, in bradykinin-induced NO signaling, and in cytokine-induced iNOS-dependent NO signaling that causes an intranuclear rise in zinc ions (Pearce et al. 2000; Spahl et al. 2003). A variety of G protein-coupled receptors generate a redox signal that then releases zinc for signaling (Sanchez-Blazques et al. 2012). Thus, in oxidative signaling associated zinc signals must be considered. Zinc protects specific redox-sensitive thiols and initiates specific responses by inhibiting enzymes or activating gene expression as part of an antioxidant response.

2.5 Targets of Free Zinc Ion Transients

Knowledge of pZn values and matching affinities of proteins targeted by transient or permanent rises in free zinc ion concentrations becomes critical when distinguishing physiological from pathophysiological changes. Only above their steady-state levels can zinc ions bind to additional proteins and modulate their biological activities. Zinc inhibits some enzymes with nanomolar affinities

(Maret et al. 1999). These proteins are generally not recognized as zinc enzymes because they are isolated with chelating and reducing agents to preserve their enzymatic activity. When investigating zinc inhibition, two methodological issues need to be addressed. First, zinc-buffered solutions should be employed to control free zinc ions at such low concentrations, and second, because the inhibition is assayed by enzymatic activity, only apparent K_i values will be obtained if the inhibition is competitive with respect to substrate. A full kinetic analysis is then required to determine K_i values. In the case of receptor protein tyrosine phosphatase beta, the inhibition is so strong (21 pM) that it suggests tonic inhibition of the enzyme (Wilson et al. 2012). With free zinc ion concentrations of 24 pM in human erythrocytes (Simons 1991), zinc inhibition of human erythrocyte Ca^{2+}-ATPase with a K_i of 80 pM (pH 7.4) also indicates at least partial inhibition under physiological conditions (Hogstrand et al. 1999). The affinities of proteins that are targets of free zinc ions are therefore only slightly lower than those of zinc enzymes, suggesting an important principle: whereas zinc enzymes need to be activated by binding zinc, the zinc-inhibited enzymes need to be activated by removing zinc (Maret 2013b). The number of enzymes that zinc inhibits is not known and will depend on the exact magnitude of zinc ion fluctuations and whether global changes or local transients are involved. Enzymes may be inhibited tonically or their activity modulated, and inhibition with zinc may occur under physiological or pathophysiological conditions. Zinc inhibits many thiol-dependent enzymes (protein tyrosine phosphatases, caspases, and cathepsins) and other enzymes that have diads or triads of catalytic His, Glu(Asp), and Cys residues as binding sites for metal ions (Maret 2013b). How zinc-inhibited cytosolic enzymes are reactivated is not known. In vitro investigations suggest that the activation involves zinc removal by thionein, the apoprotein of MT. A common observation is that zinc enhances tyrosine phosphorylation signaling. In most instances, the effect can be explained with phosphatase inhibition rather than kinase activation. Colocalization of protein tyrosine phosphatases, receptors, and zinc transporters/channels provides a way for zinc microdomains and gradients to modulate signaling complexes. Although enzyme inhibition is emerging as one principle of zinc signaling, there may be others, such as enzyme activation or modulating protein–protein interactions (Maret 2004b). In as much as it is discussed that ER-released calcium affects mitochondria, ER-released zinc could also have a significant effect on mitochondrial respiration (Ye et al. 2001).

Secreted zinc ions affect intercellular zinc signaling. Zinc ions released from zinc-rich neurons into the synapse inhibit the NMDA (*N*-methyl-D-aspartate) receptor postsynaptically. The inhibition constant is less than 10 nM (pH 7.3) (Paoletti et al. 1997). Zinc affects many other receptor proteins but affinities for zinc are often not known.

Another regulatory function, but not a zinc signal, relates to the zinc inhibition of enzymes secreted from cells. Micromolar zinc ion concentrations inhibit the zinc enzyme bovine carboxypeptidase A at a second zinc-binding site ($K_i = 0.5$ μM) and serine proteases such as kallikreins with inhibition constants ranging from 10 nM to about 10 μM (Maret 2008b). These interactions are thought to be physiologically

significant, although these zinc affinities are orders of magnitude lower than those discussed for inhibition of cytosolic enzymes. The reason for this is that seminal and prostatic fluids, where these kallikreins are found, contain about 10 mM zinc. Presumably, the enzymes are stored with an inhibitory zinc ion bound, which then dissociates and activates the enzymes once they are secreted and diluted.

2.6 Concluding Remarks

It is remarkable that dozens of proteins control cellular zinc homeostasis. These proteins redistribute zinc subcellularly and control zinc ion transients. The role of zinc as a messenger in not one but at least three different mechanisms of release with multiple functional outcomes in intracrine, paracrine, and possibly endocrine signaling leaves no doubt as to the major importance of zinc for cell biology. Its signaling functions are in addition to its functions in enzymes and in protein–DNA/RNA and protein–protein interactions in thousands of proteins. Regulation with zinc ions extends the functions of the other two redox-inert metal ions, magnesium and calcium, into a range of concentrations that are typical for hormones. Together these metals ions cover many orders of concentrations for regulation. In the series Mg^{2+}, Ca^{2+}, and Zn^{2+}, specificity and the ratio of protein-bound metal to free metal increases commensurate with increasing affinities for proteins (Maret 2001). The metal ions have different coordination chemistries and chemical properties and hence target different proteins and are involved in different processes. Given that association (on) rates are fast and not restricted, the significant different affinities dictate very different dissociation (off) rates. The effect of zinc signals is expected to be orders of magnitude longer than that of calcium signals unless there are specific mechanisms to increase the off rates. This factor makes zinc ions ideally suited for long-term changes such as holding the cell in specific states.

Sulfur donors of cysteine confer redox activity on some zinc coordination environments, thereby linking zinc redistribution and redox signaling. There is crosstalk between calcium and zinc signaling at several levels. Thus, zinc signaling is integrated into other cellular signaling networks:

$$\text{Redox signals} \leftrightarrow \text{Zinc signals} \leftrightarrow \text{Calcium signals}$$

Calcium influx into cells is necessary for exocytosis of Zn^{2+}-containing vesicles. Extracellular signals that elicit calcium signals for inducing NO are also linked to generating zinc transients. Extracellular zinc ions bind to a zinc receptor (ZnR), which is a G protein-coupled receptor identified as GPR39, triggering the release of Ca^{2+} from the ER (Hershfinkel et al. 2001; Holst et al. 2007; Yasuda et al. 2007). ZnT1 interacts with the L-type calcium channel (LTCC) β-subunit and reduces the cell-surface expression of the α-subunit (Levy et al. 2009). This action is through the Ras-ERK signaling pathways and also affects activity and surface expression of

T-type calcium channels (Mor et al. 2012). The α1D-subunit of the ER resident L-type calcium channel has a role in controlling the cellular zinc wave (Yamasaki et al. 2012).

Experimentally, there continue to be tremendous challenges. Investigating free zinc ions at their very low concentrations is demanding in terms of analytical procedures and purity of reagents, which often have orders of magnitude higher zinc concentrations than the cellular free zinc ion concentrations. Zinc is buffered in the cell but not in most biological experiments in which buffers are primarily employed to control pH. Although electrophysiology is a mainstay in the field of calcium research, the electrophysiology of zinc has not been explored. Direct and quantitative zinc measurements are necessary to refine the models of zinc homeostasis and zinc signaling.

The concept that a transition metal ion is a signaling ion has major implications for health and disease. It changes the view of the micronutrient zinc as merely being a cofactor of proteins to one including global functions in cellular control and suggests that perturbation of zinc homeostasis has much wider consequences for cell proliferation, differentiation, and cell death as nutritional zinc deficiency. The many factors affecting the proteins that buffer and muffle zinc also perturb zinc status and may elicit cellular zinc deficiency or zinc overload (accumulation), if the cell can no longer handle zinc properly. How these processes affect zinc stores as a source of signaling zinc ions and how other metal ions, either essential ones out of homeostatic control or nonessential ones such as cadmium, interact with the control and signaling of zinc needs to be addressed.

References

Aizenman E, Stout AK, Hartnett KA et al (2000) Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. J Neurochem 75:1878–1888

Andreini C, Banci L, Bertini I et al (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196–201

Bal W, Kurowska E, Maret W (2012a) The final frontier of pH and the undiscovered country beyond. PLoS One 7:e45832

Bal W, Sokolowska M, Kurowska E et al (2012b) Binding of transition metal ions to albumin: sites, affinities and rates. Biochim Biophys Acta 1830:5444–5455

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

Bozym RA, Thompson RB, Stoddard AK et al (2006) Measuring picomolar intracellular exchangeable zinc in PC-12 cells using a ratiometric fluorescence biosensor. ACS Chem Biol 1:103–111

Chen Y, Maret W (2001) Catalytic selenols couple the redox cycles of metallothionein and glutathione. Eur J Biochem 268:3346–3353

Chong CR, Auld DS (2007) Catalysis of zinc transfer by D-penicillamine to secondary chelators. J Med Chem 50:5524–5527

Colvin RA, Bush AI, Volitakis I et al (2008) Insights into Zn^{2+} homeostasis in neurons from experimental and modeling studies. Am J Physiol Cell Physiol 294:C726–C742

Colvin RA, Holmes WR, Fontaine CP et al (2010) Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. Metallomics 2:306–317
Danscher G, Stoltenberg M (2005) Zinc-specific autometallographic in vivo selenium methods: tracing of zinc-enriched (ZEN) pathways, and pools of zinc ions in a multitude of other ZEN cells. J Histochem Cytochem 53:141–153
Frederickson CJ, Koh J-Y, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6:449–462
Fukada T, Kambe T (2011) Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics 3:662–674
Fukada T, Yamasaki S, Nishida K et al (2011) Zinc homeostasis and signaling in health and diseases. J Biol Inorg Chem 16:1123–1134
Günther V, Lindert U, Schaffner W (2012) The taste of heavy metals: gene regulation by MTF-1. Biochim Biophys Acta 1823:1416–1425
Haase H, Rink L (2014) Zinc signals and immune function. Biofactors 40:27–40
Hao Q, Maret W (2006) Aldehydes release zinc from proteins. A pathway from oxidative stress/lipid peroxidation to cellular functions of zinc. FEBS J 273:4300–4310
Heinz U, Kiefer M, Tholey A et al (2005) On the competition for available zinc. J Biol Chem 280:3197–3207
Hershfinkel M, Moran A, Grossman N et al (2001) A zinc-sensing receptor triggers the release of intracellular Ca^{2+} and regulates ion transport. Proc Natl Acad Sci USA 98:11749–11754
Hirano T, Murakami M, Fukada T et al (2008) Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. Adv Immunol 97:149–176
Hogstrand C, Verbost PM, Wendelaar Bonga SE (1999) Inhibition of human Ca^{2+}-ATPase by Zn^{2+}. Toxicology 133:139–145
Hogstrand C, Kille P, Ackland ML et al (2013) A mechanism for epithelial-mesenchymal transition and anoikis resistance in breast cancer triggered by zinc channel ZIP6 and signal transducer and activator of transcription 3 (STAT3). Biochem J 455:229–237
Holst B, Egerod KL, Schild E et al (2007) GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology 148:13–20
Ippolito JA, Baird TT Jr, McGee SA et al (1995) Structure-assisted redesign of a protein zinc-binding site with femtomolar affinity. Proc Natl Acad Sci USA 92:5017–5021
Irving H, Williams RJP (1948) Order of stability of metal complexes. Nature (Lond) 162:746–747
Jacob C, Maret W, Vallee BL (1998) Control of zinc transfer between thionein, metallothionein and zinc proteins. Proc Natl Acad Sci USA 95:3489–3494
Kaltenberg J, Plum JL, Ober-Blöbaum JL et al (2010) Zinc signals promote IL-2-dependent proliferation of T-cells. Eur J Immunol 40:1496–1503
Kim AM, Bernhardt ML, Kong BY et al (2011) Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs. ACS Chem Biol 6:716–723
Krężel A, Maret W (2006) Zinc buffering capacity of a eukaryotic cell at physiological pH. J Biol Inorg Chem 11:1049–1062
Krężel A, Maret W (2007) The nanomolar and picomolar Zn(II) binding properties of metallothionein. J Am Chem Soc 129:10911–10921
Krężel A, Maret W (2008) Thionein/metallothionein control Zn(II) availability and the activity of enzymes. J Biol Inorg Chem 13:401–409
Krężel A, Hao Q, Maret W (2007) The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling. Arch Biochem Biophys 463:188–200
Laity JH, Andrews GK (2007) Understanding the mechanism of zinc-sensing by metal-responsive element binding transcription factor-1 (MTF-1). Arch Biochem Biophys 463:201–210
Levy S, Beharier O, Etzion Y et al (2009) Molecular basis for zinc transporter 1 action as an endogenous inhibitor of L-type calcium channels. J Biol Chem 284:32434–32443
Li Y, Maret W (2008) Human metallothionein metallomics. J Anal Atom Spectrom 23:1055–1062
Li Y, Maret W (2009) Transient fluctuations of intracellular zinc ions in cell proliferation. Exp Cell Res 315:2463–2470

Lu M, Fu D (2007) Structure of the zinc transporter YiiP. Science 317:1746–1748
Magneson GR, Puvathingal JM, Ray WJ (1987) The concentration of free Mg^{2+} and free Zn^{2+} in equine blood plasma. J Biol Chem 262:11140–11148
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
Maret W (2004a) Zinc and sulfur: a critical biological partnership. Biochemistry 43:3301–3309
Maret W (2004b) Protein interface zinc sites: a role of zinc in the supramolecular assembly of proteins and in transient protein–protein interactions. In: Messerschmidt A, Bode W, Cygler M (eds) Handbook of metalloproteins, vol 3. Wiley, Chichester, pp 432–441
Maret W (2006) Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxid Redox Signal 8:1419–1441
Maret W (2008a) Metallothionein redox biology in the cytoprotective and cytotoxic functions of zinc. Exp Gerontol 43:363–369
Maret W (2008b) Zinc proteomics and the annotation of the human zinc proteome. Pure Appl Chem 80:2679–2687
Maret W (2009) Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals. Biometals 22:149–157
Maret W (2011a) Redox biochemistry of mammalian metallothioneins. J Biol Inorg Chem 16:1079–1086
Maret W (2011b) Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. Biometals 24:411–418
Maret W (2012) New perspectives of zinc coordination environments in proteins. J Inorg Biochem 111:110–116
Maret W (2013a) Zinc biochemistry: from a single zinc enzyme to a key element of life. Adv Nutr 4:82–91
Maret W (2013b) Inhibitory zinc sites in enzymes. Biometals 26:197–204
Maret W, Li Y (2009) Coordination dynamics of zinc in proteins. Chem Rev 109:4682–4707
Maret W, Vallee BL (1998) Thiolate ligands in metallothionein confer redox activity on zinc clusters. Proc Natl Acad Sci USA 95:3478–3482
Maret W, Larsen KS, Vallee BL (1997) Coordination dynamics of biological zinc "clusters" in metallothioneins and in the DNA-binding domain of the transcription factor Gal4. Proc Natl Acad Sci USA 94:2233–2237
Maret W, Jacob C, Vallee BL et al (1999) Inhibitory sites in enzymes: zinc removal and reactivation by thionein. Proc Natl Acad Sci USA 96:1936–1940
McCranor BJ, Bozym RA, Vitolo MI et al (2012) Quantitative imagine of mitochondrial and cytosolic free zinc levels in an in vitro model of ischemia/reperfusion. J Bioenerg Biomembr 44:253–263
Mor M, Beharier O, Levy S et al (2012) ZnT-1 enhances the activity and surface expression of T-type calcium channels through activation of Ras-ERK signaling. Am J Physiol Cell Physiol 303:C192–C203
Nydegger I, Rumschik SM, Zhao J et al (2012) Evidence for an extracellular zinc-veneer in rodent brains from experiments with Zn-ionophores and ZnT3 knockouts. ACS Chem Neurosci 3:761–766
Ohyoshi E, Hamada Y, Nakata K et al (1999) The interaction between human and bovine serum albumin and zinc studied by a competitive spectrophotometry. J Inorg Biochem 75:213–218
Paoletti P, Ascher P, Neyton J (1997) High-affinity inhibition of NMDA NR1-NR2A receptors. J Neurosci 17:5711–5725
Park JG, Qin Y, Galati DF et al (2012) New sensors for quantitative measurement of mitochondrial Zn(2+). ACS Chem Biol 7:1636–1640
Pearce LL, Gandley RE, Han W et al (2000) Role of metallothionein in nitric oxide signaling as revealed by a green fluorescent fusion protein. Proc Natl Acad Sci USA 97:477–482
Peck EJ, Ray WJ (1971) Metal complexes of phosphoglucomutase in vivo. J Biol Chem 246:1160–1167

Qin Y, Dittmer PJ, Park JG et al (2011) Measuring steady-state and dynamic endoplasmic reticulum and Golgi Zn^{2+} with genetically encoded sensors. Proc Natl Acad Sci USA 108:7351–7356

Qiu A, Shayeghi M, Hogstrand C (2005) Molecular cloning and functional characterization of a high-affinity zinc importer (*Dr*ZIP1) from zebrafish (*Danio rero*). Biochem J 388:745–754

Ray WJ (1969) Role of bivalent cations in the phosphoglucomutase system. J Biol Chem 244:3740–3747

Rink L (ed) (2011) Zinc in human health. IOS Press, Amsterdam

Sanchez-Blazques P, Rodriguez-Munoz M, Bailon C et al (2012) GPCRs promote the release of zinc ions mediated by nNOS/NO and the redox transducer RGSZ2 protein. Antioxid Redox Signal 17:1163–1177

Sensi SL, Paoletti P, Bush AI (2009) Zinc in the physiology and pathology of the CNS. Nat Rev Neurosci 10:780–791

Sikorska M, Krężel A, Otlewski J (2012) Femtomolar Zn^{2+} affinity of LIM domain PDLIM1 protein uncovers crucial contribution of protein–protein interactions to protein stability. J Inorg Biochem 115:28–35

Simons TJB (1991) Intracellular free zinc and zinc buffering in human red blood cells. J Membr Biol 123:63–71

Spahl DU, Berendji-Gruen D, Suschek CV et al (2003) Regulation of zinc homeostasis by inducible NO synthase-derived NO: nuclear metallothionein translocation and intranuclear Zn^{2+} release. Proc Natl Acad Sci USA 100:13952–13957

Stork CJ, Li YV (2010) Zinc release from thapsigargin/IP3-sensitive stores in cultured cortical neurons. J Mol Signal 5:5

Takeda A, Nakamura M, Fujii H et al (2013) Synaptic Zn(2+) homeostasis and its significance. Metallomics 5:417–423

Tamaki M, Fujitani Y, Hara A et al (2013) The diabetes-susceptibility gene SLC30A8/ZnT8 regulates hepatic insulin clearance. J Clin Invest 123:4513–4524

Taylor KM, Kille P, Hogstrand C (2012a) Protein kinase CK2 opens the gate for zinc signaling. Cell Cycle 11:1863–1864

Taylor KM, Hiscox S, Nicholson RI et al (2012b) Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal 5(210):ra11

Thomas RC, Coles JA, Deitmer JW (1991) Homeostatic muffling. Nature (Lond) 350:564

Ueno S, Tsukamoto M, Hirano T et al (2002) Mossy fiber Zn^{2+} spillover modulates heterosynaptic *N*-methyl-D-aspartate receptor activity in hippocampal CA3 circuits. J Cell Biol 158:215–220

Vinkenborg JL, Nicholson TJ, Bellomo EA et al (2009) Genetically encoded FRET sensors to monitor intracellular Zn^{2+} homeostasis. Nat Methods 6:737–740

Wellenreuther G, Cianci M, Tucoulou R et al (2009) The ligand environment of zinc stored in vesicles. Biochem Biophys Res Commun 380:198–203

Williams RJP (1984) Zinc: what is its role in biology? Endeavour 8:65–70

Wilson M, Hogstrand C, Maret W (2012) Picomolar concentrations of free zinc(II) ions regulate receptor protein tyrosine phosphatase beta activity. J Biol Chem 287:9322–9326

Yamasaki S, Sakata-Sogawa K, Hasegawa A et al (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645

Yamasaki S, Hasegawa A, Hojyo S et al (2012) A novel role of the L-type calcium channel alpha1D subunit as a gatekeeper for intracellular zinc signaling: zinc wave. PLoS One 7: e39654

Yasuda S, Miyazaki T, Munechika K et al (2007) Isolation of Zn^{2+} as an endogenous agonist of GPR39 from fetal bovine serum. J Recept Signal Transduct Res 27:235–246

Ye B, Maret W, Vallee BL (2001) Zinc metallothionein imported into liver mitochondria modulates respiration. Proc Natl Acad Sci USA 98:2317–2322

Chapter 3
Zinc Transport Proteins and Zinc Signaling

Taiho Kambe, Tokuji Tsuji, and Kazuhisa Fukue

Abstract There is an increasing body of evidence indicating that zinc transport proteins play crucial roles in a variety of biological events. The Zrt, Irt-like protein/solute carrier family 39 (ZIP/SLC39A) and Zn transporter (ZnT)/SLC30A family are proteins that are primarily involved in zinc transport across the cellular membrane. In general, the ZIP family facilitates zinc influx into the cytosol from the extracellular space and release from intracellular compartments, and the ZnT family facilitates zinc mobilization in the opposite direction. A number of articles have shown that zinc movement by both transporter families contributes to zinc signaling in cellular events such as signal transduction, immunological response, cell differentiation, cell development, and cancer progression. In addition, recent reports have revealed that some types of calcium channels and other membrane proteins are employed in zinc permeation in the cell. In this chapter, we review the current understanding of the molecular functions, and the regulation of expression and activity of these zinc transport proteins, from the perspective of zinc signaling with a focus on recent advances.

Keywords Calcium channel • Zinc • Zinc signaling • Zinc transporter • ZIP (Zrt Irt-like protein) • ZnT (zinc transporter)

3.1 Introduction: Zinc Transport Proteins

Zinc is a charged divalent cation in physiological conditions. Thus, it cannot cross the cytoplasmic membrane and membranes of the intracellular compartments by passive diffusion. Zinc transport therefore requires a set of membrane proteins that are indispensable for zinc to fulfill crucial roles in multifarious biological events. During the past two decades there have been significant advances in our understanding of zinc transport proteins. In metazoa, the Zrt, Irt-like protein/solute carrier family 39 (ZIP/SLC39A) and the Zn transporter (ZnT)/SLC30A family are primary zinc transport proteins (Gaither and Eide 2001; Kambe et al. 2004; Eide 2006; Cousins et al. 2006). Moreover, some types of calcium (Ca) channels

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and other membrane proteins contribute to cellular zinc movement (Frederickson et al. 2005; Sensi et al. 2011; Bouron and Oberwinkler 2013). In contrast to copper and iron, the divalent zinc cation does not require a redox reaction during the membrane transport process (Kambe et al. 2008b; Kambe 2013). Thus, the expression levels of active zinc transporters and zinc permeable channel proteins at the cellular sites where they normally operate directly define the net cellular zinc transport.

Zinc transport proteins are involved in many cellular responses through modulating enzyme activation/inactivation, transcriptional activation/repression, membrane trafficking, and protein stability (Maret and Li 2009; Fukada and Kambe 2011; Maret 2013). Increasing evidence has shown that these functions are mediated by altering zinc ion concentration and distribution, that is, zinc transport proteins directly contribute to zinc signaling (Hirano et al. 2008; Haase and Rink 2009; Hogstrand et al. 2009; Fukada et al. 2011). Here, functions of the zinc transport proteins, such as ZIPs and ZnTs, and other membrane proteins including Ca channels involved in zinc transport, are reviewed from the point of view of zinc signaling.

3.2 Two Solute Carrier Proteins: ZIPs and ZnTs

ZIP and ZnT transporters belong to the solute carrier proteins and thus function as secondary active transporters (Gaither and Eide 2001; Kambe et al. 2004; Eide 2006; Cousins et al. 2006; Lichten and Cousins 2009; Fukada and Kambe 2011). Under physiological conditions, ZIP transporters import zinc into the cytosol from the extracellular space or intracellular compartments, and ZnT transporters mobilize zinc in the opposite direction (recently, it has been argued that ZIPs are channels, but in this chapter ZIP proteins are described as transporters) (Fig. 3.1). ZIP and ZnT members were identified by genetic, functional screenings and amino acid sequence similarities, resulting in more than 20 members (Kambe et al. 2006). They are expressed in a cell- and tissue-specific or zinc-regulatory manner, are developmentally regulated. Several ZIP transporters and most of the ZnT transporters are localized to intracellular compartments such as the secretory and endosomal-lysosomal compartments (Hennigar and Kelleher 2012; Kambe 2011), where they execute their specific functions (Fig. 3.2). Thus, in addition to zinc mobilization into and out of the cytosol across the cytoplasmic membrane, zinc mobilization into and out of these compartments by ZIP and ZnT transporters is also involved in zinc signaling. Before outlining the molecular functions of the ZIP and ZnT transporters in zinc signaling, their fundamental information is briefly reviewed.

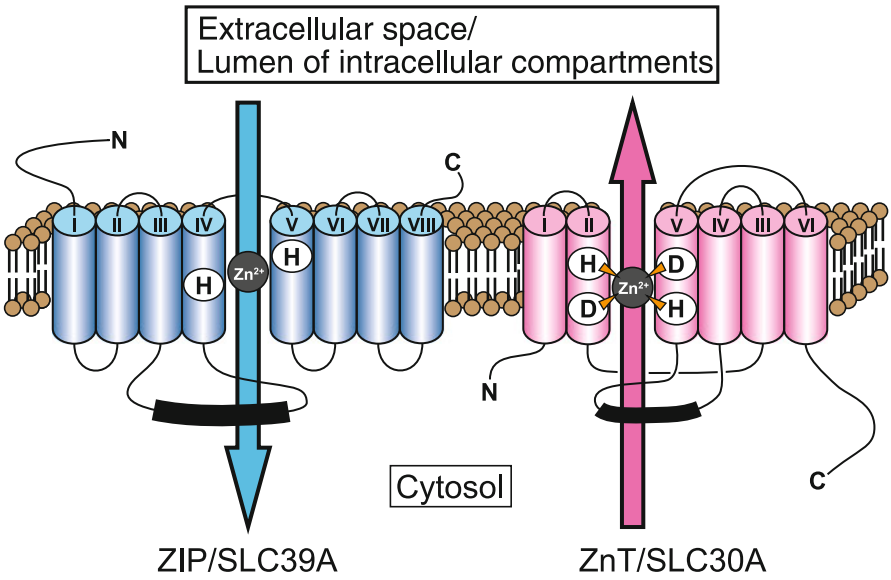

Fig. 3.1 Predicted structures and zinc transport directions of ZIP and ZnT transporters: predicted topologies of ZIP (*left*) and ZnT (*right*) transporters. The His-rich cluster of ZIP and the His-rich loop of ZnT are indicated by *bold lines*. The highly conserved histidine (*H*) residues in transmembrane domain (TMD) *IV* and *V* of ZIP transporters are thought to be involved in zinc transport activity. The highly conserved aspartic acid (*D*) and H residues in TMD *II* and *V* of ZnT transporters are thought to form zinc-binding sites in TMDs based on the homology to YiiP. ZIP transporters mobilize zinc from the extracellular space or the lumen of intracellular compartments to the cytosol, and ZnT transporters mobilize zinc in the opposite direction

3.2.1 ZIP Transporters

Fourteen ZIP transporters operate in mammals (Jeong and Eide 2013). They can be divided into four subfamilies, I, II, LIV-1, and gufA, based on their phylogenetic relationships (Gaither and Eide 2001; Taylor and Nicholson 2003; Kambe et al. 2004). The name "ZIP" (ZRT, IRT-like protein) (Eng et al. 1998), which was named after the first identified members, the *Saccharomyces cerevisiae* Zrt1 and Zrt2 zinc transporters (Zhao and Eide 1996a, b) and the *Arabidopsis thaliana* IRT1 iron transporter (Eide et al. 1996), suggests that ZIP transporters can mobilize several divalent metals. Actually, some of the ZIP transporters have been shown to be involved in iron and manganese transport (Jenkitkasemwong et al. 2012; Fujishiro et al. 2012). In this context, a particularly interesting aspect is the recent evidence of their contribution to iron metabolism as a transporter of non-transferrin-bound iron (NTBI) in the liver (Liuzzi et al. 2006; Pinilla-Tenas et al. 2011; Beker Aydemir et al. 2012).

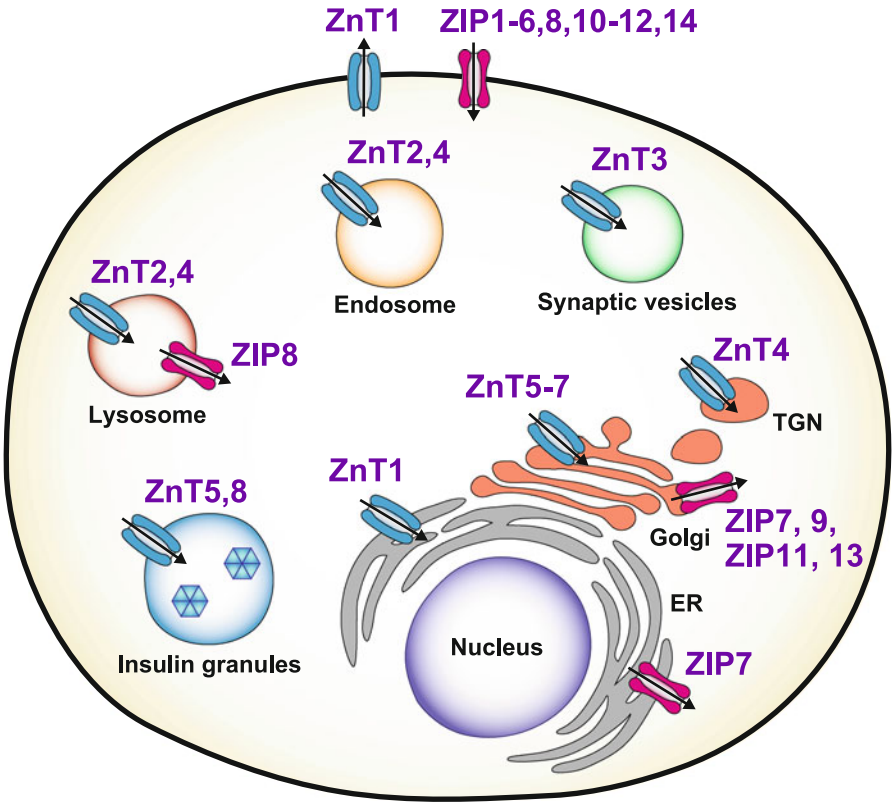

Fig. 3.2 Subcellular localization and direction of zinc transport of ZIP and ZnT transporters shown according to available information. *Red-purple* and *blue* indicate ZIP and ZnT transporters, respectively. A number of ZIP and ZnT transporters are expressed in a cell type-specific manner: ZnT3 is expressed in synaptic vesicles in a subset of glutamatergic neurons, and ZnT8 is expressed in insulin granules of pancreatic β cells. Most ZIPs and ZnTs are thought to form homodimers, except for ZnT5 and ZnT6, which form heterodimers (see text). *TGN trans*-Golgi network, *ER* endoplasmic reticulum

All ZIP members are predicted to have eight transmembrane domains (TMDs) with a similar membrane topology in which the amino- and carboxyl-terminal ends are located outside the cytoplasmic membrane or in the lumen of intracellular compartments (Taylor and Nicholson 2003; Kambe et al. 2004; Fukada and Kambe 2011). ZIP transporters are thought to form functional dimers, although there is not a lot of evidence to support this idea (Bin et al. 2011). Most ZIP transporters have a cytosolic His-rich cluster between TMDs III and IV, which may play a role in cytosolic zinc binding (Fig. 3.1). The most conserved portion among the members is found in TMDs IV and V, both of which are partially amphipathic and contain conserved His residues (Eng et al. 1998; Fukada and Kambe 2011)

(Fig. 3.3). It has been suggested that these domains may comprise an aqueous pore through which zinc ions pass and the conserved His residues may constitute an intramembranous zinc-binding site, but only little information about the transport mechanism of ZIP transporters has been elucidated. Using a purified bacterial ZIP homologue in reconstituted proteoliposomes has revealed a nonsaturable and electrogenic zinc transport (Lin et al. 2010). In contrast, several ZIP transporters have been shown to facilitate zinc transport in the presence of bicarbonate ions, when overexpressed in the cells (Gaither and Eide 2000; He et al. 2006). Further investigation is required to characterize the molecular mechanism of zinc transport by ZIP transporters.

3.2.2 ZnT Transporters

Ten ZnT transporters have been molecularly characterized in mammals thus far (Huang and Tepaamorndech 2013). However, ZnT9 (referred to as GAC63) was found to function as a nuclear coactivator, and thus generally nine ZnT transporters are thought to be operative in mammals (Kambe 2012; Fukada and Kambe 2011). They belong to the cation diffusion facilitator (CDF) family, which is a zinc efflux protein family conserved in diverse organisms from bacteria to mammals. Despite the term "diffusion facilitator," CDF transporters do not serve as diffusion facilitators, but function as secondary active transporters. In fact, CDF transporters, including ZnTs, have been shown to function as a Zn^{2+}/H^{+} antiporter (Ohana et al. 2009; MacDiarmid et al. 2002; Kawachi et al. 2008). The Zn^{2+}/H^{+} antiport mode is reasonable for ZnTs, because most ZnTs are localized to acidic compartments such as endosomes/lysosomes (e.g., ZnT2, -3, -4, and -8). The CDF transporters are classified into three groups—Zn-CDF, Zn/Fe-CDF, and Mn-CDF—based on their phylogenetic relationships, and ZnT transporters all belong to the Zn-CDF group (Montanini et al. 2007; Kambe 2012) (Fig. 3.4). A more recent phylogenetic analysis has divided them into four groups (Gustin et al. 2011): (1) ZnT1 and ZnT10; (2) ZnT2, ZnT3, ZnT4, and ZnT8; (3) ZnT5 and ZnT7; and (4) ZnT6 (Kambe et al. 2006; Kambe 2012).

An X-ray structure of ZnT transporters has not been elucidated. However, the crystal structure of YiiP, an *Escherichia coli* homologue, provides a framework that allows exploration of the molecular basis of the ZnT transporters structure. YiiP forms a homodimer with six TMDs, and both the N- and C-termini are located in the cytosol (Lu and Fu 2007; Lu et al. 2009). Four TMDs, I, II, IV, and V, form a compact four-helix bundle with an inner core forming a channel, and the remaining TMDs III and VI cross over in an antiparallel configuration outside the bundle (Lu and Fu 2007; Lu et al. 2009). One His and three Asp residues within TMDs II and V form a zinc-binding site in the TMDs. Zinc binding to the C-terminal cytosolic domain triggers hinge movements, pivoting around four salt bridges situated at the juncture of the cytosolic domains and the TMDs. These salt bridges interlock the TMDs at the dimer interface, thereby modulating coordination

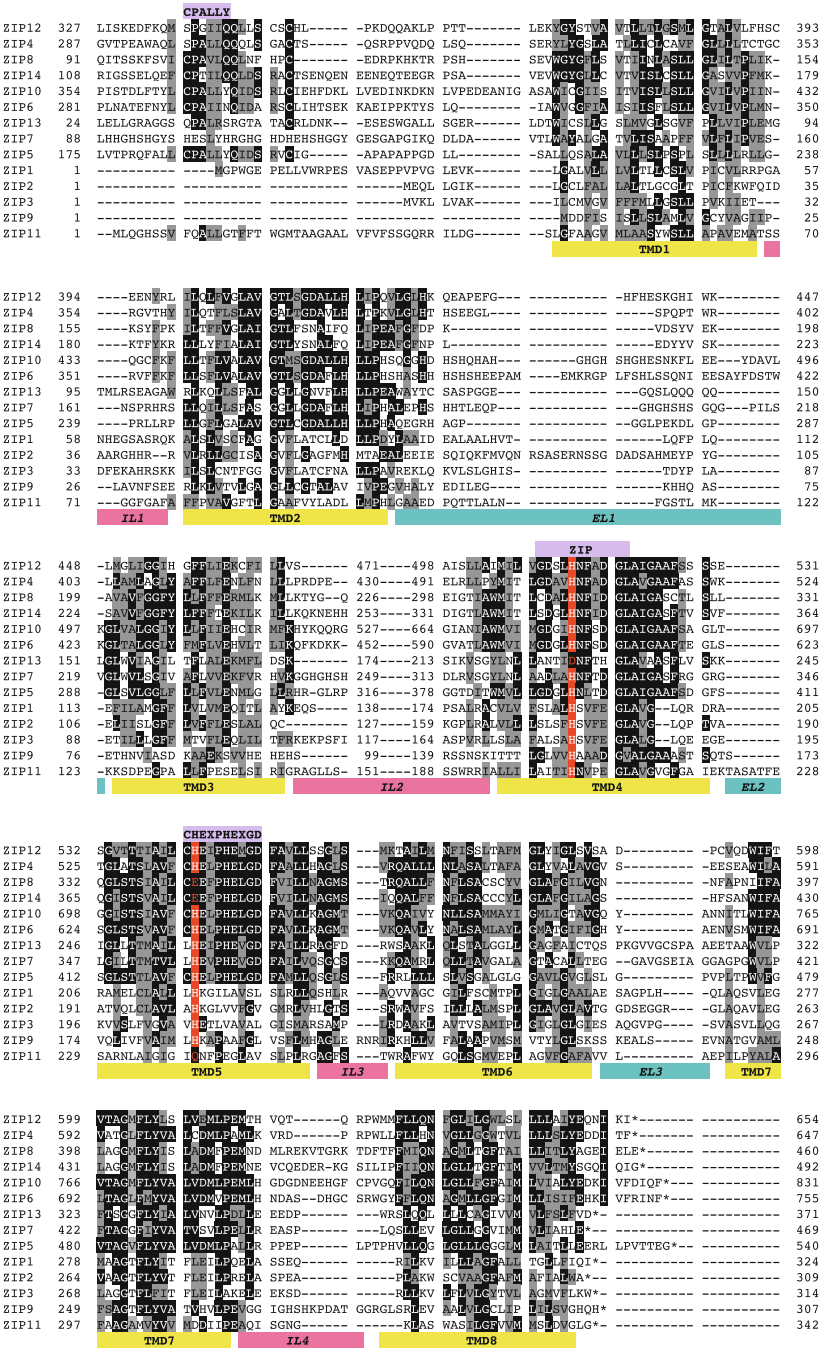

Fig. 3.3 Sequence alignment of ZIP transporters. The order of alignment is according to the similarities among subfamilies, which was performed with amino acid sequences of ZIP transporter proteins using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/) with some modifications.

geometry of the zinc-binding site in the TMDs for zinc transport. Based on the information of YiiP, ZnT transporters have been predicted to have a similar topology with six TMDs and intracellular N- and C-termini (Kambe et al. 2004; Kambe 2012; Fukada and Kambe 2011) (Fig. 3.1). The highly conserved His and Asp residues (two of each) within TMDs II and V are critical for zinc transport activity (Ohana et al. 2009; Fukunaka et al. 2011; Itsumura et al. 2013). In ZnT6, two of the four residues are changed to hydrophobic residues (Kambe 2012); therefore, ZnT6 does not have zinc transport activity and is instead thought to be not functional until it forms heterodimers with ZnT5 (Fukunaka et al. 2009). Moreover, in ZnT10, the His residue in TMD II is substituted by Asn, which may explain why ZnT10 can be used in manganese metabolism (Tuschl et al. 2012; Quadri et al. 2012; Kambe 2012). The distinctive cytosolic His-rich loop between TMDs IV and V has been suggested to be employed in zinc binding (Suzuki et al. 2005b) and modulate zinc recognition, acting as a selectivity filter or sensor of the cytosolic zinc level (Kawachi et al. 2008, 2012).

3.2.3 Physiology and Pathogenesis of ZIPs and ZnTs

A number of ZIP and ZnT transporters have been shown to be involved in human genetic diseases. Moreover, knockout (KO) mice have been generated for almost half of these transporters. Here, phenotypes of these diseases, and mutant and KO mice and other model organisms are briefly reviewed to unveil the fundamental importance of ZIPs and ZnTs in zinc biology. The details of the mice phenotypes have been described in other reviews (Kambe et al. 2008b; Fukada and Kambe 2011).

3.2.3.1 Phenotypes of Inherited Human Diseases, and Mutant and KO Mice and other Model Organisms of ZIP Transporters

There are two ZIP genes with mutations that are related to genetic diseases. *ZIP4* has been identified as the gene responsible for the rare autosomal genetic disease, acrodermatitis enteropathica (AE) (Kury et al. 2002; Wang et al. 2002). AE is

Fig. 3.3 (continued) The putative transmembrane domains (*TMD*), intracellular loops (*IL*), and external loops (*EL*) are shown below the alignment in *yellow*, *pink*, and *turquoise*, respectively. "Extracellular" corresponds to the extracellular space and the lumen of the intracellular compartments. Residues highlighted in *black* and *gray* are highly conserved and semi-conserved, respectively. Residues highlighted in *red* indicate the positions of residues important for zinc transport. Conserved sequences in ZIP transporters [CPALLY, ZIP, and CHEXPHEXGD motif (Taylor and Nicholson 2003)] are indicated in *lavender*. The amino acid sequences at the N-terminal end of the LIV-1 subfamily members (the first seven proteins) are not displayed in the alignment, and the loop region corresponding to IL2 between TMDIII and TMDIV is omitted from the figure because of high sequence variations. "–" denotes a gap in the alignment

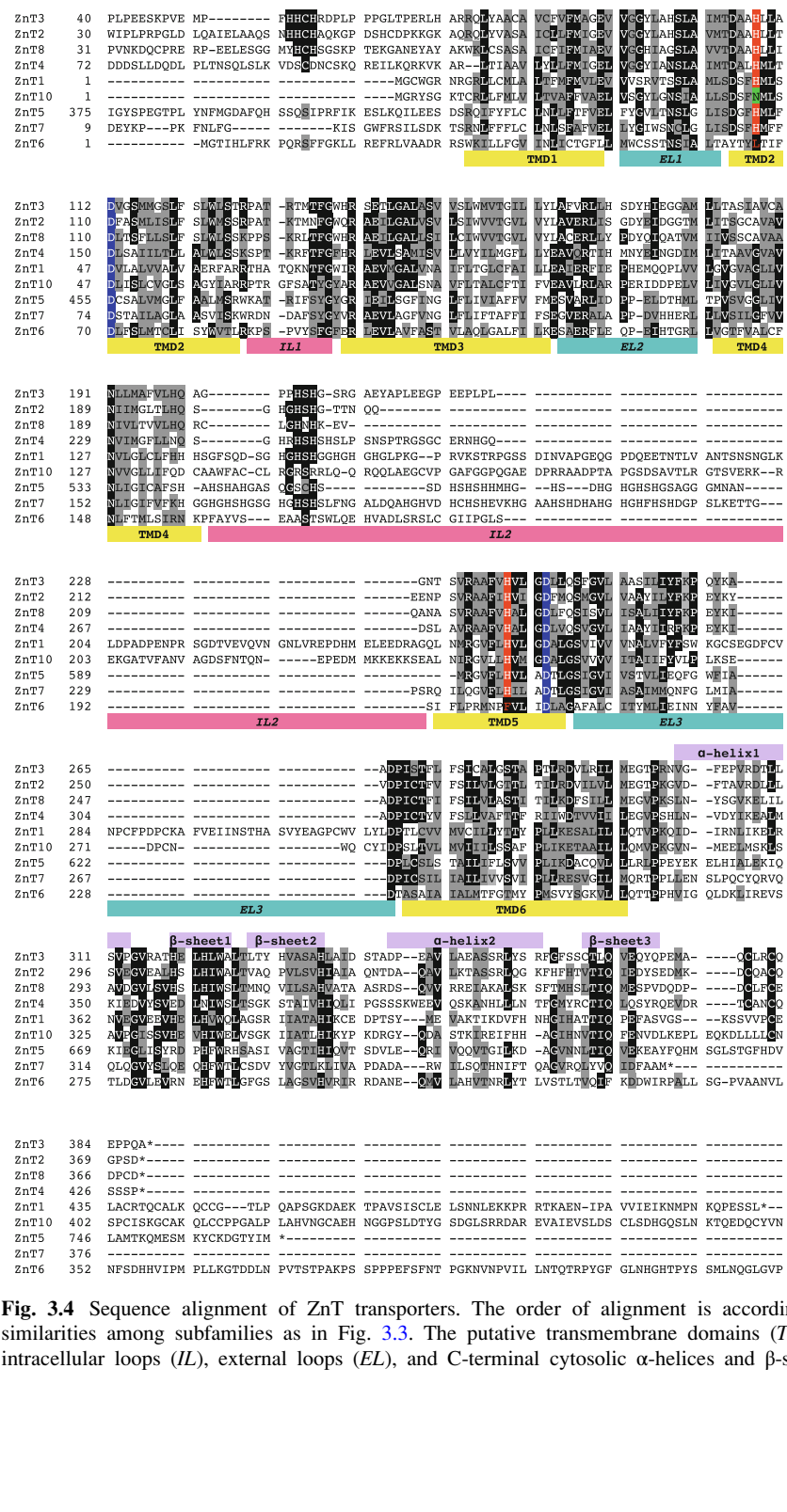

Fig. 3.4 Sequence alignment of ZnT transporters. The order of alignment is according to similarities among subfamilies as in Fig. 3.3. The putative transmembrane domains (*TMD*), intracellular loops (*IL*), external loops (*EL*), and C-terminal cytosolic α-helices and β-sheets

caused by defective intestinal zinc absorption, characterized by eczematous dermatitis, alopecia, and diarrhea (Maverakis et al. 2007; Schmitt et al. 2009). Mutations of the *ZIP13* gene have been shown to result in the spondylocheiro dysplastic form of Ehlers–Danlos syndrome (SCD-EDS) (Giunta et al. 2008; Fukada et al. 2008). SCD-EDS is characterized by abnormalities in hard and connective tissue development (see Chap. 12).

KO mice and other model organisms clearly show the pathophysiological importance of ZIP functions. *Zip1*-, *Zip2*-, and *Zip3*-KO mice do not show an obvious phenotype of zinc deficiency but are more likely to develop abnormal embryos when dietary zinc is limited (Dufner-Beattie et al. 2005, 2006; Peters et al. 2007). Similar phenotypes are also observed in *Zip1*, *Zip2*, and *Zip3* triple-KO mice (Kambe et al. 2008a). A recent study has revealed that *Zip3*-KO shows zinc retention in the secreted milk pool, indicating its functions in zinc reuptake from the alveolar lumen in lactating mammary glands (Kelleher et al. 2009). Moreover, *Zip1* and *Zip3* double-KO mice attenuate seizure-induced CA1 neurodegeneration (Qian et al. 2011), suggesting their involvement in zinc entry-induced neural degeneration. Complete *Zip4*-KO mice are embryonically nonviable, showing its indispensability during development (Dufner-Beattie et al. 2007). Furthermore, intestine-specific conditional *Zip4*-KO shows pivotal roles of Zip4 in intestinal integrity (Geiser et al. 2012). Recently, complete *Zip5*-KO mice and tissue-specific *Zip5*-KO mice have been generated (Geiser et al. 2013), clearly showing that Zip5 participates in the control of zinc excretion. The in vivo functions of Zip6 and Zip7 have not been yet found in mouse KO studies, but their importance has been confirmed in model organisms. In *Drosophila*, a Zip6 mutant (FOI) shows abnormal cell migration (Pielage et al. 2004) and defects in gonad formation (Mathews et al. 2006). In zebrafish, using an antisense *Zip6* morpholino (MO), ZIP6 (zLiv1) has been shown to be essential for gastrulation by controlling epithelial-mesenchymal transition (Yamashita et al. 2004). In a Zip7 *Drosophila* mutant (Catsup) showing semidominant lethality (Stathakis et al. 1999), catecholamine-related functions, and membrane protein trafficking including Notch and epidermal growth factor receptor are impaired (Stathakis et al. 1999; Groth et al. 2013). Morpholino-antisense gene knockdown of *Zip7* in zebrafish has demonstrated its requirement for eye, brain, and skeleton formation during early embryonic development (Yan et al. 2012). The hypomorphic *Zip8* mice show a fetal and postnatal lethal phenotype because of hypoplasia of multiple organs such as spleen, liver, kidney, and lung

Fig. 3.4 (continued) based on the crystal structure of YiiP are indicated in *yellow*, *pink*, *turquoise*, and *lavender*, respectively. Residues highlighted in *red* and *yellow* indicate H and D, respectively, essential for zinc binding in TMDs. Residues highlighted in *black* and *gray* are highly conserved and semi-conserved, respectively. The asparagine residue in TMD II of ZnT10, which has been speculated to be involved in recognition of manganese, is indicated in *green*. The amino acid sequences at the C-terminal end of ZnT6 (30 aa) and ZnT10 (4 aa) are not displayed in the alignment. The TMDs indicated in ZnT5, which is predicted to have fifteen TMDs, correspond to TMDs X and XV. "–" denotes a gap in the alignment

(Galvez-Peralta et al. 2012). The neonates also show defects in hematopoiesis. Thus, Zip8 plays indispensable roles in both multiple organ organogenesis and hematopoiesis from early embryogenesis (Galvez-Peralta et al. 2012). Recent analysis using *Zip12* antisense MO knockdown in *Xenopus* indicated its important role in nervous system development, including neurulation and neuronal differentiation. (Chowanadisai et al. 2013). *Zip13*-KO mice show phenotypes similar to those of SCD-EDS patients, such as delayed growth and skeletal and connective tissue abnormalities (Fukada et al. 2008). *Zip14*-KO mice have recently been generated, exhibiting growth retardation and impaired gluconeogenesis (Hojyo et al. 2011) (see Chap. 12). *Zip14*-KO mice also show greater body fat, hypoglycemia, and higher insulin levels, as well as increased liver glucose (Beker Aydemir et al. 2012), and decreased hepatocyte proliferation during liver regeneration (Aydemir et al. 2012).

3.2.3.2 Phenotypes of Inherited Human Diseases, and Mutant and KO Mice and other Model Organisms of ZnT Transporters

ZnT2 has been identified as the gene responsible for transient neonatal zinc deficiency, which is caused by low-zinc breast milk (Chowanadisai et al. 2006; Lasry et al. 2012; Itsumura et al. 2013). The nonsynonymous single nucleotide polymorphism (SNP), rs13266634, of the *ZnT8* gene increases the risk for type 2 diabetes mellitus (Sladek et al. 2007). The rs13266634 SNP has also been shown to be a determinant of humoral autoreactivity to ZnT8, which is found in approximately 60–80 % of type 1 diabetes mellitus patients at the onset of the disease (Wenzlau et al. 2008; Kawasaki et al. 2008). Homozygous mutations of the *ZnT10* gene are involved in parkinsonism, which is characterized by hepatic cirrhosis, polycythemia, dystonia, and hypermanganesemia. Thus, ZnT10 is probably involved in manganese transport.

In addition to *Zip*-KO mice, a number of *Znt*-KO mice have been generated. *Znt1*-KO mice are embryonically nonviable from an early stage (Andrews et al. 2004), revealing its indispensability during early development. In Drosophila, gut-specific silencing of *Znt1* has increased lethality under zinc-deficient conditions (Wang et al. 2009). *Znt3*-KO mice show lack of zinc in synaptic vesicles (Cole et al. 1999). Older *Znt3*-KO mice exhibit deficits in learning (Adlard et al. 2010), contextual discrimination and spatial working memory (Sindreu et al. 2011), and fear memory (Martel et al. 2010). A spontaneous *Znt4* mutant mouse, named lethal milk, is characterized by reduced milk zinc levels; thereby, pups nursed by affected dams die before weaning (Huang and Gitschier 1997). *Znt5*-KO mice show poor growth, osteopenia, and male-specific sudden cardiac death because of the bradyarrhythmia (Inoue et al. 2002). *Znt5*-KO mice also show defects in high-affinity immunoglobin E receptor (FcεRI)-induced cytokine production in mast cells, because of impaired nuclear factor kappa-light-chain enhancer of activated B cell (NF-κB) activation through protein kinase C (PKC) translocation to the plasma membrane (Nishida et al. 2009) (see Chap. 5). *Znt7*-KO mice display poor growth,

decreased adiposity, and reduced absorption of dietary zinc, although no typical symptoms of zinc deficiency are found (Huang et al. 2007). Male *Znt7*-KO mice fed with a high-fat diet have severe glucose intolerance and show insulin resistance (Huang et al. 2012). *Znt8*-KO mice have been generated in a number of research groups (Pound et al. 2009; Lemaire et al. 2009; Nicolson et al. 2009; Wijesekara et al. 2010; Hardy et al. 2012; Tamaki et al. 2013) (see Chap. 13). The importance of ZnT8 for the formation of zinc-insulin crystals has been consistent, but phenotypes related to glucose tolerance, insulin processing/secretion, and body weight have been variable among these mice. The molecular mechanism that causes these differences in *Znt8*-KO mice may give a clue about how the rs13266634 SNP in the *ZnT8* gene affects the pathogenesis of type 2 diabetes mellitus.

3.2.4 ZIP and ZnT Transporters and Zinc Signaling

Zinc mobilized by ZIP and ZnT transporters is engaged in zinc signaling. The best characterized zinc transporter related to zinc signaling is ZIP7 (Taylor et al. 2012) (Table 3.1). ZIP7 participates in the activation of signaling pathways including Akt and extracellular signal-regulated kinase (ERK) 1/2 by releasing zinc from the endoplasmic reticulum (ER) when phosphorylated by CK2 protein kinase in response to extracellular signals such as epidermal growth factor (EGF) (Taylor et al. 2012) (see Chap. 14). The zinc release from the perinuclear area including the ER is called a "zinc wave," which was first found in mast cells stimulated by FcεRI (Yamasaki et al. 2007) (see Chap. 5). A similar release of zinc from intracellular organelles and vesicles has been found with a number of ZIP transporters; ZIP13 releases zinc from the Golgi, which is important in the nuclear translocation of Smad proteins, thereby operating in the bone morphogenetic protein (BMP)/transforming growth factor-β (TGF- β) signaling pathway (Fukada et al. 2008) (see Chap. 12). ZIP13 has also been shown to be involved in zinc release from intracellular vesicle stores to maintain ER homeostasis by controlling ER stress (Jeong et al. 2012). ZIP9 functions in zinc release from the Golgi and is required for the regulation of Akt and ERK activation in the B cell-receptor signaling pathway (Matsuura et al. 2009; Taniguchi et al. 2013). ZIP8, when located to lysosomes, mediates lysosomal zinc release and is shown to control TCR-mediated T-cell activation, probably through inhibiting calcineurin, and thus sustaining cAMP response element-binding protein (CREB) activity (Aydemir et al. 2009).

Needless to say, zinc mobilized from the extracellular space into the cytosol by ZIP transporters is important for zinc signaling. ZIP8 plays a critical role in zinc signaling, when localized to the plasma membrane, by regulating host defense through inhibition of NF-κB via zinc-mediated down-modulation of IκB kinase (IKK) activity (Liu et al. 2013) (see Chap. 11). The closest homologue, ZIP14, facilitates G protein-coupled receptor-mediated cAMP-CREB signaling by suppressing the basal phosphodiesterase (PDE) activity in the growth plate, pituitary gland, and liver through mediating the zinc influx (Hojyo et al. 2011)

Table 3.1 Subcellular localization of ZIP transporters and their zinc signaling functions

Protein	Primary subcellular localization	Zinc signaling functions	References
ZIP1	Plasma membrane	–	
ZIP2	Plasma membrane	–	
ZIP3	Plasma membrane	–	
ZIP4	Plasma membrane	CREB signaling in pancreatic cancer	Zhang et al. (2010)
ZIP5	Plasma membrane	–	
ZIP6	Plasma membrane	Activation of Snail	Yamashita et al. (2004), Hogstrand et al. (2013)
		Phosphorylation of ERK1/2, inactivation of GSK-3β	Zhao et al. (2007), Hogstrand et al. (2013)
ZIP7	ER, Golgi apparatus	CK2-mediated "zinc wave" for Akt and ERK1/2 activation	Taylor et al. (2012), Hogstrand et al. (2009)
ZIP8	Plasma membrane, lysosomes	T-cell activation by sustaining CREB activity, inhibition of NF-κB signaling	Aydemir et al. (2009)
			Liu et al. (2013)
ZIP9	Golgi apparatus	B-cell receptor signaling	Taniguchi et al. (2013)
ZIP10	Plasma membrane	–	
ZIP11	Plasma membrane	–	
ZIP12	Plasma membrane	CREB activation	Chowanadisai et al. (2013)
ZIP13	Golgi apparatus, vesicles	BMP/TGF-β signaling	Fukada et al. (2008)
ZIP14	Plasma membrane	GPCR-mediated cAMP-CREB signaling	Hojyo et al. (2011)
		Inhibition of IRS-1/2 phosphorylation	Beker Aydemir et al. (2012)

(see Chap. 12). Moreover, zinc (and iron) mediated by ZIP14 is involved in inhibition of insulin receptor substrate (IRS) 1/2 phosphorylation through interleukin (IL) 6-mediated STAT3 signaling and cAMP-induced suppressors of cytokine signaling (SOCSs) (Beker Aydemir et al. 2012). Zinc mobilized by ectopically expressed ZIP4 is important for CREB activity (Zhang et al. 2010), which increases miR-373 expression, leading to promotion of pancreatic cancer growth (Zhang et al. 2013). ZIP6 negatively regulates Toll-like receptor (TLR) signaling, and thus a decrease in the intracellular zinc level caused by downregulation of ZIP6 is an important maturation signal in dendritic cells (Kitamura et al. 2006). ZIP6 also

Table 3.2 Subcellular localization of ZnT transporters and their zinc signaling functions

Protein	Primary subcellular localization	Zinc signaling functions	References
ZnT1	Plasma membrane	Activation of Ras/Raf/MEK/ERK pathway	Bruinsma et al. (2002), Jirakulaporn and Muslin (2004)
	ER	Downregulation of signaling via MTF-1, c-Jun, and Elk	Lazarczyk et al. (2008)
ZnT2	Endosomes/ lysosomes	–	
ZnT3	Synaptic vesicles	Modulation of synaptic transmission, ERK signaling in presynaptic neurons	Sindreu et al. (2011)
ZnT4	Endosomes/lysosomes, TGN	–	
ZnT5	Early secretory pathway	NF-κB signaling via PKC in mast cells	Nishida et al. (2009)
		Regulation of PKC/NF-κB and PI3K/Akt pathways	Zhang et al. (2012)
ZnT6	Early secretory pathway	–	
ZnT7	Early secretory pathway	Regulation of PKC/NF-κB, PI3K/Akt, and MAPK/ERK pathways	Zhang et al. (2012), Liang et al. (2013)
ZnT8	Insulin granules	Insulin clearance from bloodstream	Tamaki et al. (2013)
ZnT10	Golgi apparatus, recycling endosomes	ERK signaling	Patrushev et al. (2012)

has a role in T cell-receptor (TCR)-mediated T-cell activation by controlling Src homology region 2 domain-containing phosphatase-1 (SHP-1) recruitment to the TCR activation complex (Yu et al. 2011). Moreover, ZIP6 is involved in expression and phosphorylation of ERK1/2 (Zhao et al. 2007) and can inactivate glycogen synthase kinase 3β (GSK-3β) directly or indirectly via Akt by increasing the zinc influx, which results in activation of the zinc-finger transcription factor Snail in tumor cells (Hogstrand et al. 2013). In zebrafish, ZIP6 regulates the nuclear translocation of Snail, which is indispensable for the epithelial-mesenchymal transition (EMT) during early development (Yamashita et al. 2004). ZIP12, which is highly expressed in brain, has been shown to play crucial roles in neuronal differentiation, and neurite extension via CREB activation, by mobilizing zinc into neurons (Chowanadisai et al. 2013).

Important biological functions of zinc signaling associated with ZnTs are also abundant (Table 3.2), although the molecular mechanism is less clear in some cases. ZnT1 promotes Raf-1 activation by binding to the amino-terminal regulatory portion of Raf-1, which may be regulated by removing zinc from the Raf-1 cysteine-rich domain, because zinc inhibits the binding between Raf-1 and ZnT1

(Jirakulaporn and Muslin 2004). In nematodes, the ZnT1 orthologue, CDF1, plays a crucial role in fine-tuning Ras-mediated signaling (Bruinsma et al. 2002); thereby, the signaling functions of ZnT1 in the Ras/Raf/MEK/ERK pathway are highly conserved among metazoa. The Ras-ERK activation by ZnT1 also contributes to the enhancement of T-type Ca channel activity by increasing its surface expression (Mor et al. 2012), and in the brain it is important for protection of cells from simulated ischemia–reperfusion (Beharier et al. 2012). Moreover, ZnT1 has been shown to downregulate transcription factors stimulated by metal regulatory transcription factor 1 (MTF1), c-Jun, and Elk, when localized to the ER, by forming a hetero-complex with EVER1 and EVER2 in keratinocytes (Lazarczyk et al. 2008). In dendritic cells, ZnT1 controls TLR signaling by decreasing the intracellular zinc level (Kitamura et al. 2006). ZnT3 is important in ERK1/2 MAPK signaling via inhibition of the zinc-sensitive MAPK tyrosine phosphatase activity in presynaptic neurons, although the contribution of ZnT3 is indirect, because the effects occur after zinc, which ZnT3 transports into presynaptic vesicles, is synaptically released and then taken up again in presynaptic neurons (Sindreu et al. 2011). ZnT5 is employed in zinc signaling by NF-κB activation through PKC translocation to the plasma membrane (Nishida et al. 2009) (see Chap. 5). ZnT5 and ZnT7 contributes to maintain secretory pathway homeostasis and regulate zinc-requiring enzyme activities (Ishihara et al. 2006; Suzuki et al. 2005a; Fukunaka et al. 2011). ZnT5 and ZnT7 have also been suggested to be involved in PKC/NF-κB and phosphatidyl inositide 3-kinase (PI3K)/Akt signaling (Zhang et al. 2012). ZnT7 has been shown to protect cells from oxidative stress-induced apoptosis via activating the PI3K/Akt-mediated survival pathway and the MAPK/ERK pathway (Liang et al. 2013). Zinc is accumulated by ZnT8 in insulin granules for insulin crystallization in pancreatic β cells (Chimienti et al. 2005). Zinc released from β cells in response to increased glucose levels decreases insulin secretion from β cells and reduces insulin clearance from the bloodstream by the liver, which indicates that ZnT8 is involved in zinc signaling in both an autocrine and endocrine fashion (Tamaki et al. 2013). ZnT10 functions in the regulation of ERK signaling by controlling cellular zinc levels (Patrushev et al. 2012). All the information clearly shows that ZIP- and ZnT-mediated zinc is indispensable in zinc signaling.

3.3 Zinc Permeable Channels and Membrane Proteins

Membrane transport proteins other than ZIP and ZnT transporters have also been shown to contribute to cellular zinc mobilization across the cellular membrane (Frederickson et al. 2005; Sensi et al. 2011; Bouron and Oberwinkler 2013) (Fig. 3.5). However, comprehensive understanding is limited, and many cases need to be investigated more closely to elucidate their importance in zinc physiology and pathogenesis. In this section, membrane transport channels that have been shown to be involved in zinc transport are summarized.

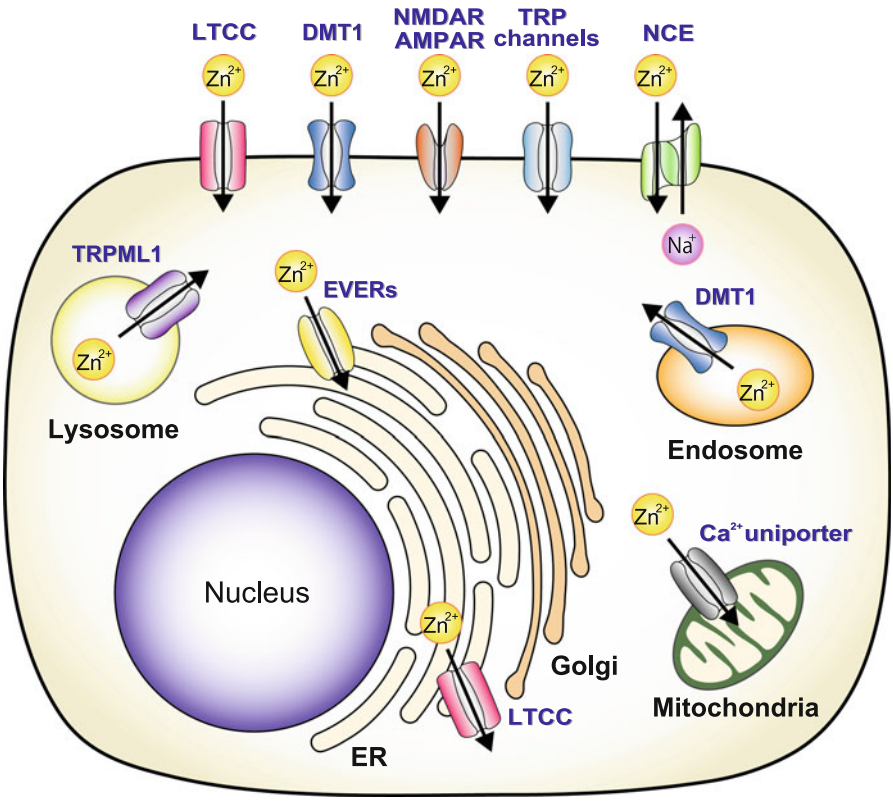

Fig. 3.5 Subcellular localization and direction of zinc mobilization of Ca channels and other membrane proteins involved in zinc transport shown according to available information. The L-type calcium channel (*LTCC*) localized to the ER is lacking the β-subunit. The contribution of divalent metal transporter 1 (*DMT1*) to zinc transport is thought to be minor. *NCE* Na^{+}–Ca^{2+} exchanger

3.3.1 Ca Channels

Ca ions are the most common second messengers in eukaryotic cells. Thus, all cells finely tune the Ca signal using broad groups of channels and pump proteins (Rizzuto and Pozzan 2003). The Ca permeable channels are generally divided into a number of groups: (1) voltage-gated Ca channels, (2) receptor-operated Ca channels, (3) second messenger-operated channels, and (4) transient receptor potential (TRP) channels. These groups are subdivided into a great diversity of protein families based on molecular, biophysical, pharmacological, and functional properties. Ca channels such as Ca release-activated Ca channels, InsP3 receptor, and ryanodine receptor channels increase the Ca levels in the cytosol, thus eliciting Ca signaling, whereas sarcoplasmic/endoplasmic reticulum Ca ATPases (SERCAs)

and plasma membrane Ca ATPases (PMCAs) remove Ca from the cytosol, thus terminating Ca signaling (Feske et al. 2012). The tuning of Ca influx versus Ca efflux/mobilization depends on the stimulus and the cell type.

Among the Ca channels, proteins belonging to voltage-gated Ca channels (VGCCs), glutamate-gated receptors, and TRP channels are thought to be involved in zinc transport (Bouron and Oberwinkler 2013) and to contribute to the cellular zinc metabolism in specific cellular events.

VGCCs are known as selective Ca channels, but many evidences have indicated that they can permeate zinc. Most VGCCs are large protein complexes composed of a pore-forming α-subunit and one or more auxiliary β-subunits that regulate channel properties (Buraei and Yang 2013). The L-type Ca channel (LTCC), a member of VGCC, is involved in some forms of pathological death of neurons as a zinc entry route from the extracellular space (Sensi et al. 1997; Kim et al. 2000), but probably contributes to a physiological zinc entry route in pancreatic β cells under stimulatory conditions during the depolarization of the plasma membrane (Gyulkhandanyan et al. 2006). In mast cells, the pore-forming α1D subunit of LTCC has been shown to be located to the ER, probably because of very low expression of the β-subunit, and thus is operative for zinc release from the ER after stimulation through FcεRI (Yamasaki et al. 2012): this is an important mechanism of the "zinc wave" in mast cells (Yamasaki et al. 2007) (see Chap. 5).

Glutamate-gated receptors are known to bind extracellular zinc, and thus their activities are modulated by synaptic zinc (see Chaps. 8 and 9). Some glutamate receptors also function as zinc permeable channels in postsynaptic neurons, mainly in pathological aspects such as ischemic injury. Ca permeable *α*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite-type glutamate receptors and *N*-Methyl-D-aspartate (NMDA)-type glutamate receptors provide zinc entry routes in postsynaptic neurons and astrocytes during neuronal excitation (Frederickson et al. 2005; Sensi et al. 2009). Zinc inhibits NMDA receptors, which seems somewhat paradoxical considering its zinc permeation property. An interesting finding has been reported about the zinc-permeable activity of AMPA receptor channels. Prion protein facilitates zinc uptake mediated by AMPA receptors containing GluA1, but lacking GluA2 subunits, in neuronal cells (Watt et al. 2012). In this case, the N-terminal polybasic region of the prion protein is important for interaction with the AMPA receptor, and the octapeptide repeat of the prion protein (Walter et al. 2007), which is known to bind with zinc, is required for facilitating zinc uptake by AMPA receptors (Watt et al. 2012). Moreover, zinc permeation by nicotinic acetylcholine receptors has been shown at higher concentrations of zinc (Ragozzino et al. 2000), but the pathophysiological meaning is unclear.

The mammalian TRP channels superfamily comprises 28 members, which are divided into seven subfamilies: TRPA, TRPC, TRPM, TRPN, TRPV, TRPP, and TRPML (Venkatachalam and Montell 2007). A number of TRP channel proteins have been shown to operate in zinc transport. TRPA1, which is a cation channel primarily expressed in sensory nerves, is activated by the psychoactive component in marijuana, environmental irritants, pungent compounds (Venkatachalam and Montell 2007), and zinc (Banke and Wickenden 2009). TRPA1 can operate as a

zinc influx route for extracellular zinc, and the cytosolic aspartate residue at position 915 of TRPA1 is indispensable for its zinc mobilization (Hu et al. 2009). The D915A TRPA1 mutant cannot permeate zinc or be activated by extracellular zinc; however it can be activated by intracellular zinc (Hu et al. 2009), indicating its physiological relevance in zinc mobilization. TRPC6, but not TRPC3, facilitates intracellular zinc accumulation when overexpressed (Gibon et al. 2011). TRPM7 is well known as a magnesium (Mg) conducting channel in addition to Ca (Schmitz et al. 2003; Ryazanova et al. 2010). TRPM7 appears to permeate zinc in the presence of Ca and Mg (Monteilh-Zoller et al. 2003) and has been shown to contribute to zinc-induced neurotoxicity (Inoue et al. 2010). TRPM7 is known to form heterodimers with TRPM6, which can also permeate zinc (Li et al. 2006). TRPM1, another member of the TRPM subfamily, is not permeable to zinc (Lambert et al. 2011), which excludes the possibility that all the members of this subfamily are involved in zinc transport. TRPV5 and TRPV6 have been shown to contribute to a zinc influx route when overexpressed in cells, but their physiological relevance in mammals remains unknown (Qiu and Hogstrand. 2004; Kovacs et al. 2013).

TRPML1 functions as a nonselective cation channel including Ca and other metals such as iron (Dong et al. 2008). Loss of TRPML1 causes the lysosomal storage disorder, mucolipidosis IV (MLIV), resulting in large hyperacidic lysosomes, membranous vacuoles, mitochondrial fragmentation, and autophagic dysfunction (Dong et al. 2008). The lysosomes of TRPML1-knockdown cells accumulate chelatable zinc ions, suggesting TRPML1 is involved in zinc release from lysosomes (Eichelsdoerfer et al. 2010). A recent study has reported that TRPML1 works in concert with ZnT4 to regulate zinc translocation between the cytosol and lysosomes (Kukic et al. 2013) and is thereby important for proper cell function.

3.3.2 Other Membrane Proteins

A number of reports show the involvement of membrane proteins in zinc transport; representative examples are described here. Transmembrane channel-like (TMC) proteins constitute eight members in mammals, which are thought to operate as ion channels (Hahn et al. 2009). The ER membrane proteins, EVER1 and EVER2, described earlier, are identical to TMC6 and TMC8, which contribute to the regulation of intracellular zinc distribution by forming complexes with ZnT1 (Lazarczyk et al. 2009). EVER proteins are also probably involved in the activation-dependent regulation of zinc concentration in T cells (Lazarczyk et al. 2012).

Divalent metal transporter 1 (DMT1) is well known as an essential iron transporter (Gunshin et al. 1997). Because DMT1 can transport various divalent cations, DMT1 generally is thought to be involved in zinc transport. However, some reports have shown that DMT1 can transport zinc poorly (Mackenzie et al. 2007; Illing et al. 2012). A putative member of the Na^+–Ca^{2+} exchanger superfamily can mobilize zinc in or out of the cytosol, depending on the Na^+ gradient

Table 3.3 Subcellular localization of Ca channels and their zinc signaling functions

Protein	Primary subcellular localization	Zinc signaling functions	References
L-type Ca channel	ER	"Zinc wave"-mediated by FcεRI in mast cells	Yamasaki et al. (2012)
AMPA receptors	Plasma membrane	Inhibition of zinc-sensitive tyrosine phosphatases	Watt et al. (2012)

(Sensi et al. 2009). Mobilization of zinc into mitochondria is thought to be conducted through a cation-permeable channel or the mitochondrial Ca uniporter, which can prevent cytosolic zinc accumulation in neurons undergoing excitotoxicity (Sensi et al. 2009).

Although they are not transport proteins, membrane proteins may contribute to the regulation of zinc transport activity. An intriguing example is presenilin. Presenilin 1 and 2 are serpentine integral membrane proteins with nine TMDs known for their role as the catalytic subunit of γ-secretase, which is involved in proteolysis of type I integral membrane proteins, including amyloid precursor protein and Notch receptor (Tomita and Iwatsubo 2013). Zinc uptake from the extracellular space is decreased in cells lacking presenilins, and thus presenilins may be involved in the regulation of the zinc uptake process (Greenough et al. 2011).

3.3.3 Zinc Signaling via Ca Channels

There is little information indicating that Ca channels and other membrane proteins directly contribute to zinc signaling (Table 3.3). However, an important evidence has been reported for the "zinc wave" process, in which the pore-forming α1D-subunit of LTCC plays a role in zinc release from the ER, as described earlier (Yamasaki et al. 2012) (see Chap. 5). Recently, it has been reported that under control of prion protein, zinc mediated by Ca-permeable AMPA receptors regulates the activity of zinc-sensitive tyrosine phosphatases (Watt et al. 2012). The enhanced zinc uptake by prion protein is lost in familial-associated mutants of prion protein (Watt et al. 2012), which suggests that zinc mobilization regulated by prion protein via AMPA receptors may affect learning and memory and promote neuronal survival. This kind of zinc signaling may have some roles in many cases of zinc permeation through Ca channels and other membrane proteins. Moreover, zinc influx into the cytosol of the postsynaptic cells, probably via NMDA, AMPA, or VGCCs, regulates Src activity, which contributes to the activation of receptor tyrosine kinase TrkB and synaptic potentiation (Huang et al. 2008). Zinc influx via these receptors and channels also contributes to activation in multiple intracellular pathways leading to necrotic, apoptotic, and autophagic neuronal death (Sensi et al. 2009).

3.4 Concluding Remarks and Perspectives

A growing body of evidence indicates that zinc transport proteins play pivotal roles in zinc signaling. However, the molecular basis explaining how zinc signaling conducted by zinc transport proteins controls each specific event is far from being complete and thus should be intensively investigated. The impaired zinc signaling caused by loss or reduced functions of zinc transport proteins can be partially rescued by zinc supplements in some cases, but not in other cases (Fukada et al. 2008; Hojyo et al. 2011; Taylor et al. 2012; Taniguchi et al. 2013; Liu et al. 2013), which suggests that zinc signaling conducted by zinc transport proteins is controlled by sophisticated regulation mechanisms. One mechanism may be that the concentration and distribution of zinc ions mobilized by zinc transport proteins is strictly controlled spatially and temporally, and the other may be that zinc transport process is modulated by other specific proteins for strict regulation (Taylor et al. 2012). The latter may include the case in which a putative zinc chaperone protein supports zinc signaling by specifying zinc handling to zinc transport proteins or target proteins (Fukada and Kambe 2011; Fujimoto et al. 2013). Considering the involvement of zinc signaling in multifarious pathophysiological processes, the answers to these questions would provide novel insight and may lead to therapeutic progress.

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References

Adlard PA, Parncutt JM, Finkelstein DI, Bush AI (2010) Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease? J Neurosci 30:1631–1636

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

Aydemir TB, Liuzzi JP, McClellan S, Cousins RJ (2009) Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells. J Leukoc Biol 86:337–348

Aydemir TB, Sitren HS, Cousins RJ (2012) The zinc transporter Zip14 influences c-Met phosphorylation and hepatocyte proliferation during liver regeneration in mice. Gastroenterology 142:1536–1546

Banke TG, Wickenden AD (2009) Intracellular zinc irritates TRPA1. Nat Chem Biol 5:141–142

Beharier O, Dror S, Levy S, Kahn J, Mor M, Etzion S et al (2012) ZnT-1 protects HL-1 cells from simulated ischemia-reperfusion through activation of Ras-ERK signaling. J Mol Med (Berl) 90:127–138

Beker Aydemir T, Chang SM, Guthrie GJ, Maki AB, Ryu MS, Karabiyik A et al (2012) Zinc transporter ZIP14 functions in hepatic zinc, iron and glucose homeostasis during the innate immune response (endotoxemia). PLoS One 7:e48679

Bin BH, Fukada T, Hosaka T, Yamasaki S, Ohashi W, Hojyo S et al (2011) Biochemical characterization of human ZIP13 protein: a homo-dimerized zinc transporter involved in the spondylocheiro dysplastic Ehlers–Danlos syndrome. J Biol Chem 286:40255–40265

Bouron A, Oberwinkler J (2014) Contribution of calcium-conducting channels to the transport of zinc ions. Pflugers Arch. 466:381–387

Bruinsma JJ, Jirakulaporn T, Muslin AJ, Kornfeld K (2002) Zinc ions and cation diffusion facilitator proteins regulate Ras- mediated signaling. Dev Cell 2:567–578

Buraei Z, Yang J (2013) Structure and function of the beta subunit of voltage-gated Ca^{2+} channels. Biochim Biophys Acta 1828:1530–1540

Chimienti F, Favier A, Seve M (2005) ZnT-8, a pancreatic beta-cell-specific zinc transporter. Biometals 18:313–317

Chowanadisai W, Graham DM, Keen CL, Rucker RB, Messerli MA (2013) Neurulation and neurite extension require the zinc transporter ZIP12 (slc39a12). Proc Natl Acad Sci USA 110:9903–9908

Chowanadisai W, Lonnerdal B, Kelleher SL (2006) Identification of a mutation in SLC30A2 (ZnT-2) in women with low milk zinc concentration that results in transient neonatal zinc deficiency. J Biol Chem 281:39699–39707

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

Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. J Biol Chem 281:24085–24089

Dong XP, Cheng X, Mills E, Delling M, Wang F, Kurz T et al (2008) The type IV mucolipidosis-associated protein TRPML1 is an endolysosomal iron release channel. Nature (Lond) 455:992–996

Dufner-Beattie J, Huang ZL, Geiser J, Xu W, Andrews GK (2005) Generation and characterization of mice lacking the zinc uptake transporter ZIP3. Mol Cell Biol 25:5607–5615

Dufner-Beattie J, Huang ZL, Geiser J, Xu W, Andrews GK (2006) Mouse ZIP1 and ZIP3 genes together are essential for adaptation to dietary zinc deficiency during pregnancy. Genesis 44:239–251

Dufner-Beattie J, Weaver BP, Geiser J, Bilgen M, Larson M, Xu W et al (2007) The mouse acrodermatitis enteropathica gene Slc39a4 (Zip4) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency. Hum Mol Genet 16:1391–1399

Eichelsdoerfer JL, Evans JA, Slaugenhaupt SA, Cuajungco MP (2010) Zinc dyshomeostasis is linked with the loss of mucolipidosis IV-associated TRPML1 ion channel. J Biol Chem 285:34304–34308

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

Eide DJ (2006) Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta 1763:711–722

Eng BH, Guerinot ML, Eide D, Saier MH Jr (1998) Sequence analyses and phylogenetic characterization of the ZIP family of metal ion transport proteins. J Membr Biol 166:1–7

Feske S, Skolnik EY, Prakriya M (2012) Ion channels and transporters in lymphocyte function and immunity. Nat Rev Immunol 12:532–547

Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6:449–462

Fujimoto S, Itsumura N, Tsuji T, Anan Y, Tsuji N, Ogra Y et al (2013) Cooperative functions of ZnT1, metallothionein and ZnT4 in the cytoplasm are required for full activation of TNAP in the early secretory pathway. PLoS One 8:e77445

Fujishiro H, Yano Y, Takada Y, Tanihara M, Himeno S (2012) Roles of ZIP8, ZIP14, and DMT1 in transport of cadmium and manganese in mouse kidney proximal tubule cells. Metallomics 4:700–708

Fukada T, Civic N, Furuichi T, Shimoda S, Mishima K, Higashiyama H et al (2008) The zinc transporter SLC39A13/ZIP13 is required for connective tissue development; its involvement in BMP/TGF-beta signaling pathways. PLoS One 3:e3642

Fukada T, Kambe T (2011) Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics 3:662–674

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signaling in health and diseases: zinc signaling. J Biol Inorg Chem 16:1123–1134

Fukunaka A, Kurokawa Y, Teranishi F, Sekler I, Oda K, Ackland ML et al (2011) Tissue nonspecific alkaline phosphatase is activated via a two-step mechanism by zinc transport complexes in the early secretory pathway. J Biol Chem 286:16363–16373

Fukunaka A, Suzuki T, Kurokawa Y, Yamazaki T, Fujiwara N, Ishihara K et al (2009) Demonstration and characterization of the heterodimerization of ZnT5 and ZnT6 in the early secretory pathway. J Biol Chem 284:30798–30806

Gaither LA, Eide DJ (2000) Functional expression of the human hZIP2 zinc transporter. J Biol Chem 275:5560–5564

Gaither LA, Eide DJ (2001) Eukaryotic zinc transporters and their regulation. Biometals 14:251–270

Galvez-Peralta M, He L, Jorge-Nebert LF, Wang B, Miller ML, Eppert BL et al (2012) ZIP8 zinc transporter: indispensable role for both multiple-organ organogenesis and hematopoiesis in utero. PLoS One 7:e36055

Geiser J, De Lisle RC, Andrews GK (2013) The zinc transporter Zip5 (Slc39a5) regulates intestinal zinc excretion and protects the pancreas against zinc toxicity. PLoS One 8:e82149

Geiser J, Venken KJ, De Lisle RC, Andrews GK (2012) A mouse model of acrodermatitis enteropathica: loss of intestine zinc transporter ZIP4 (Slc39a4) disrupts the stem cell niche and intestine integrity. PLoS Genet 8:e1002766

Gibon J, Tu P, Bohic S, Richaud P, Arnaud J, Zhu M et al (2011) The over-expression of TRPC6 channels in HEK-293 cells favours the intracellular accumulation of zinc. Biochim Biophys Acta 1808:2807–2818

Giunta C, Elcioglu NH, Albrecht B, Eich G, Chambaz C, Janecke AR et al (2008) Spondylocheiro dysplastic form of the Ehlers–Danlos syndrome: an autosomal-recessive entity caused by mutations in the zinc transporter gene SLC39A13. Am J Hum Genet 82:1290–1305

Greenough MA, Volitakis I, Li QX, Laughton K, Evin G, Ho M et al (2011) Presenilins promote the cellular uptake of copper and zinc and maintain copper chaperone of SOD1-dependent copper/zinc superoxide dismutase activity. J Biol Chem 286:9776–9786

Groth C, Sasamura T, Khanna MR, Whitley M, Fortini ME (2013) Protein trafficking abnormalities in *Drosophila* tissues with impaired activity of the ZIP7 zinc transporter catsup. Development (Camb) 140:3018–3027

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF et al (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature (Lond) 388:482–488

Gustin JL, Zanis MJ, Salt DE (2011) Structure and evolution of the plant cation diffusion facilitator family of ion transporters. BMC Evol Biol 11:76

Gyulkhandanyan AV, Lee SC, Bikopoulos G, Dai F, Wheeler MB (2006) The Zn^{2+}-transporting pathways in pancreatic beta-cells: a role for the L-type voltage-gated Ca^{2+} channel. J Biol Chem 281:9361–9372

Haase H, Rink L (2009) Functional significance of zinc-related signaling pathways in immune cells. Annu Rev Nutr 29:133–152

Hahn Y, Kim DS, Pastan IH, Lee B (2009) Anoctamin and transmembrane channel-like proteins are evolutionarily related. Int J Mol Med 24:51–55

Hardy A, Wijesekara N, Genkin I, Prentice KJ, Bhattacharjee A, Kong D et al (2012) Effects of high fat diet feeding on Znt8-null mice: differences between beta cell and global knockout of Znt8. Am J Physiol Endocrinol Metab 302:E1084–E1096

He L, Girijashanker K, Dalton TP, Reed J, Li H, Soleimani M et al (2006) ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of transporter properties. Mol Pharmacol 70:171–180

Hennigar SR, Kelleher SL (2012) Zinc networks: the cell-specific compartmentalization of zinc for specialized functions. Biol Chem 393:565–578
Hirano T, Murakami M, Fukada T, Nishida K, Yamasaki S, Suzuki T (2008) Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. Adv Immunol 97:149–176
Hogstrand C, Kille P, Ackland ML, Hiscox S, Taylor KM (2013) A mechanism for epithelial-mesenchymal transition and anoikis resistance in breast cancer triggered by zinc channel ZIP6 and STAT3 (signal transducer and activator of transcription 3). Biochem J 455:229–237
Hogstrand C, Kille P, Nicholson RI, Taylor KM (2009) Zinc transporters and cancer: a potential role for ZIP7 as a hub for tyrosine kinase activation. Trends Mol Med 15:101–111
Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin BH, Koseki H et al (2011) The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. PLoS One 6:e18059
Hu H, Bandell M, Petrus MJ, Zhu MX, Patapoutian A (2009) Zinc activates damage-sensing TRPA1 ion channels. Nat Chem Biol 5:183–190
Huang L, Gitschier J (1997) A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat Genet 17:292–297
Huang L, Kirschke CP, Lay YA, Levy LB, Lamirande DE, Zhang PH (2012) Znt7-null mice are more susceptible to diet-induced glucose intolerance and insulin resistance. J Biol Chem 287:33883–33896
Huang L, Tepaamorndech S (2013) The SLC30 family of zinc transporters: a review of current understanding of their biological and pathophysiological roles. Mol Aspects Med 34:548–560
Huang L, Yu YY, Kirschke CP, Gertz ER, Lloyd KK (2007) Znt7 (Slc30a7)-deficient mice display reduced body zinc status and body fat accumulation. J Biol Chem 282:37053–37063
Huang YZ, Pan E, Xiong ZQ, McNamara JO (2008) Zinc-mediated transactivation of TrkB potentiates the hippocampal mossy fiber-CA3 pyramid synapse. Neuron 57:546–558
Illing AC, Shawki A, Cunningham CL, Mackenzie B (2012) Substrate profile and metal-ion selectivity of human divalent metal-ion transporter-1. J Biol Chem 287:30485–30496
Inoue K, Branigan D, Xiong ZG (2010) Zinc-induced neurotoxicity mediated by transient receptor potential melastatin 7 channels. J Biol Chem 285:7430–7439
Inoue K, Matsuda K, Itoh M, Kawaguchi H, Tomoike H, Aoyagi T et al (2002) Osteopenia and male-specific sudden cardiac death in mice lacking a zinc transporter gene, Znt5. Hum Mol Genet 11:1775–1784
Ishihara K, Yamazaki T, Ishida Y, Suzuki T, Oda K, Nagao M et al (2006) Zinc transport complexes contribute to the homeostatic maintenance of secretory pathway function in vertebrate cells. J Biol Chem 281:17743–17750
Itsumura N, Inamo Y, Okazaki F, Teranishi F, Narita H, Kambe T et al (2013) Compound heterozygous mutations in SLC30A2/ZnT2 results in low milk zinc concentrations: a novel mechanism for zinc deficiency in a breast-fed infant. PLoS One 8:e64045
Jenkitkasemwong S, Wang CY, Mackenzie B, Knutson MD (2012) Physiologic implications of metal-ion transport by ZIP14 and ZIP8. Biometals 25:643–655
Jeong J, Eide DJ (2013) The SLC39 family of zinc transporters. Mol Aspects Med 34:612–619
Jeong J, Walker JM, Wang F, Park JG, Palmer AE, Giunta C et al (2012) Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers–Danlos syndrome. Proc Natl Acad Sci USA 109:E3530–E3538
Jirakulaporn T, Muslin AJ (2004) Cation diffusion facilitator proteins modulate Raf-1 activity. J Biol Chem 279:27807–27815
Kambe T (2011) An overview of a wide range of functions of ZnT and Zip zinc transporters in the secretory pathway. Biosci Biotechnol Biochem 75:1036–1043
Kambe T (2012) Molecular architecture and function of ZnT transporters. Curr Top Membr 69:199–220
Kambe T (2013) Regulation of zinc transport. In: Culotta V, Scott RS (eds) Encyclopedia of inorganic and bioinorganic chemistry: metals in cells. Wiley, Chichester, pp 301–309

Kambe T, Geiser J, Lahner B, Salt DE, Andrews GK (2008a) Slc39a1 to 3 (subfamily II) Zip genes in mice have unique cell-specific functions during adaptation to zinc deficiency. Am J Physiol Regul Integr Comp Physiol 294:R1474–R1481

Kambe T, Suzuki T, Nagao M, Yamaguchi-Iwai Y (2006) Sequence similarity and functional relationship among eukaryotic ZIP and CDF transporters. Genomics Proteomics Bioinformatics 4:1–9

Kambe T, Weaver BP, Andrews GK (2008b) The genetics of essential metal homeostasis during development. Genesis 46:214–228

Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M (2004) Overview of mammalian zinc transporters. Cell Mol Life Sci 61:49–68

Kawachi M, Kobae Y, Kogawa S, Mimura T, Kramer U, Maeshima M (2012) Amino acid screening based on structural modeling identifies critical residues for the function, ion selectivity and structure of *Arabidopsis* MTP1. FEBS J 279:2339–2356

Kawachi M, Kobae Y, Mimura T, Maeshima M (2008) Deletion of a histidine-rich loop of AtMTP1, a vacuolar Zn^{2+}/H^{+} antiporter of *Arabidopsis thaliana*, stimulates the transport activity. J Biol Chem 283:8374–8383

Kawasaki E, Uga M, Nakamura K, Kuriya G, Satoh T, Fujishima K et al (2008) Association between anti-ZnT8 autoantibody specificities and SLC30A8 Arg325Trp variant in Japanese patients with type 1 diabetes. Diabetologia 51:2299–2302

Kelleher SL, Lopez V, Lonnerdal B, Dufner-Beattie J, Andrews GK (2009) Zip3 (Slc39a3) functions in zinc reuptake from the alveolar lumen in lactating mammary gland. Am J Physiol Regul Integr Comp Physiol 297:R194–R201

Kitamura H, Morikawa H, Kamon H, Iguchi M, Hojyo S, Fukada T et al (2006) Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nat Immunol 7:971–977

Kim AH, Sheline CT, Tian M, Higashi T, McMahon R, Cousins RJ et al (2000) L-type Ca^{2+} channel-mediated Zn^{2+} toxicity and modulation by ZnT-1 in PC12 cells. Brain Res 886:99–107

Kovacs G, Montalbetti N, Franz MC, Graeter S, Simonin A, Hediger MA (2013) Human TRPV5 and TRPV6: key players in cadmium and zinc toxicity. Cell Calcium 54:276–286

Kukic I, Lee JK, Coblentz J, Kelleher SL, Kiselyov K (2013) Zinc-dependent lysosomal enlargement in TRPML1-deficient cells involves MTF-1 transcription factor and ZnT4 (Slc30a4) transporter. Biochem J 451:155–163

Kury S, Dreno B, Bezieau S, Giraudet S, Kharfi M, Kamoun R et al (2002) Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. Nat Genet 31:239–240

Lambert S, Drews A, Rizun O, Wagner TF, Lis A, Mannebach S et al (2011) Transient receptor potential melastatin 1 (TRPM1) is an ion-conducting plasma membrane channel inhibited by zinc ions. J Biol Chem 286:12221–12233

Lasry I, Seo YA, Ityel H, Shalva N, Pode-Shakked B, Glaser F et al (2012) A dominant negative heterozygous G87R mutation in the zinc transporter, ZnT-2 (SLC30A2), results in transient neonatal zinc deficiency. J Biol Chem 287:29348–29361

Lazarczyk M, Cassonnet P, Pons C, Jacob Y, Favre M (2009) The EVER proteins as a natural barrier against papillomaviruses: a new insight into the pathogenesis of human papillomavirus infections. Microbiol Mol Biol Rev 73:348–370

Lazarczyk M, Dalard C, Hayder M, Dupre L, Pignolet B, Majewski S et al (2012) EVER proteins, key elements of the natural anti-human papillomavirus barrier, are regulated upon T-cell activation. PLoS One 7:e39995

Lazarczyk M, Pons C, Mendoza JA, Cassonnet P, Jacob Y, Favre M (2008) Regulation of cellular zinc balance as a potential mechanism of EVER-mediated protection against pathogenesis by cutaneous oncogenic human papillomaviruses. J Exp Med 205:35–42

Lemaire K, Ravier MA, Schraenen A, Creemers JW, Van de Plas R, Granvik M et al (2009) Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. Proc Natl Acad Sci USA 106:14872–14877

Li M, Jiang J, Yue L (2006) Functional characterization of homo- and heteromeric channel kinases TRPM6 and TRPM7. J Gen Physiol 127:525–537

Liang D, Xiang L, Yang M, Zhang X, Guo B, Chen Y et al (2013) ZnT7 can protect MC3T3-E1 cells from oxidative stress-induced apoptosis via PI3K/Akt and MAPK/ERK signaling pathways. Cell Signal 25:1126–1135

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr 29:153–176

Lin W, Chai J, Love J, Fu D (2010) Selective electrodiffusion of zinc ions in a Zrt-, Irt-like protein, ZIPB. J Biol Chem 285:39013–39020

Liu MJ, Bao S, Galvez-Peralta M, Pyle CJ, Rudawsky AC, Pavlovicz RE et al (2013) ZIP8 regulates host defense through zinc-mediated inhibition of NF-kappaB. Cell Rep 3:386–400

Liuzzi JP, Aydemir F, Nam H, Knutson MD, Cousins RJ (2006) Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. Proc Natl Acad Sci USA 103:13612–13617

Lu M, Chai J, Fu D (2009) Structural basis for autoregulation of the zinc transporter YiiP. Nat Struct Mol Biol 16:1063–1067

Lu M, Fu D (2007) Structure of the zinc transporter YiiP. Science 317:1746–1748

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

Mackenzie B, Takanaga H, Hubert N, Rolfs A, Hediger MA (2007) Functional properties of multiple isoforms of human divalent metal-ion transporter 1 (DMT1). Biochem J 403:59–69

Maret W (2013) Zinc biochemistry: from a single zinc enzyme to a key element of life. Adv Nutr 4:82–91

Maret W, Li Y (2009) Coordination dynamics of zinc in proteins. Chem Rev 109:4682–4707

Martel G, Hevi C, Friebely O, Baybutt T, Shumyatsky GP (2010) Zinc transporter 3 is involved in learned fear and extinction, but not in innate fear. Learn Mem 17:582–590

Mathews WR, Ong D, Milutinovich AB, Van Doren M (2006) Zinc transport activity of fear of intimacy is essential for proper gonad morphogenesis and DE-cadherin expression. Development (Camb) 133:1143–1153

Matsuura W, Yamazaki T, Yamaguchi-Iwai Y, Masuda S, Nagao M, Andrews GK et al (2009) SLC39A9 (ZIP9) regulates zinc homeostasis in the secretory pathway: characterization of the ZIP subfamily I protein in vertebrate cells. Biosci Biotechnol Biochem 73:1142–1148

Maverakis E, Fung MA, Lynch PJ, Draznin M, Michael DJ, Ruben B et al (2007) Acrodermatitis enteropathica and an overview of zinc metabolism. J Am Acad Dermatol 56:116–124

Montanini B, Blaudez D, Jeandroz S, Sanders D, Chalot M (2007) Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity. BMC Genomics 8:107

Monteilh-Zoller MK, Hermosura MC, Nadler MJ, Scharenberg AM, Penner R, Fleig A (2003) TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. J Gen Physiol 121:49–60

Mor M, Beharier O, Levy S, Kahn J, Dror S, Blumenthal D et al (2012) ZnT-1 enhances the activity and surface expression of T-type calcium channels through activation of Ras-ERK signaling. Am J Physiol Cell Physiol 303:C192–C203

Nicolson TJ, Bellomo EA, Wijesekara N, Loder MK, Baldwin JM, Gyulkhandanyan AV et al (2009) Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. Diabetes 58:2070–2083

Nishida K, Hasegawa A, Nakae S, Oboki K, Saito H, Yamasaki S et al (2009) Zinc transporter Znt5/Slc30a5 is required for the mast cell-mediated delayed-type allergic reaction but not the immediate-type reaction. J Exp Med 206:1351–1364

Ohana E, Hoch E, Keasar C, Kambe T, Yifrach O, Hershfinkel M et al (2009) Identification of the Zn^{2+} binding site and mode of operation of a mammalian Zn^{2+} transporter. J Biol Chem 284:17677–17686

Patrushev N, Seidel-Rogol B, Salazar G (2012) Angiotensin II requires zinc and downregulation of the zinc transporters ZnT3 and ZnT10 to induce senescence of vascular smooth muscle cells. PLoS One 7:e33211

Peters JL, Dufner-Beattie J, Xu W, Geiser J, Lahner B, Salt DE et al (2007) Targeting of the mouse Slc39a2 (Zip2) gene reveals highly cell-specific patterns of expression, and unique functions in zinc, iron, and calcium homeostasis. Genesis 45:339–352

Pielage J, Kippert A, Zhu M, Klambt C (2004) The *Drosophila* transmembrane protein fear-of-intimacy controls glial cell migration. Dev Biol 275:245–257

Pinilla-Tenas JJ, Sparkman BK, Shawki A, Illing AC, Mitchell CJ, Zhao N et al (2011) Zip14 is a complex broad-scope metal-ion transporter whose functional properties support roles in the cellular uptake of zinc and nontransferrin-bound iron. Am J Physiol Cell Physiol 301:C862–C871

Pound LD, Sarkar SA, Benninger RK, Wang Y, Suwanichkul A, Shadoan MK et al (2009) Deletion of the mouse Slc30a8 gene encoding zinc transporter-8 results in impaired insulin secretion. Biochem J 421:371–376

Qian J, Xu K, Yoo J, Chen TT, Andrews G, Noebels JL (2011) Knockout of Zn transporters Zip-1 and Zip-3 attenuates seizure-induced CA1 neurodegeneration. J Neurosci 31:97–104

Qiu A, Hogstrand C (2004) Functional characterisation and genomic analysis of an epithelial calcium channel (ECaC) from pufferfish, *Fugu rubripes*. Gene (Amst) 342:113–123

Quadri M, Federico A, Zhao T, Breedveld GJ, Battisti C, Delnooz C et al (2012) Mutations in SLC30A10 cause parkinsonism and dystonia with hypermanganesemia, polycythemia, and chronic liver disease. Am J Hum Genet 90:467–477

Ragozzino D, Giovannelli A, Degasperi V, Eusebi F, Grassi F (2000) Zinc permeates mouse muscle ACh receptor channels expressed in BOSC 23 cells and affects channel function. J Physiol 529(Pt 1):83–91

Rizzuto R, Pozzan T (2003) When calcium goes wrong: genetic alterations of a ubiquitous signaling route. Nat Genet 34:135–141

Ryazanova LV, Rondon LJ, Zierler S, Hu Z, Galli J, Yamaguchi TP et al (2010) TRPM7 is essential for Mg^{2+} homeostasis in mammals. Nat Commun 1:109

Schmitt S, Kury S, Giraud M, Dreno B, Kharfi M, Bezieau S (2009) An update on mutations of the SLC39A4 gene in acrodermatitis enteropathica. Hum Mutat 30:926–933

Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R et al (2003) Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. Cell 114:191–200

Sensi SL, Canzoniero LM, Yu SP, Ying HS, Koh JY, Kerchner GA et al (1997) Measurement of intracellular free zinc in living cortical neurons: routes of entry. J Neurosci 17:9554–9564

Sensi SL, Paoletti P, Bush AI, Sekler I (2009) Zinc in the physiology and pathology of the CNS. Nat Rev Neurosci 10:780–791

Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, Hershfinkel M (2011) The neurophysiology and pathology of brain zinc. J Neurosci 31:16076–16085

Sindreu C, Palmiter RD, Storm DR (2011) Zinc transporter ZnT-3 regulates presynaptic Erk1/2 signaling and hippocampus-dependent memory. Proc Natl Acad Sci USA 108:3366–3370

Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D et al (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature (Lond) 445:881–885

Stathakis DG, Burton DY, McIvor WE, Krishnakumar S, Wright TR, O'Donnell JM (1999) The catecholamines up (Catsup) protein of *Drosophila melanogaster* functions as a negative regulator of tyrosine hydroxylase activity. Genetics 153:361–382

Suzuki T, Ishihara K, Migaki H, Matsuura W, Kohda A, Okumura K et al (2005a) 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

Suzuki T, Ishihara K, Migaki H, Nagao M, Yamaguchi-Iwai Y, Kambe T (2005b) Two different zinc transport complexes of cation diffusion facilitator proteins localized in the secretory

pathway operate to activate alkaline phosphatases in vertebrate cells. J Biol Chem 280:30956–30962
Tamaki M, Fujitani Y, Hara A, Uchida T, Tamura Y, Takeno K et al (2013) The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. J Clin Invest 123:4513–4524
Taniguchi M, Fukunaka A, Hagihara M, Watanabe K, Kamino S, Kambe T et al (2013) Essential role of the zinc transporter ZIP9/SLC39A9 in regulating the activations of Akt and Erk in B-cell receptor signaling pathway in DT40 cells. PLoS One 8:e58022
Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P (2012) Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal 5:ra11
Taylor KM, Nicholson RI (2003) The LZT proteins; the LIV-1 subfamily of zinc transporters. Biochim Biophys Acta 1611:16–30
Tomita T, Iwatsubo T (2013) Structural biology of presenilins and signal peptide peptidases. J Biol Chem 288:14673–14680
Tuschl K, Clayton PT, Gospe SM Jr, Gulab S, Ibrahim S, Singhi P et al (2012) Syndrome of hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia caused by mutations in SLC30A10, a manganese transporter in man. Am J Hum Genet 90:457–466
Venkatachalam K, Montell C (2007) TRP channels. Annu Rev Biochem 76:387–417
Walter ED, Stevens DJ, Visconte MP, Millhauser GL (2007) The prion protein is a combined zinc and copper binding protein: Zn^{2+} alters the distribution of Cu^{2+} coordination modes. J Am Chem Soc 129:15440–15441
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
Wang X, Wu Y, Zhou B (2009) Dietary zinc absorption is mediated by ZnT1 in *Drosophila melanogaster*. FASEB J 23:2650–2661
Watt NT, Taylor DR, Kerrigan TL, Griffiths HH, Rushworth JV, Whitehouse IJ et al (2012) Prion protein facilitates uptake of zinc into neuronal cells. Nat Commun 3:1134
Wenzlau JM, Liu Y, Yu L, Moua O, Fowler KT, Rangasamy S et al (2008) A common nonsynonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes. Diabetes 57:2693–2697
Wijesekara N, Dai FF, Hardy AB, Giglou PR, Bhattacharjee A, Koshkin V et al (2010) Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion. Diabetologia 53:1656–1668
Yamasaki S, Hasegawa A, Hojyo S, Ohashi W, Fukada T, Nishida K et al (2012) A novel role of the L-type calcium channel alpha(1D) subunit as a gatekeeper for intracellular zinc signaling: zinc wave. PLoS One 7:e39654
Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E et al (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645
Yamashita S, Miyagi C, Fukada T, Kagara N, Che YS, Hirano T (2004) Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature (Lond) 429:298–302
Yan G, Zhang Y, Yu J, Yu Y, Zhang F, Zhang Z et al (2012) Slc39a7/zip7 plays a critical role in development and zinc homeostasis in zebrafish. PLoS One 7:e42939
Yu M, Lee WW, Tomar D, Pryshchep S, Czesnikiewicz-Guzik M, Lamar DL, Li G et al (2011) Regulation of T cell receptor signaling by activation-induced zinc influx. J Exp Med 208:775–785
Zhang X, Liang D, Guo B, Deng W, Chi ZH, Cai Y et al (2012) Zinc transporter 5 and zinc transporter 7 induced by high glucose protects peritoneal mesothelial cells from undergoing apoptosis. Cell Signal 25:999–1010
Zhang Y, Bharadwaj U, Logsdon CD, Chen C, Yao Q, Li M (2010) ZIP4 regulates pancreatic cancer cell growth by activating IL-6/STAT3 pathway through zinc finger transcription factor CREB. Clin Cancer Res 16:1423–1430

Zhang Y, Yang J, Cui X, Chen Y, Zhu VF, Hagan JP et al (2013) A novel epigenetic CREB-miR-373 axis mediates ZIP4-induced pancreatic cancer growth. EMBO Mol Med 5:1322–1334

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 L, Chen W, Taylor KM, Cai B, Li X (2007) LIV-1 suppression inhibits HeLa cell invasion by targeting ERK1/2-Snail/Slug pathway. Biochem Biophys Res Commun 363:82–88

Chapter 4
Oxidative Stress and Neuronal Zinc Signaling

Hirokazu Hara and Elias Aizenman

Abstract Zinc (Zn^{2+}) is an abundant transition metal that is found in the central nervous system (CNS) at relatively high concentrations. A small fraction of this metal is located within synaptic vesicles in a subpopulation of excitatory neurons along with glutamate, as "chelatable" Zn^{2+}, and can be synaptically released in an activity-dependent fashion. Vesicular Zn^{2+} can thus act as a neurotransmitter or neuromodulator under physiological conditions. However, under certain pathological conditions excessive synaptically released Zn^{2+} can translocate into postsynaptic neurons, inducing cell death. There is mounting additional evidence that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are critically involved in the pathophysiology of many neuronal diseases, including ischemic stroke. The majority of neuronal Zn^{2+} exists not within synaptic vesicles, but tightly bound to Zn^{2+}-binding proteins including metallothionein (MT), as well as transcription factors with zinc-finger domains. Under pathological conditions, oxidative and nitrosative stress causes oxidation and *S*-nitrosylation of cysteine thiols in Zn^{2+}-containing proteins, liberating the bound metal. Oxidative stress-induced elevation of intracellular Zn^{2+} can activate various deleterious cell-signaling pathways, sometimes with disastrous consequences. As such, Zn^{2+}-containing proteins such as MT serve as redox-sensitive molecular switches for zinc signals, playing a critical role in the regulation of neuronal function and viability.

Keywords Cerebral ischemia • Metallothionein • Neurotoxicity • Oxidative stress • Potassium channel • Zinc

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4.1 Introduction

Reactive oxygen species (ROS) are by-products of the cellular metabolism of oxygen in aerobic cells. These molecules are powerful oxidants and can lead to oxidative damage of cellular components, resulting in cytotoxicity. To limit oxidative damage, cells have several antioxidant defense systems, including antioxidant metabolites, as well as enzymes having ROS-scavenging activity. Injurious oxidative stress is thus caused by an imbalance between the production of ROS and the ability of antioxidant defense systems to protect cells. Mounting evidence suggests that ROS are closely associated with pathophysiological processes present in many neurological disorders including ischemic stroke, amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease (Grunblatt et al. 2000; Moro et al. 2005; Butterfield et al. 2006; Boillee and Cleveland 2008; Clark and Simon 2009). Because neuronal cells are nondividing and thus especially vulnerable to the deleterious consequences of oxidative stress, excessive ROS generated under pathological conditions can lead to extensive and permanent neuronal damage. As such, ROS are generally regarded as harmful molecular moieties in the nervous system. Recent reports, however, have demonstrated that ROS can serve as signaling molecules, altering the redox state of specific target molecules that affect cellular function (Stamler et al. 2001; Spickett et al. 2006). These redox-signaling events include stress responses, which can sometimes trigger cellular protective mechanisms (Shih et al. 2003; Itoh et al. 2004; Hara et al. 2006).

Zinc (Zn^{2+}) is an abundant transition metal in bioorganic molecules and is essential for life (Geiser et al. 2012). Zn^{2+} is required for the function of many enzymes and the structural stability of a large number of proteins, including transcription factors containing so-called zinc-finger domains (Shi and Berg 1996). In all, approximately 3,000 proteins, roughly 10 % of the translational output of the human genome, contain Zn^{2+} as either a regulatory or a structural component (Maret and Li 2009). In the central nervous system (CNS), approximately 10 % of the Zn^{2+} content is located within synaptic vesicles of a subpopulation of excitatory neurons, along with glutamate. This population of Zn^{2+} is labile or "chelatable" [i.e., easily visualized with traditional autometallographic methods such as Timm's staining (Haug et al. 1971)] and is synaptically co-released with glutamate in an activity-dependent manner (Assaf and Chung 1984; Howell et al. 1984). Therefore, this metal is now generally considered a neuromodulator, perhaps even a neurotransmitter (Paoletti et al. 1997; Besser et al. 2009; Perez-Rosello et al. 2013).

Under certain pathological conditions such as cerebral ischemia and severe epilepsy, excess vesicular Zn^{2+} released into the synaptic cleft has been shown to enter postsynaptic neurons through a variety of ion channels including calcium (Ca^{2+}) permeable glutamate receptors (Weiss et al. 1993; Sensi et al. 1999) and voltage-gated Ca^{2+} channels (Sensi et al. 1997; Canzoniero et al. 1999), leading to the activation of neuronal cell death-inducing cellular pathways. Most of the Zn^{2+} in the CNS, however, exists in a tightly bound form to metal-binding proteins, including metallothionein (MT). As such, cytoplasmic levels of free or unbound

Zn^{2+} are virtually nonexistent, perhaps reaching the low picomolar or even femtomolar range (Canzoniero et al. 1997; Outten and O'Halloran 2001; Thompson et al. 2002). MT and other Zn^{2+}-binding proteins, however, are very susceptible to oxidative changes within cells. In fact, MT itself has a very negative redox potential [below −366 mV (Maret and Vallee 1998)], and even mild oxidative stress can promptly lead to the liberation of Zn^{2+} from this protein. This rise of intracellular Zn^{2+} may serve as a messenger signal to regulate a wide array of cell functions (Aizenman et al. 2000b; Bossy-Wetzel et al. 2004; Yamashita et al. 2004; Yamasaki et al. 2007). In this chapter, we focus on the molecular mechanism of oxidative stress-induced zinc signaling and role of this metal in neuronal cell death.

4.2 Basis of Oxidative Stress

Oxygen is an essential component for prokaryotic and eukaryotic aerobic metabolism. ROS are generated as by-products of oxygen consumption under normal physiological conditions. Generally, the primary intracellular source of the generation of ROS is the mitochondria. Approximately 95 % of the oxygen is metabolized by cytochrome *c* oxidase in the mitochondria, which transfers four electrons to oxygen, with two molecules of water produced as the product. However, about 1–2 % of oxygen is not completely consumed, leading to the formation of ROS (Boveris and Chance 1973).

4.2.1 Reactive Oxygen and Nitrogen Species

A free radical is defined as any molecular species capable of independent existence containing one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. ROS, oxygen-derived reactive species, generally include oxygen radicals [e.g., superoxide ion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$)], as well as certain nonradical species [e.g., hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$)].

Superoxide ion ($O_2^{\bullet-}$), which is produced by the one-electron reduction of oxygen, is generated by electron leakage from the mitochondrial electron-transport chain, cytochrome P450, and redox-cycling enzymes such as xanthine oxidase and NADPH oxidase. In some cases, superoxide is intentionally produced in activated phagocytic cells to attack and kill invading bacteria. Hydrogen peroxide (H_2O_2), a reduced form by two electrons, is produced by the dismutation of superoxide ion. Although superoxide has extremely limited permeability through lipid membranes, hydrogen peroxide has the capability to cross the membrane freely and diffuse considerable distances (Takahashi and Asada 1983; Antunes and Cadenas 2000). Hydroxyl radical ($OH^{\bullet}$) is generated from hydrogen peroxide through the Fenton reaction, catalyzed by Fe^{2+}. This molecule, the most reactive radical species in this

group, can attack and damage a very large number of molecules found in cells. As such, the half-life of this species is very short.

Reactive nitrogen species (RNS), including nitric oxide (NO), are nitrogen-containing oxidants and are sometimes considered to be a subset of ROS. NO is a free radical gaseous molecule that has a wide array of biological functions. NO is synthesized from L-arginine by NO synthases (NOSs). There are three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NO can very rapidly react with superoxide to form peroxynitrite ($ONOO^-$), a potentially highly damaging free radical that has also been shown to generate hydroxyl radicals (Beckman et al. 1990).

4.2.2 Antioxidant Defense Systems

Unregulated levels of ROS cause damage to cellular components, including nucleic acids, proteins, and lipids, leading to cell injury. To counteract these deleterious effects of ROS, cells have various antioxidant defense systems involving antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. SODs are specific antioxidant enzymes that catalyze the dismutation of superoxide to hydrogen peroxide and oxygen. There are three isoforms of SODs: copper/zinc SOD (Cu/Zn-SOD, cytosolic isoform), manganese SOD (Mn-SOD, mitochondrial isoform), and extracellular-SOD (EC-SOD, extracellular isoform). GPx, at the expense of glutathione (GSH), and catalase convert hydrogen peroxide to water. Besides these antioxidant enzymes, there are nonenzymic antioxidant compounds such as α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C), and GSH. However, in spite of these various antioxidant defense systems, oxidative stress can and does arise as a consequence of an imbalance between ROS or RNS production and antioxidant defense systems.

4.2.3 Redox Modification of Proteins

There is significant evidence that certain kinds of oxidants reversibly influence protein activity, and, as such, the concept of protein oxidation as a functional posttranslational modification is a topic generating some attention (Stamler et al. 2001; Forman et al. 2004; Spickett et al. 2006; Winterbourn and Hampton 2008). For example, Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II), which is usually activated by elevation of intracellular Ca^{2+}, has been shown to be also activated via a Ca^{2+}-independent mechanism. As such, the activity of CaMK II can be regulated via oxidation of methionine residues, located in the autoinhibitory motif of the enzyme, with the oxidation normally reversed by methionine sulfoxide reductase A (Erickson et al. 2008; He et al. 2011). Methionine residues possess reactive sulfur-containing side chains that are susceptible to

oxidation (Hoshi and Heinemann 2001). Oxidants are also known to react with protein cysteine thiols, and indeed, oxidation and reduction of thiol-containing proteins are thought to be a primary mechanism by which ROS/RNS modulate signaling pathways. Therefore, endogenously generated reactive species can, in theory, be considered second messengers under certain circumstances. Cysteine thiols are initially oxidized to cysteine sulfenic acid (–SOH) by ROS such as hydrogen peroxide. In general, sulfenic acids can either quickly form disulfide bonds or can be further oxidized to sulfinic ($–SO_2H$) or sulfonic ($–SO_3H$) acids. Oxidation to sulfenic acid and formation of disulfide bonds are reversible modifications, whereas further oxidation to sulfinic and sulfonic acids is generally considered an irreversible modification of cysteine residues (Spickett et al. 2006).

Previously, we and others showed that *N*-methyl-D-aspartate (NMDA) receptor activation is regulated via oxidation of cysteine residues (Aizenman et al. 1989, 1990; Tang and Aizenman 1993; Sullivan et al. 1994; Brimecombe et al. 1999). Oxygen free radicals generated by xanthine and xanthine oxidase, or the oxidizing agents, 5,5′-dithiobis(2-nitrobenzoic acid), decrease NMDA-evoked cation currents in cortical neurons in culture. In contrast, the sulfydryl reducing agent dithiothreitol enhances NMDA-evoked currents. Sullivan et al. (1994) demonstrated that two cysteine residues (Cys774 and Cys798) on the NMDA receptor subunit (NR) 1 contribute to redox modification of the NMDA receptor. Although there are now many examples of ion channels being modified by reversible oxidation processes, a recent report described that the oxidation of the potassium channel Kv2.1 promotes oligomerization of the channels, contributing to apoptosis in neuronal cells (Cotella et al. 2012; Wu et al. 2013). The formation of disulfide bridges between cytoplasmic residues Cys73 and Cys710 caused by oxidants is involved in the oligomerization. This voltage-dependent channel is highlighted at this juncture, because, as we shall see later on, it is a critical component of zinc signaling processes mediating neuronal cell death.

NO is a free radical gaseous molecule that has been known to have pleiotrophic effects on biological systems. NO is generated from L-arginine by NO synthase and serves as a signaling molecule. The three NOS isoforms, which are neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS, are differentially expressed in various tissues. Both nNOS, predominantly expressed central and peripheral neurons, and eNOS, expressed in endothelial tissues of blood vessels, are Ca^{2+} dependent. In contrast, activation of iNOS, primarily expressed in immune and glial cells, is Ca^{2+} independent. NO and its derivatives can rapidly react with cysteine thiols to generate *S*-nitrosothiols. These NO-mediated thiol modifications can lead to significant changes in protein structure and function, and it has been established that transcription factor, membrane channels, and metabolic enzymes can be readily modified by these reactions (Denninger and Marletta 1999; Stamler et al. 2001; Spickett et al. 2006). Thus, *S*-nitrosylation represents an important signaling mechanism as many proteins have been identified as targets for this form of modification (Jaffrey et al. 2001). For example, the activity of SHP-1 and SHP-2 protein tyrosine phosphatases (PTPs) has been shown to be suppressed by NO (Forman et al. 2004). The redox-sensitive cysteine in a conserved motif in the active

site of these PTPs undergoes *S*-nitrosylation, leading to inhibition of their phosphatase enzymatic function. NO has also been reported to inhibit the DNA-binding activity of transcription factors containing zinc fingers (Matthews et al. 1996; Tabuchi et al. 1996; Nikitovic et al. 1998; Hara and Adachi 2002). This structure is one class of Zn^{2+}-binding motifs consisting of cysteine and histidine residues (Shi and Berg 1995). A large group of proteins have also been reported to have other different Zn^{2+}-binding motifs, including RING, LIM, and PHD-finger domains (Matthews and Sunde 2002; Krishna et al. 2003), and are thus potential targets for oxidant-mediated regulation of their function. The common feature of these structures (e.g., Cys_4 and Cys_2His_2) is that Zn^{2+} coordination is in most cases tetrahedral in geometry, providing the structural stabilization of protein. Although Zn^{2+} is a redox inert metal, zinc/cysteine coordination in the zinc-finger structures is thought to act as a redox-sensitive molecular switch (Krezel et al. 2007; Kroncke and Klotz 2009).

Finally, nitrotyrosination is the reaction of tyrosine residues in proteins with peroxynitrite to form 3-nitrotyrosine, which has been used as a biomarker of nitrosative stress under pathological conditions. The formation of 3-nitrotyrosine has been widely reported to be present in a wide range of neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (Beckman et al. 1993; Smith et al. 1997; Beal 2002).

4.3 Oxidative Stress in Cerebral Ischemia

4.3.1 ROS Generation in Cerebral Ischemia

Stroke is an acute cerebrovascular event. About 85 % of all strokes are the result of ischemia caused by occlusion of a major cerebral artery by a thrombus or embolism. Interruption of blood flow results in a major decrease in the supply of oxygen and nutrients to the affected region, leading to brain damage. Focal cerebral ischemia has been studied extensively using an animal model of middle cerebral artery occlusion (MCAO), causing a reduction of cerebral blood flow in both the striatum and the cortex. Reperfusion following transient MCAO is a good model of focal cerebral ischemia/reperfusion (I/R) injury. ROS have been implicated in the pathophysiology associated with the restoration of blood flow following a cerebrovascular accident (Chan 2001; Warner et al. 2004; Kahles and Brandes 2012). As such, overexpression of Cu/Zn-SOD and antioxidants protect against ischemic brain injury following ischemia and reperfusion (Chan et al. 1998; Amemiya et al. 2005). In addition, in barbiturate-anesthetized rats, transient MCAO followed by reperfusion has been reported to robustly produce ROS compared to permanent MCAO (Peters et al. 1998). These findings suggest the possibility that ROS play a major role in brain injury caused by cerebral ischemia, especially following I/R.

Mitochondria have been thought of as a principal source of ROS generation in ischemic stroke. Indeed, infusion of the complex I inhibitor rotenone into the hippocampus is shown to inhibit ROS generation during reperfusion after in vivo ischemia (Piantadosi and Zhang 1996). Furthermore, studies using mitochondrial Mn-SOD transgenic and knockout mice revealed that $O_2^{\cdot-}$ derived from mitochondria contributes to neuronal cell death after cerebral ischemia (Keller et al. 1998; Murakami et al. 1998; Kim et al. 2002). Overexpression of Mn-SOD protects against neuronal cell death in MCAO models. In contrast, knockout of Mn-SOD increases infarct volume. In addition to mitochondria, pro-oxidant enzymes such as xanthine oxidase and NADPH oxidase are likely involved in ROS generation caused by cerebral I/R (Kahles and Brandes 2012). It has been observed that expression levels of the NADPH oxidase isoforms NOX2 and NOX4 increases in brain microvessels and endothelial cells after ischemic stroke. Deletion of the NOX2 gene, which is normally predominantly expressed in neutrophils, has also been reported to reduce infarct volume after MCAO (Walder et al. 1997). Moreover, as described next, overload of Zn^{2+} into neurons is thought to contribute to neuronal cell death following cerebral ischemia. Related to the aforementioned discussion, Noh and Koh (2000) showed that exposure of cortical neurons to a high concentration of Zn^{2+} induces and activates NADPH oxidase.

4.3.2 NO Generation in Cerebral Ischemia

As already described, there are three isoforms, nNOS, eNOS, and iNOS. All three isoforms of NOS have shown to be involved in neuronal cell death following cerebral ischemia. During and following injury, excitotoxic concentrations of glutamate released from excitatory synaptic terminals lead to the activation of NMDA and Ca^{2+}-permeable AMPA receptors, promoting influx of Ca^{2+}. The rise of intracellular Ca^{2+} induces activation of nNOS, resulting in NO production. In nNOS knockout mice, infarct volumes are decreased after MCAO and neurological deficits are much less pronounced when compared to wild-type mice (Huang and Lo 1998). Moreover, nNOS inhibitors protect against global ischemia in gerbils and provides some reduction in infarct volume following transient MCAO in rats (O'Neill et al. 2000). Importantly, PDZ domains, which mediate protein–protein interaction, have been shown to be critically relevant in NMDA-mediated neuronal cell death process (Aarts and Tymianski 2004; Cui et al. 2007). PSD-95 and nNOS bind to NMDA receptors via the C-terminal PDZ domain of NMDA receptor subunit GluN2 (Kornau et al. 1995; Christopherson et al. 1999). PSD-95 plays a critical role in NO-induced toxicity via direct interaction with NMDA receptors, whereby Ca^{2+} influx via the receptor channel can activate nNOS, but only when the synthase is tethered to the receptor (Sattler et al. 1999). In fact, a peptide mimicking the NR2B PDZ domain (TAT-NR2B9c) has been shown to inhibit pro-death NMDA receptor signaling, leading to protection against MNDA-induced excitotoxicity (Soriano et al. 2008). Importantly, a small-scale clinical trial with

TAT-NR2B9c suggests the possibility that the peptide may be useful in attenuating brain tissue damage in humans, although clearly these findings are only preliminary (Hill et al. 2012). It must be mentioned, however, that eNOS knockout mice develop significantly larger vertebral infarcts after MCAO (Huang et al. 1996). Therefore, NO derived from eNOS may have contrasting, beneficial effects on neuronal injury caused by cerebral ischemia (Samdani et al. 1997).

Inducible iNOS differs fundamentally from nNOS and eNOS as it is not activated by Ca^{2+}. Under normal conditions the expression levels of iNOS are very low. The activity of iNOS is normally regulated in response to pathogen recognition and cytokine release. Because the activation of iNOS occurs later than that of nNOS and eNOS, iNOS is thought to contribute to delayed neuronal cell death in stroke and other forms of injury. Indeed, infarct volume in iNOS knockout mice was similar to that of wild-type mice 24 h after permanent ischemia. However, a reduction of infarct volume was observed in iNOS knockout mice at later times (Iadecola et al. 1997). These findings do suggest, however, that selective iNOS inhibition may be an important component for useful therapeutic intervention following a stroke.

4.4 Zinc Neurotoxicity

4.4.1 Entry of Extracellular Zn^{2+} into Neurons

In the CNS, "chelatable" Zn^{2+} is present in the synaptic vesicles of excitatory neurons along with glutamate. "Zincergic" neurons are widely distributed in the cortex, hippocampus, and amygdala and have been suggested to play a role in learning and memory cellular processes (Frederickson et al. 2000; Takeda 2001). Zn^{2+}-containing neurons are also located in the olfactory bulb and the auditory brainstem (Frederickson et al. 1988; Perez-Clausell et al. 1989). Zn^{2+} is released in an activity-dependent manner into the synaptic cleft and serves as a neuromodulator or perhaps even as a neurotransmitter. This metal allosterically inhibits NMDA and γ-aminobutyric acid receptors (Peters et al. 1987; Christine and Choi 1990; Li et al. 1994; Ruiz et al. 2004), while potentiating glycine receptor function (Lynch et al. 1998; Hirzel et al. 2006). Synaptically released Zn^{2+} has also been reported to activate a postsynaptic, Zn^{2+}-sensing metabotropic receptor, GPR39 (Besser et al. 2009). Through this receptor, synaptic Zn^{2+} promotes postsynaptic insertion of the potassium-chloride co-transporter 2 (KCC2), thereby enhancing synaptic inhibition (Chorin et al. 2011; Saadi et al. 2012). More recently, synaptically released Zn^{2+} was reported to stimulate postsynaptic synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG) via activation of GPR39 (Perez-Rosello et al. 2013). 2-AG suppresses neurotransmitter release through activation of cannabinoid receptor 1 (CB1R) in presynaptic terminals. Finally, synaptic Zn^{2+}

may influence the activity of other proteins following its activity-dependent release, including T-type Ca^{2+} channels (Grauert et al. 2014).

In contrast to the physiological roles for Zn^{2+}, under pathological conditions, such as transient global ischemia and epileptic seizures, excess Zn^{2+} can be released from vesicles along with glutamate into the extracellular space. As elevations of extracellular Zn^{2+} have been shown to cause neuronal cell injury (Fig. 4.1), the extracellular Zn^{2+} chelator Ca-EDTA can block neurotoxicity following transient global cerebral ischemia (Tonder et al. 1990; Koh et al. 1996). Zn^{2+} released into the synaptic cleft has been shown to enter into postsynaptic neurons through a variety of channels and transporters, including voltage-gated Ca^{2+} channels (VGCCs), NMDA receptors, Ca^{2+}-permeable AMPA receptors, as well as TRP channels (Weiss et al. 1993; Sensi et al. 1997, 1999; Marin et al. 2000; Inoue et al. 2010). Of interest, a recent in vivo study revealed that hippocampus CA1 damage following kainic acid injection was attenuated in Zip-1 and -3 null mice (Qian et al. 2011). Therefore, Zip family transporters might also be involved in Zn^{2+} translocation and neurotoxicity following ischemia.

Spreading depression (SD) is an intense depolarization wave that spreads throughout brain tissue (Leo 1944). SD-like events occurring after ischemia and trauma are shown to be involved in spreading brain injury (Leao 1947). Although Ca^{2+} influx through Ca^{2+} channels has been tightly linked to SD-related damage, more recent studies have suggested that Zn^{2+} entry through L-type Ca^{2+} channels or NMDA receptors may substantially contribute to SD-related injurious processes (Dietz et al. 2009). Moreover, Carter et al. (2011) have recently reported that Zn^{2+} release and accumulation during SD caused by oxygen-glucose deprivation is abolished in brain slices prepared from ZnT3 knockout mice. These findings suggest the possibility that SD-induced Zn^{2+} release contributes to neuronal injury following cerebral ischemia.

4.4.2 *Zn^{2+}-Induced Energy Failure and Mitochondrial Dysfunction*

There is widespread evidence that exposure of cortical neurons to Zn^{2+} causes energy failure and mitochondrial dysfunction, leading to neuronal cell death (Fig. 4.1) (Dineley et al. 2003). Elevation of intracellular Zn^{2+} levels has been shown to reduce cellular nicotinamide adenine dinucleotide (NAD^+) levels, leading to inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is an important enzyme in the regulation of glycolysis (Sheline et al. 2000; Cai et al. 2006). In addition, Zn^{2+} also directly inhibits GAPDH (Krotkiewska and Banas 1992) and partially inhibits phosphofructokinase (Ikeda et al. 1980). These events provoke inhibition of glycolysis, resulting in decrease in ATP levels and energy failure. Restoration of NAD^+ can attenuate neuronal cell death induced by

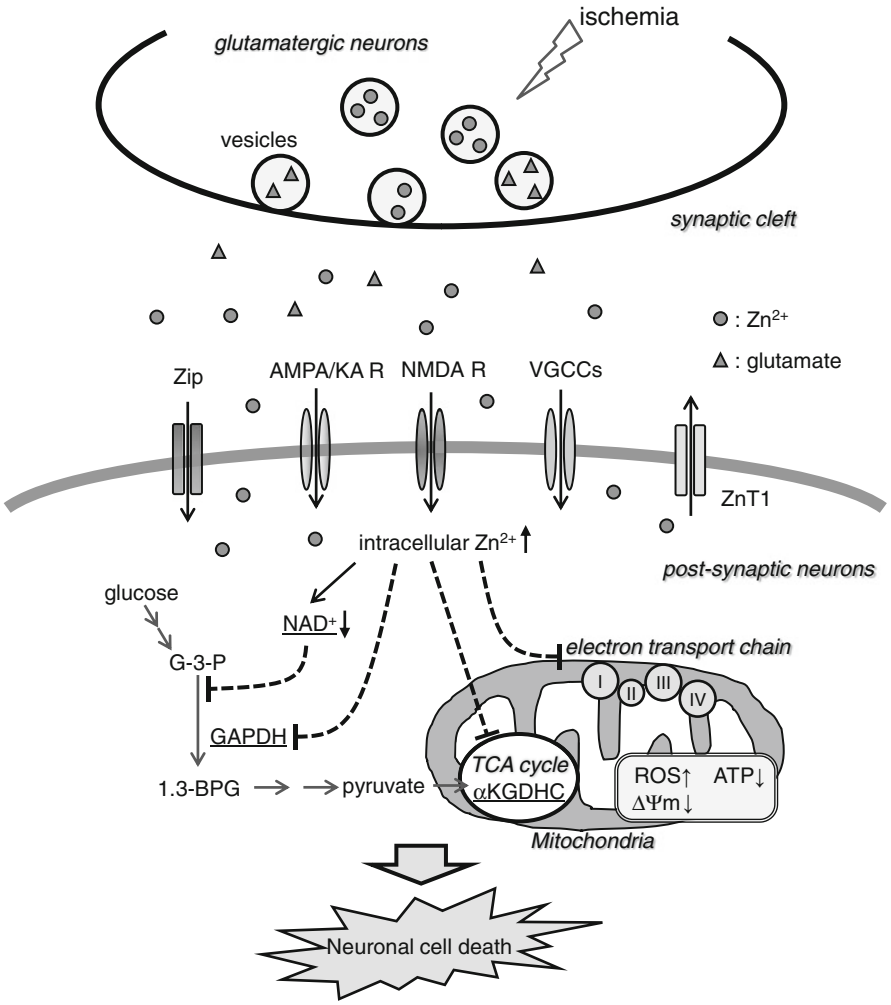

Fig. 4.1 Synaptic Zn^{2+}-dependent neuronal cell death. During injurious conditions, such as cerebral ischemia, excess Zn^{2+} can be released from glutamatergic neurons to the synaptic cleft along with glutamate. Released Zn^{2+} has been shown to translocate into postsynaptic neurons through a variety of channels, including voltage-gated Ca^{2+} channels (VGCCs), the *N*-methyl-D-aspartate (NMDA) receptor, and AMPA/kinate (KA) receptors, as well as zinc transporters. Elevation of intracellular Zn^{2+} inhibits glycolysis, the tricarboxylic acid (TCA) cycle, and the mitochondrial electron-transport chain, leading to energy failure and, ultimately, neuronal cell death. *G3P* glyceraldehyde-3-phosphate, *1,3-BPG* 1,3-bisphosphoglycerate, *α-KGDHC* α-ketoglutarate dehydrogenase complex

Zn^{2+} (Cai et al. 2006). Zn^{2+} also inhibits α-ketoglutarate dehydrogenase complex (α-KGDHC) of the TCA cycle (Brown et al. 2000). In sum, these studies strongly suggest that zinc neurotoxicity is caused, at least in part, by suppressing cellular energy production.

In addition to the aforementioned studies, Zn^{2+} influx through Ca^{2+}-AMPA channels was shown to induce neuronal cell death via mitochondria-mediated generation of ROS (Sensi et al. 1999). As such, the vitamin E-derivative antioxidant trolox was shown to ameliorate Zn^{2+}-induced neurotoxicity (Kim et al. 1999). These observations indicate the possibility that ROS generation caused by Zn^{2+} exposure also contributes to Zn^{2+} toxicity. Mitochondria are thought to be the primary source of Zn^{2+}-generated ROS and play a critical role in Zn^{2+}-induced neuronal cell death, primarily by producing an increase in intramitochondrial Zn^{2+} levels and mitochondrial dysfunction (Sensi et al. 2000). Zn^{2+} has also been shown to inhibit the mitochondrial electron-transport chain (Skulachev et al. 1967). For example, the cytochrome bc_1 complex, also known as complex III of the respiratory chain, can be reversibly inhibited by Zn^{2+} by interfering with proton transfer reactions at the Q_p center of the complex (Link and von Jagow 1995). Importantly, mitochondrial dysfunction caused by inhibition of the electron-transfer chain often leads to ROS production (Sensi et al. 1999; Dineley et al. 2005).

In addition to inhibition of respiration, some reports show that Zn^{2+} taken up by mitochondria induces reduction of the mitochondrial membrane potential and opening of the mitochondrial permeability transition pore (mPTP) in isolated nonneuronal and neuronal mitochondria (Brierley and Knight 1967; Wudarczyk et al. 1999; Jiang et al. 2001). The mPTP allows the passage of any molecule of $<$1,500 Da across the mitochondrial membrane. Opening of the mPTP causes mitochondrial swelling and release of cytochrome *c* (Cyt-*c*), as well as apoptosis-inducing factor (Kroemer and Reed 2000). Additionally, entry of Zn^{2+} into the mitochondria through the Ca^{2+} uniporter causes mPTP-dependent mitochondrial swelling, as well as release of Cyt-*c* and apoptosis-inducing factor (Jiang et al. 2001). Of interest, Bonanni et al. (2006) demonstrated that Zn^{2+}-dependent activation of large, multi-conductance channels were present in mitochondria isolated from ischemic rat brain. A contrasting study (Dineley et al. 2005; Devinney et al. 2009), however, reported that Zn^{2+} was unable to induce mPTP opening in rat liver mitochondria. Thus, although it is clear that Zn^{2+} can alter mitochondrial function in a number of ways, the specific actions of this metal on the organelle under injurious conditions require further study.

4.4.3 Intracellular Zn^{2+} Liberation and Neuronal Cell Death

As already described, extracellular Zn^{2+} has been shown to be elevated in focal and global cerebral ischemia. Influx of Zn^{2+} from the extracellular space into neurons through multiple pathways induces accumulation of neuronal Zn^{2+}, leading to Zn^{2+} toxicity. Zn^{2+} is accumulated in synaptic vesicles via the action of the vesicular Zn^{2+}-specific transporter (ZnT3) (Wenzel et al. 1997). In fact, vesicular Zn^{2+} is conspicuously absent in mice with a genetic ablation of *ZnT3* (Wenzel et al. 1997). In a critically important study, Lee et al. (2000) demonstrated that Zn^{2+} continued to remain elevated in the hippocampus of $ZnT3^{-/-}$ mice treated with kainate

intraperitoneally, leading to Zn^{2+}-dependent cell death. This key observation suggested that synaptic vesicular Zn^{2+} may not be either necessary or sufficient for the cytoplasmic accumulation of the metal in dying neurons, and, importantly, that synaptic source of Zn^{2+} may not be unique for this injurious process. Because Zn^{2+} accumulation and Zn^{2+} neurotoxicity occur even in the absence of vesicular Zn^{2+}, it was hypothesized that Zn^{2+} released from intracellular stores could play a critical role in neural injury (Aizenman et al. 2000b).

4.4.4 Metallothioneins

As mentioned earlier, the majority (80–90 %) of Zn^{2+} in the brain is tightly bound to a large number of proteins. There has been evidence that ROS and RNS react with Zn^{2+}/thiol clusters to induce the release of Zn^{2+} from various proteins, most notably MT, but also including proteins such as protein kinase C, as well as zinc-finger transcription factors (Kroncke et al. 1994; Maret 1994; Knapp and Klann 2000). MTs are a family of small cysteine-rich metal-binding proteins with a major role in regulation of intracellular Zn^{2+} homeostasis and heavy metal detoxification. In the adult mammalian CNS, there are three principal MT isoforms: MT-I, MT-II, and MT-III. MT-I and MT-II are widely expressed in most tissues and MT-III is predominantly expressed in the CNS. MT-III was first isolated and identified as a growth inhibitory factor (GIF) in a series of Alzheimer's disease-directed studies (Uchida et al. 1991; Tsuji et al. 1992). An additional MT isoform has been described, MT-IV; its expression is restricted to stratified squamous epithelia including upper stomach and skin (Quaife et al. 1994). MTs are formed by peptide chains of 61–68 amino acids, structurally resembling a dumbbell-shaped molecule, with metal-binding domains (α and β) at either end (Romero-Isart and Vasak 2002). MTs contain 20 cysteine residues, which can coordinate up to seven zinc ions. In the α-domain, four Zn^{2+} ions are bound to 11 cysteines, whereas in β-domain, three Zn^{2+} ions are bound to 9 cysteines. In this way, each Zn^{2+} ion is bound tetrahedrally to four cysteines. In general, the zinc cluster in the C-terminal α-domain tends to be more stable than that in the β-domain.

Biochemical analysis of the association of Zn^{2+} ion with the apoprotein, thionein, has revealed that there are Zn^{2+}-interacting sites with different binding affinity (Krezel and Maret 2007b). The sites are classified into strong, moderate, and weak groups, depending on the binding affinity to Zn^{2+} ions (stability constant log K_d 11.8, ~10, and 7.7, respectively). As four Zn^{2+} ions can be tightly bound to the strong Zn^{2+}-interacting sites, thionein functions as a potent metal chelator (Fig. 4.2). In contrast, Zn^{2+} ions bound to MT with weak and moderate strength can be transferred to thionein and, importantly, to other Zn^{2+}-binding proteins. As such, MT can also serve as a Zn^{2+} donor. Differential fluorescent labeling of cysteine clusters of MT revealed that tissues contain almost as much thionein as metal-bound MT. The different binding properties of MT provide a role for buffering Zn^{2+} ions at a wide range of intracellular Zn^{2+} concentrations. Because

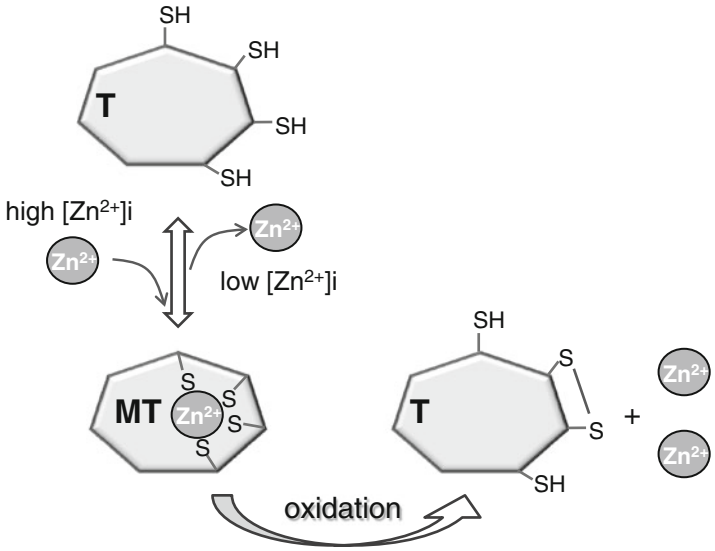

Fig. 4.2 Metallothionein (*MT*) regulates intracellular zinc dynamics. The MT/thionein (*T*) system controls intracellular Zn^{2+} concentrations in a wide range of physiological conditions. However, because cysteine residues in MT are highly redox sensitive, MT is readily oxidized by extracellular or intracellular oxidants, resulting in the unregulated liberation of previously coordinated Zn^{2+}. In turn, the liberated Zn^{2+} can initiate various deleterious cell signaling pathways

this protein has a high cysteine content (20/mol) and cysteine thiols in thinoein are highly reactive, this molecule likely contributes to the total cellular thiol redox buffering capacity (Fig. 4.2). Indeed, some studies have shown that MT has functions to scavenge ROS and can serve as an important cellular antioxidant (Lazo et al. 1998; Reinecke et al. 2006; Krezel and Maret 2007a; Chiaverini and De Ley 2010).

4.4.5 Zn^{2+}-Mediated Gene Expression

Zn^{2+} has been shown to trigger MT expression by inducing the binding of metal regulatory element-binding transcription factor 1 (MTF-1) to specific DNA motifs termed metal responsive elements (MRE), located in the 5′-flanking regulatory region of Zn^{2+}-inducible genes, including MT-I, MT-II, and several zinc transporters (ZnTs), such as ZnT1 (Andrews 2000; Langmade et al. 2000). MTF-1 is a zinc-finger protein in the Cys_2His_2 family of transcription factors and has six zinc-finger domains. Binding to the MRE and transcriptional activation is reversibly regulated by interaction of Zn^{2+} with the zinc-finger domains in MTF-1 (Andrews 2000). Although under normal conditions MTF-1 is preferentially sequestered in the cytoplasm, various stimuli (e.g., heavy metals and oxidative stress-induced Zn^{2+}

release) promote translocation of MTF-1 to the nucleus to induce gene transcription. Cadmium (Cd^{2+}), known as a strong MT inducer, can activate MTF-1, but does so by displacing Zn^{2+} from MT. Using a cell-free MTF-1-dependent transcription system, Zhang et al. (2003) demonstrated that Zn^{2+}-saturated MT is indispensable for Cd^{2+}-induced MTF-1/MRE activation. These results suggest that zinc is the only metal that can directly mediate MTF-1 activation and nuclear translocation. Because Cd^{2+} binds to MT with a much higher affinity than Zn^{2+}, upon entry of Cd^{2+} into cells, Cd^{2+} displaces bound Zn^{2+} from Zn^{2+}-saturated MT or other Zn^{2+}-containing proteins. Similarly, hydrogen peroxide, a known inducer of MTF-1 activation, does so by oxidation of thiol of cysteine residues in MT (Zhang et al. 2003), leading to promotion of Zn^{2+} release from MT (described next). As such, liberation of Zn^{2+} by Cd^{2+} and hydrogen peroxide is necessary for MTF-1-mediated transactivation of *MT* gene by these stressors. Because MTF-1 serves as a selective intracellular Zn^{2+} sensor, a method using a MRE-driven luciferase construct was developed (Chen et al. 1998; Giedroc et al. 2001) and has been utilized to monitor alterations in intracellular Zn^{2+} levels (Daniels et al. 2002; Bi et al. 2004). We have showed that this system can be applied effectively to detect liberated Zn^{2+} caused by oxidative stress in primary cortical neurons (Hara and Aizenman 2004; Aras et al. 2009).

4.4.6 Redox-Dependent Zn^{2+} Release

Oxidative and nitrosative stress can modify cysteine residues in the Zn^{2+}/thiolate complex of Zn^{2+}-containing proteins (Stamler et al. 2001; Forman et al. 2004; Spickett et al. 2006; Winterbourn and Hampton 2008). As such, Zn^{2+}/thiolate clusters provide a redox-active coordination environment for the redox-inert Zn^{2+} ion (Maret 1994, 2000; St Croix et al. 2002; Krezel and Maret 2007a). Because the redox potential of the clusters is very negative (Maret and Vallee 1998), MT is oxidized easily even under mild oxidative stress and releases Zn^{2+} ions. Therefore, oxidants release Zn^{2+}, whereas reductants restore the full potential of the sulfur ligands to bind Zn^{2+} (Fig. 4.2). Zn^{2+} release from Zn^{2+}/thiolate clusters of MT can be induced by several ROS species. MT-III is significantly more reactive to NO than MT-I and MT-II and readily forms *S*-nitrosothiols, while released Zn^{2+} levels from MT-III induced by other ROS/RNS (e.g., hydrogen peroxide) are similar to those generated by MT-I/II (Chen et al. 2002). It was reported that basic and acidic amino acids in the vicinity of target cysteine residue facilitate its *S*-nitrosylation by acid–base catalysis. Because MT-III contains such sequences in its α- and β-domains, MT-III tends to be preferentially *S*-nitrosylated. Lee et al. (2003) observed that when sodium nitroprusside (SNP), a NO donor, was injected into the lateral ventricle of wild-type mice, Zn^{2+} accumulation and neuronal cell death occurred in the C1A region of the hippocampus. Moreover, Zn^{2+} chelation was shown to block SNP-induced Zn^{2+} accumulation and neuronal cell death (Lee et al. 2003). Because it has also been observed that neuronal cell death is

significantly decreased in MT-III$^{-/-}$ mice, the metal-binding protein may represent a significant source of the Zn^{2+}-mediating damage under these conditions (Lee et al. 2003).

Thiol oxidants and NO have been shown to induce the release of Zn^{2+} from MTs in a variety of experimental paradigms. Pierce et al. (2000) constructed a fusion protein consisting of MT sandwiched between two mutant green fluorescent proteins (CFP and YFP), termed FRET-MT [based on the Ca^{2+}-sensitive cameleon probe (Miyawaki et al. 1999)], and showed that MT is an important component of intracellular redox signaling using this FRET-MT system. Our group also assayed for intracellular Zn^{2+} release induced by the thiol-oxidizing agent, 2,2′-dithiodipyridine (DTDP), and peroxynitrite using the same FRET-MT detection system in cultured cortical neurons (Zhang et al. 2004). When neurons were exposed to these agents, change of emission intensity ratio in FRET-MT transfected neurons was observed, reflecting Zn^{2+} liberation from the MT-containing chimeric indicator.

4.5 Signaling Pathway in Liberated Zn^{2+}-Induced Neuronal Apoptosis

Oxidants induce the liberation of intracellular Zn^{2+} in neurons and other systems. The liberated Zn^{2+}, in turn, was observed to lead to the activation of signaling pathways leading to neuronal apoptosis (Aizenman et al. 2000b; McLaughlin et al. 2001).

4.5.1 Zinc Signaling in Neuronal Death

Our group demonstrated that DTDP-liberated Zn^{2+} induced neuronal apoptosis via apoptosis signal-regulating kinase 1 (ASK1)-mediated p38 activation, enhancement of potassium currents, enabling caspase activation (Aras and Aizenman 2005; Knoch et al. 2008) (Fig. 4.3). Treatment with the K^+ channel blocker tetraethylammonium (TEA) or high extracellular K^+ can attenuate DTDP-induced neuronal apoptosis. We observed a robust, delayed increase in TEA-sensitive potassium channel currents following a brief exposure to DTDP. Because DTDP-induced enhancement of potassium currents is blocked by p38 inhibitors, p38 is an upstream regulator of this process. In contrast, a spin-trap agent can prevent DTDP-induced neuronal cell death and p38 activation, suggesting an additional ROS-mediated step in the signaling cascade, likely the result of 12-lipoxygenase activation (McLaughlin et al. 2001; Zhang et al. 2004). Free Zn^{2+} can also be taken up by mitochondria, resulting in changes in mitochondrial membrane potential and additional ROS generation (Sensi et al. 1999, 2000; Dineley et al. 2003, 2005).

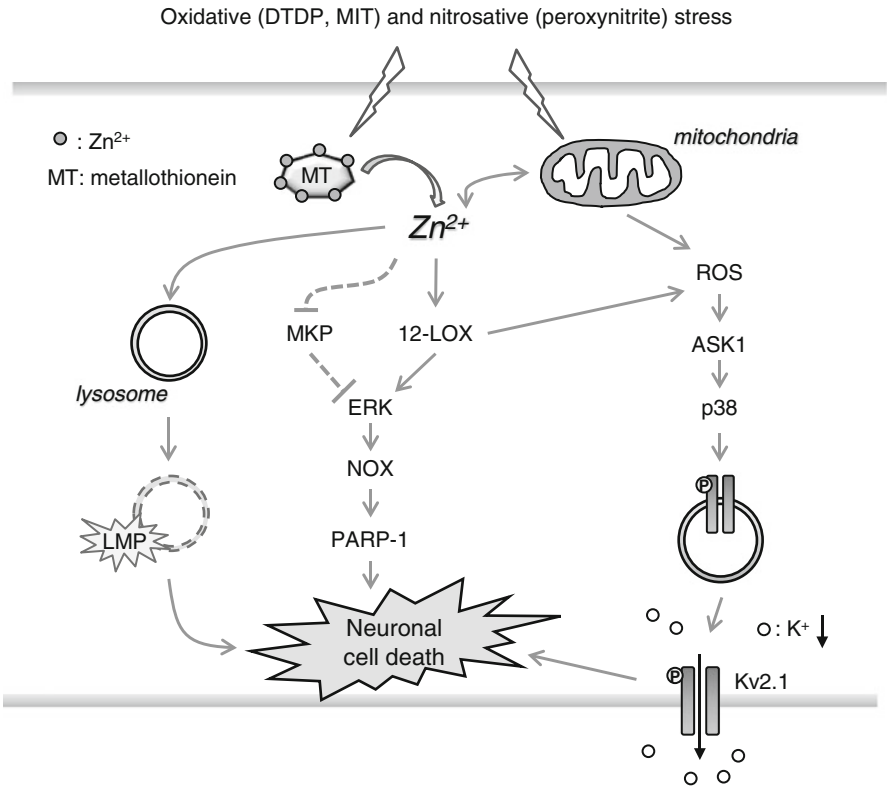

Fig. 4.3 Oxidative stress-induced Zn^{2+} signaling in neuronal cell death. Oxidative and nitrosative stress induce Zn^{2+} release from MT or mitochondria. Liberated Zn^{2+} provokes mitochondrial dysfunction as well as activation of ASK1 and p38 MAPK. The latter phosphorylates Kv2.1, leading to an apoptotic K^+ current surge. Interestingly, different stimuli have been shown to trigger activation of other signaling pathways by Zn^{2+}. For example, the biocide MIT, which is known to induce oxidative stress, activates a 12-LOX-mediated ERK–, but not a p38-dependent signaling pathway, leading to reactive oxygen species (*ROS*) production, DNA damage, and overactivation of PARP-1. In addition, elevation of intracellular Zn^{2+} caused by oxidative stress can promote neuronal cell death via lysosomal membrane permeability (*LMP*). *MKP* MAPK phosphatase

Therefore, ROS are required for DTDP-induced p38 activation. ASK1 is a ubiquitously expressed MAP kinase kinase kinase and has been shown to play a critical role in apoptosis in response to various stimuli. Under normal conditions, ASK1 is bound to thioredoxin (Trx) and its kinase activity is inactivated. Under stress conditions, however, ROS oxidize sulfhydryl groups of Trx and oxidized Trx is dissociated from ASK1, leading to activation of ASK1 and its downstream kinases, primarily p38 and c-jun-N-terminal kinase (JNK) (Ichijo et al. 1997; Saitoh et al. 1998). We found that dominant-negative ASK1 or overexpression of Trx prevented DTDP-induced surge of potassium currents (Aras and Aizenman 2005), directly implicating this upstream kinase system in Zn^{2+}-triggered enhancement of apoptotic-enabling potassium currents.

4.5.2 Involvement of Kv2.1 in DTDP-Induced Neurotoxicity

Cellular K^+ efflux has been shown to be an essential step in the apoptotic cascade in various types of cells (Yu et al. 1997; Bortner and Cidlowski 1999; Aizenman et al. 2000b; McLaughlin et al. 2001). In neurons, the loss of potassium is mediated by a dramatic enhancement in potassium currents. When cultured cortical neurons are exposed to serum deprivation, staurosporine (Yu et al. 1997), or β-amyloid peptides (Yu et al. 1998), a prominent surge of voltage-gated K^+ current is observed. Appropriately, apoptosis caused by these various stimuli can be prevented by TEA, a K^+ channel blocker.

Kv2.1 is the principal component of delayed rectifying K^+ currents in several neuronal populations (Murakoshi and Trimmer 1999; Malin and Nerbonne 2002; Guan et al. 2013). We observed that dominant-negative constructs of Kv2.1 can effectively decrease basal delayed rectifier currents in cortical neurons and, more importantly, completely abrogate the apoptotic surge of potassium currents and increasing neuronal viability (Pal et al. 2003). These observations strongly indicate that Kv2.1-encoded channels are responsible for the apoptotic surge of K^+ currents we have observed following DTDP exposure and other forms of cell injury (Aizenman et al. 2000b; McLaughlin et al. 2001; Knoch et al. 2008), entirely consistent with the protective actions of TEA or elevated extracellular K^+ concentration.

In a separate set of studies, we reported that the enhancement of K^+ currents during apoptosis was caused by the translocation of new Kv2.1 channels to the plasma membrane (Pal et al. 2006). This delivery of Kv2.1 was sensitive to botulinum neurotoxins, suggesting that *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins support this trafficking of Kv2.1. We have more recently shown that Ca^{2+}-dependent activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) is required for DTDP-induced Kv2.1 activation (McCord and Aizenman 2013) (Fig. 4.4). DTDP also promotes intracellular Ca^{2+} release and activation of CaMK II, which regulates exocytosis via a specific interaction with the SNARE protein syntaxin. Subsequently, CaMK II promotes interaction of Kv2.1-encoded K^+ channel Kv2.1 with syntaxin, leading to enhancement of K^+ currents. Of note, syntaxin has a well-described and specific association with the proximal C-terminus of Kv2.1 (Leung et al. 2003; Singer-Lahat et al. 2007). These novel findings have uncovered a crucial cooperative convergence of Zn^{2+}- and Ca^{2+}-mediated signals to regulate oxidative-induced neuronal cell death.

4.5.3 Phosphorylation of Kv2.1

Phosphorylation and dephosphorylation of a number of cytoplasmic residues, Kv2.1, strongly affects channel gating, influencing cellular excitability (Murakoshi et al. 1997; Misonou et al. 2004; Mohapatra et al. 2007). In addition to this type of

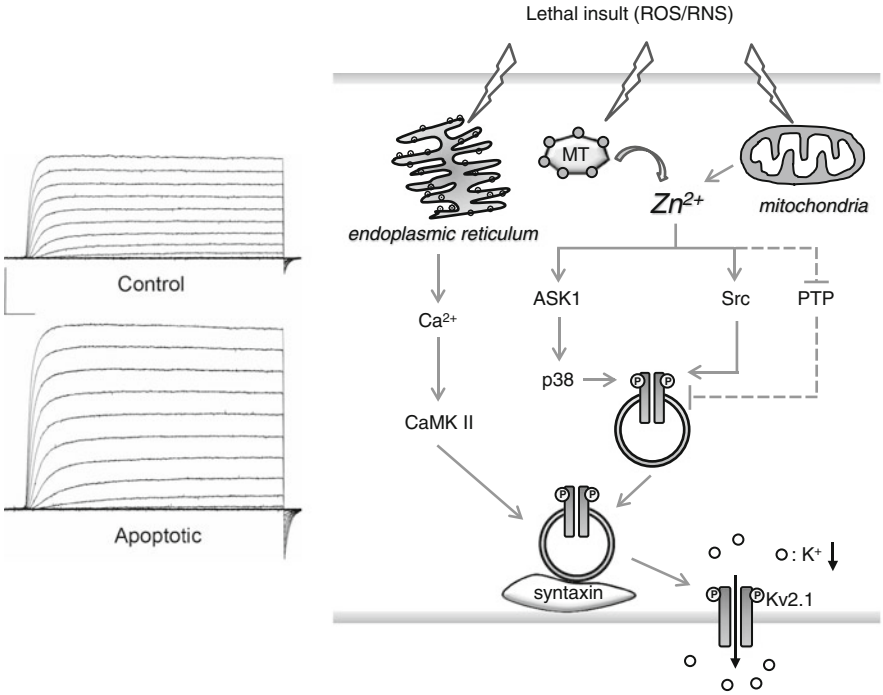

Fig. 4.4 Regulation of Kv2.1 channel by zinc signaling. *Left panel:* Oxidative stress-induced Zn^{2+} liberation triggers a surge of K^+ currents. CHO cells transfected with Kv2.1 were exposed to either vehicle (*Control*) or 25 μM DTDP (*Apoptotic*) for 5 min and recorded 3 h later. Calibration is 5 nA and 20 ms. *Right panel*: Liberated Zn^{2+} activates several signaling pathways and promotes membrane insertion of new Kv2.1 channels. The channel is phosphorylated by p38 MAPK and Src kinase, whereas Zn^{2+} prevents dephosphorylation via inhibition of protein phosphatase. The dual phosphorylated channels are inserted in the cell membrane via a syntaxin-dependent process, leading to enhanced K^+ currents and cytoplasmic K^+ efflux, enabling apoptosis. Translocation of the channel is also regulated by Ca^{2+}-dependent activation of CaMKII, which interacts with the syntaxin/Kv2.1 complex

regulation of Kv2.1 function, phosphorylation events are also at the heart of Kv2.1-mediated apoptotic currents. The MAPK p38 directly phosphorylates C-terminal serine residue 800 (S800) in Kv2.1, a process essential for the expression of the apoptotic K^+ current surge (Redman et al. 2007). Moreover, Kv2.1 N-terminal tyrosine 124 (Y124) must also be phosphorylated for the apoptotic process to ensue. Phosphorylation at Y124 is ensured not only by Src kinase activity, but by Zn^{2+}-dependent inhibition of tyrosine phosphatase ε (Cyto-PTPε) (Redman et al. 2009) (Fig. 4.4). This kinase/phosphatase was previously shown to be specific for the Y124 residue, a process that was tightly coupled to the regulation of Kv2.1-mediated current amplitude (Tiran et al. 2003, 2006). Previous reports have shown that Zn^{2+} can directly act on the highly conserved active site of protein tyrosine phosphatases at nanomolar concentrations (Maret et al. 1999; Haase and Maret 2003).

Together, these findings suggest that two amino acid residues on Kv2.1, Y124 and S800, are critical determinants of neuronal viability by regulating the expression of apoptotic K^+ currents (Fig. 4.4).

4.5.4 Phosphatase Inhibition

Phosphorylation signaling is tightly regulated through the coordinated action of protein kinases and phosphatases. Several phosphatases such as protein tyrosine phosphatases 1B (PTP-1B) are redox sensitive and inhibited by direct thiol oxidation. PTPs have conserved $C(X)_5R$ motif at their active sites, in which the cysteine thiol possesses a low pK_a (~5) and is highly susceptible to reduction and oxidation (Tanner et al. 2011). Not surprisingly, Zn^{2+} can readily interact with these thiols to inhibit phophatase activity (Maret 2013). In addition to Cyto-PTPε, Zn^{2+} has been reported to inhibit other types of protein phosphatases, including PTP-1B and MAPK phosphatase (Haase and Maret 2003; Ho et al. 2008). For example, intracellular Zn^{2+} elevation inhibits PTP-1B and augments tyrosine phosphorylation of insulin/insulin-like growth factor receptors (Haase and Maret 2003). Moreover, Ho et al. (2008) observed that glutamate-induced oxidative stress inhibits ERK2-directed phosphatases, resulting in sustained ERK1/2 activation, the process being mediated by oxidant-induced Zn^{2+} liberation. Interestingly, as Zn^{2+} failed to affect JNK3 phosphatase activity, endogenous Zn^{2+} might be more selective for ERK2-directed phosphatase inhibition under these conditions.

4.5.5 Microglia-Mediated Neuronal Cell Death

There is growing evidence that inflammation is involved in the pathogenesis of neurodegeneration in many CNS disorders (Perry et al. 2010). Microglia, resident immune cells in the CNS, serve beneficial functions in neuronal maintenance and innate immunity. However, microglia can be activated in response to neuronal injury and release injurious signals such as NO, superoxide, and proinflammatory cytokines, leading to enhanced neuronal injury (Weinstein et al. 2010). The induction of iNOS is mediated via activation of Toll-like receptor 4 (TLR4) in microglia, and, indeed, several reports have demonstrated that TLR4 participates in the exacerbation of ischemic brain injury (Caso et al. 2007; Hua et al. 2007; Yang et al. 2010). Lipopolysaccharide (LPS) activates TLR4 and initiates several signaling pathways within microglia, including activation of nuclear factor-κB (NF-κB) and MAPKs (JNK, p38, and ERK). Damaged or dying cells after cerebral ischemia release damage-associated molecular pattern molecules (DAMPs), such as high mobility group box 1, as well as heat shock proteins, which are known to be endogenous TLR4 ligands that promote neuroinflammation (Kim et al. 2006; Marsh et al. 2009; Zhang et al. 2011). Recently, peroxiredoxin family proteins

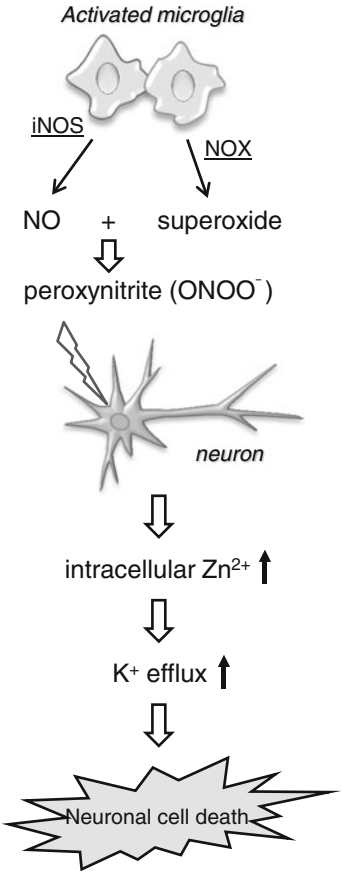

Fig. 4.5 Activated microglia-mediated Zn^{2+}-dependent neuronal cell death. Activated microglia induce iNOS expression, which in turn produces NO. NO rapidly reacts with NADPH oxidase-produced superoxide to form $ONOO^-$. This reactive nitrogen species (RNS) induces liberation of intraneuronal Zn^{2+} and activation of Kv2.1-enabled apoptosis

were shown to be released extracellularly from necrotic brain cells and induce expression of inflammatory cytokines through activation of Toll-like receptor 2 (TLR2) and TLR4, promoting neural cell death (Shichita et al. 2012).

As described earlier, NO produced by iNOS rapidly reacts with superoxide to form $ONOO^-$, a highly reactive and cytotoxic oxidant (Beckman et al. 1990; Espey et al. 2002). Because $ONOO^-$ can rapidly permeate plasma membranes and target a large number of molecules, it has been closely associated with injurious processes in neurons. We observed that activated microglial-derived $ONOO^-$ promoted the liberation of Zn^{2+} from intracellular stores in cultured neurons, resulting in a pronounced Zn^{2+}-dependent K^+ current surge (Knoch et al. 2008) (Fig. 4.5). The K^+ current surge was abolished by apocynin, an NADPH inhibitor, and 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride, a $ONOO^-$ decomposition catalyst. Importantly, activated microglia-dependent neurotoxicity was prevented by neuronal overexpression of MT-III, which provided not only additional antioxidant levels within cells, but likely also additional binding sites for the liberated Zn^{2+}.

Of interest, Zn^{2+} itself has been shown to directly activate microglia. Kauppinen et al. (2008) observed that Zn^{2+} exposure to cultured mouse microglia caused morphological changes and triggered NO and cytokine productions via NAPHD oxidase, poly(ADP-ribose) polymerase 1 (PARP-1), or NF-κB activation. In addition, microglial activation observed following cerebral ischemia-reperfusion was shown to be suppressed by injection of the Zn^{2+} chelator Ca-EDTA (Kauppinen et al. 2008). Thus, a positive feedback model emerges in which ischemic injury leads to microglial activation causing DAMPs and subsequent ROS/RNS production, which in turn leads to the intraneuronal liberation of Zn^{2+}. In addition to activating cell death signaling pathways in neurons, the accumulation of free Zn^{2+} may overwhelm homeostatic buffering mechanisms that lead to the release of Zn^{2+} from neurons, further activating microglia. A late, possibly secondary involvement of Zn^{2+}-mediated injury processes in the ischemic brain has been reported by various investigators (Calderone et al. 2004; Carter et al. 2010).

4.6 Additional Zinc Signaling Injury Pathway

4.6.1 12-Lipoxygenase (12-LOX)

Lipoxygenases (LOXs) are dioxygenases that incorporate molecular oxygen into specific positions of polyunsaturated fatty acids and, as such, are generally classified as 5-, 12-, or 15-LOXs (Shimizu and Wolfe 1990). 12-LOX, which is predominantly expressed in the brain, has been implicated in neuronal oxidative injury in various models. For example, glutamate-induced glutathione depletion causes 12-LOX activation and ROS generation, ultimately inducing neuronal cell death (Li et al. 1997). Furthermore, 12-LOX is also involved in peroxynitrite-induced, Zn^{2+}-dependent neurotoxicity in cortical neurons (Zhang et al. 2004) (Fig. 4.3). As TPEN, a Zn^{2+} chelator, was observed to block the membrane translocation and the activity of 12-LOX, Zn^{2+} liberation occurs upstream of 12-LOX activation, via a yet to be defined signaling pathway. As explained in an earlier section, ROS produced by 12-LOX are likely responsible for ASK1 activation via thioredoxin oxidation (Aras and Aizenman 2011), a critical step in p38 phosphorylation of Kv2.1. Other studies also reported that oxidative and nitrosative stressors could trigger intracellular Zn^{2+} liberation in astrocytes (Malaiyandi et al. 2004) and oligodendrocytes (Zhang et al. 2006), contributing to deleterious signaling pathways in those cells via 12-LOX activation. Of note, however, although p38 MAPK plays an important role in peroxynitrite-induced/12-LOX-dependent cell death, this MAPK appears not to be involved in oligodendrocyte toxicity. Rather, 12-LOX activation appears to occur downstream from the phosphorylation of another MAPK, ERK, which itself is activated by the liberated Zn^{2+} (Fig. 4.3). This is a unique pathway not commonly observed in neurons, although a connection between

12-LOX activity and ERK has been reported to occur in other types of neuronal toxicity, as described below.

Methylisothiazolinone (MIT), a commonly used industrial and household biocide that also triggers intracellular Zn^{2+} liberation, can lead to neuronal cell death (Du et al. 2002). Curiously, the mechanism of cell death triggered by this compound is remarkably different from that activated by either DTDP or $ONOO^-$. Indeed, the elevation of intracellular Zn^{2+} induced by MIT activates ERK, but not p38, but in this case MAPK activation occurs downstream from 12-LOX (Du et al. 2002). MIT was also observed to induce a decrease in intracellular GSH levels. Although in this case intracellular GSH depletion is also directly correlated with 12-LOX activation, TPEN was observed to inhibit MIT-induced GSH depletion. At this point we are not sure how to reconcile both sets of studies, but clearly there are different signaling mechanisms at play in oligodendrocytes versus neurons. We must mention that MIT-induced toxicity is dependent on subsequent NADPH oxidase activation, ROS generation, and DNA damage, leading to overactivation of PARP-1, NAD^+ depletion, and nonapoptotic cell death (Fig. 4.3). All these signaling pathway components are Zn^{2+}- and ERK dependent. Thus, Zn^{2+} can activate both caspase-dependent and caspase-independent cell death pathways in neurons following its liberation from intracellular binding sites.

4.6.2 Preconditioning

Preconditioning is a very interesting cellular phenomenon whereby exposure to sublethal injury triggers an adaptive response that results in tolerance to a subsequent, normally lethal stimulus. Ischemic preconditioning (IPC) in the brain can be triggered, for example, by brief (10-min) ischemic events, such as transient MCAO (Dirnagl et al. 2003; Stetler et al. 2009). Preconditioning stimuli such as this one can substantially protect brain tissue, even from subsequent permanent MCAO, at least within a certain time window (Barone et al. 1998). Although all the mechanisms underlying neuronal tolerance have yet to be completely understood, it is generally believed that preconditioning induces modest activation of molecules that are normally associated with cell damage (e.g., ROS). These signaling molecules trigger adaptive rather than deleterious responses in neurons (Ravati et al. 2000; Thompson et al. 2012). Of note, our group (McLaughlin et al. 2003), as well as others (Garnier et al. 2003; Tanaka et al. 2004; Lee et al. 2008), observed that ischemic preconditioning induces sublethal activation of caspase 3 both in vivo and in vitro.

Sublethal increases in intracellular Zn^{2+} are also important contributors to the adaptive response of neurons during preconditioning. Treatment of cultured cortical neurons with transient chemical ischemia (KCN in glucose-free conditions) can render neurons tolerant to excitotoxic injury within 24 h (Aizenman et al. 2000a; McLaughlin et al. 2003). Preconditioning leads to the liberation of intracellular Zn^{2+}, and Zn^{2+} itself, in the presence of an ionophore, can serve alone as a

preconditioning stimulus (Aras et al. 2009). Koh and coworkers further noted that Zn^{2+} was necessary for sublethal caspase activation in preconditioned neurons (Lee et al. 2008). These findings suggest that intracellular Zn^{2+} elevation during preconditioning process is an essential step in the expression of neuronal tolerance. Indeed, pretreatment with exogenous, nontoxic levels of Zn^{2+} has been reported to protect against neuronal cell death in both in vivo and in vitro cerebral ischemia models (Matsushita et al. 1996; Lee et al. 2008). Because oxidative stress caused by cerebral ischemia/reperfusion can affect zinc homeostasis in the brain, preconditioning-derived Zn^{2+} may upregulate the expression of MT or zinc transporters that could contribute to Zn^{2+} buffering during the normal lethal stimulus. Consistent with this observation, we observed that NMDA-triggered intraneuronal Zn^{2+} elevations were significantly dampened in preconditioned neurons (Aras et al. 2009).

4.6.3 Autophagy

In addition to the aforementioned mechanisms, labile Zn^{2+} liberated by oxidative stress has recently been shown to cause lysosomal changes, leading to neuronal cell death (Hwang et al. 2008; Lee and Koh 2010; Lee et al. 2010). The lysosome is a cytosolic vesicle containing numerous acidic hydrolases that are involved in degradation of cellular components. It has been reported that ischemic brain injury and epileptic stimuli cause lysosomal membrane disintegration in neuronal cells (Hwang et al. 2008; Windelborn and Lipton 2008). Hwang et al. (2008) showed that hydrogen peroxide induces the translocation of labile Zn^{2+} into lysosomes and Zn^{2+}-laden lysosomes undergo membrane disintegration, resulting in lysosomal membrane permeability (LMP) (Fig. 4.3). LMP, in turn releases the lysosomal enzyme cathepsin D, resulting in cathepsin D-dependent cell death (Boya and Kroemer 2008). Because TPEN can attenuate oxidative stress-induced LMP and subsequent cell death, Zn^{2+} is thought to play a critical, upstream role in this process. In astrocytes, exposure to hydrogen peroxide produces an increase in autophagic vacuoles positive for microtubule-associated protein 1 light chain 3 lipidated form (LC3-II), an autophagosomal marker, leading to autophagosome formation (Lee et al. 2009). Under oxidative stress conditions, accumulation of Zn^{2+} in the autophagic vacuoles is observed. As these events are also blocked by TPEN, intracellular Zn^{2+} accumulation induced by oxidative stress is likely upstream from autophagy itself. Interestingly, Lee et al. (2010) showed that MT-III has an important role in regulation of lysosomal function in astrocytes.

4.7 Concluding Remarks

Oxidative stress substantially contributes to neuronal injury after ischemic stroke. However, clinical trials have revealed that antioxidants have limited therapeutic benefit for ischemic stroke. Although intravenous administration of thrombolytic drugs such as recombinant tissue plasminogen activator (t-PA) has been shown to have therapeutic efficacy in this disease, there is a very narrow therapeutic window for the use of this drug, as well as an increased risk for intracranial bleeding. Therefore, it is imperative to develop alternative therapeutic strategies for stroke and related conditions. More than a decade of experimental evidence has demonstrated that intracellular Zn^{2+} is associated with ischemic neuronal cell death. Oxidative stress induces elevation of intracellular Zn^{2+} levels, which, in turn, activates multiple signaling pathways that can lead to the demise of neurons. Therefore, conversion of oxidative stress into a zinc signal is likely a key component of the molecular mechanisms contributing to ischemic neuronal damage. As such, a better understanding of the signaling pathways triggered by oxidative stress-induced zinc signals may provide novel therapeutic targets for cerebral ischemia in the coming years.

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References

Aarts MM, Tymianski M (2004) Molecular mechanisms underlying specificity of excitotoxic signaling in neurons. Curr Mol Med 4:137–147

Aizenman E, Lipton SA, Loring RH (1989) Selective modulation of NMDA responses by reduction and oxidation. Neuron 2:1257–1263

Aizenman E, Hartnett KA, Reynolds IJ (1990) Oxygen free radicals regulate NMDA receptor function via a redox modulatory site. Neuron 5:841–846

Aizenman E, Sinor JD, Brimecombe JC et al (2000a) Alterations of *N*-methyl-D-aspartate receptor properties after chemical ischemia. J Pharmacol Exp Ther 295:572–577

Aizenman E, Stout AK, Hartnett KA et al (2000b) Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. J Neurochem 75:1878–1888

Amemiya S, Kamiya T, Nito C et al (2005) Anti-apoptotic and neuroprotective effects of edaravone following transient focal ischemia in rats. Eur J Pharmacol 516:125–130

Andrews GK (2000) Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem Pharmacol 59:95–104

Antunes F, Cadenas E (2000) Estimation of H_2O_2 gradients across biomembranes. FEBS Lett 475:121–126

Aras MA, Aizenman E (2005) Obligatory role of ASK1 in the apoptotic surge of K^+ currents. Neurosci Lett 387:136–140

Aras MA, Aizenman E (2011) Redox regulation of intracellular zinc: molecular signaling in the life and death of neurons. Antioxid Redox Signal 15:2249–2263

Aras MA, Hara H, Hartnett KA et al (2009) Protein kinase C regulation of neuronal zinc signaling mediates survival during preconditioning. J Neurochem 110:106–117

Assaf SY, Chung SH (1984) Release of endogenous Zn^{2+} from brain tissue during activity. Nature (Lond) 308:734–736

Barone FC, White RF, Spera PA et al (1998) Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. Stroke 29:1937–1950, Discussion 1950–1951

Beal MF (2002) Oxidatively modified proteins in aging and disease. Free Radic Biol Med 32:797–803

Beckman JS, Beckman TW, Chen J et al (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624

Beckman JS, Carson M, Smith CD et al (1993) ALS, SOD and peroxynitrite. Nature (Lond) 364:584

Besser L, Chorin E, Sekler I et al (2009) Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus. J Neurosci 29:2890–2901

Bi Y, Palmiter RD, Wood KM et al (2004) Induction of metallothionein I by phenolic antioxidants requires metal-activated transcription factor 1 (MTF-1) and zinc. Biochem J 380:695–703

Boillee S, Cleveland DW (2008) Revisiting oxidative damage in ALS: microglia, Nox, and mutant SOD1. J Clin Invest 118:474–478

Bonanni L, Chachar M, Jover-Mengual T et al (2006) Zinc-dependent multi-conductance channel activity in mitochondria isolated from ischemic brain. J Neurosci 26:6851–6862

Bortner CD, Cidlowski JA (1999) Caspase independent/dependent regulation of K(+), cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis. J Biol Chem 274:21953–21962

Bossy-Wetzel E, Talantova MV, Lee WD et al (2004) Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K^+ channels. Neuron 41:351–365

Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134:707–716

Boya P, Kroemer G (2008) Lysosomal membrane permeabilization in cell death. Oncogene 27:6434–6451

Brierley GP, Knight VA (1967) Ion transport by heart mitochondria. X. The uptake and release of Zn2+ and its relation to the energy-linked accumulation of magnesium. Biochemistry 6:3892–3901

Brimecombe JC, Potthoff WK, Aizenman E (1999) A critical role of the *N*-methyl-D-aspartate (NMDA) receptor subunit (NR) 2A in the expression of redox sensitivity of NR1/NR2A recombinant NMDA receptors. J Pharmacol Exp Ther 291:785–792

Brown AM, Kristal BS, Effron MS et al (2000) Zn^{2+} inhibits alpha-ketoglutarate-stimulated mitochondrial respiration and the isolated alpha-ketoglutarate dehydrogenase complex. J Biol Chem 275:13441–13447

Butterfield DA, Perluigi M, Sultana R (2006) Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. Eur J Pharmacol 545:39–50

Cai AL, Zipfel GJ, Sheline CT (2006) Zinc neurotoxicity is dependent on intracellular NAD levels and the sirtuin pathway. Eur J Neurosci 24:2169–2176

Calderone A, Jover T, Mashiko T et al (2004) Late calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death. J Neurosci 24:9903–9913

Canzoniero LM, Sensi SL, Choi DW (1997) Measurement of intracellular free zinc in living neurons. Neurobiol Dis 4:275–279

Canzoniero LM, Turetsky DM, Choi DW (1999) Measurement of intracellular free zinc concentrations accompanying zinc-induced neuronal death. J Neurosci 19:Rc31

Carter RE, Weiss JH, Shuttleworth CW (2010) Zn^{2+} chelation improves recovery by delaying spreading depression-like events. Neuroreport 21:1060–1064
Carter RE, Aiba I, Dietz RM et al (2011) Spreading depression and related events are significant sources of neuronal Zn^{2+} release and accumulation. J Cereb Blood Flow Metab 31:1073–1084
Caso JR, Pradillo JM, Hurtado O et al (2007) Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 115:1599–1608
Chan PH (2001) Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 21:2–14
Chan PH, Kawase M, Murakami K et al (1998) Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after global cerebral ischemia and reperfusion. J Neurosci 18:8292–8299
Chen X, Agarwal A, Giedroc DP (1998) Structural and functional heterogeneity among the zinc fingers of human MRE-binding transcription factor-1. Biochemistry 37:11152–11161
Chen Y, Irie Y, Keung WM et al (2002) S-nitrosothiols react preferentially with zinc thiolate clusters of metallothionein III through transnitrosation. Biochemistry 41:8360–8367
Chiaverini N, De Ley M (2010) Protective effect of metallothionein on oxidative stress-induced DNA damage. Free Radic Res 44:605–613
Chorin E, Vinograd O, Fleidervish I et al (2011) Upregulation of KCC2 activity by zinc-mediated neurotransmission via the mZnR/GPR39 receptor. J Neurosci 31:12916–12926
Christine CW, Choi DW (1990) Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. J Neurosci 10:108–116
Christopherson KS, Hillier BJ, Lim WA et al (1999) PSD-95 assembles a ternary complex with the *N*-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. J Biol Chem 274:27467–27473
Clark J, Simon DK (2009) Transcribe to survive: transcriptional control of antioxidant defense programs for neuroprotection in Parkinson's disease. Antioxid Redox Signal 11:509–528
Cotella D, Hernandez-Enriquez B, Wu X et al (2012) Toxic role of K^+ channel oxidation in mammalian brain. J Neurosci 32:4133–4144
Cui H, Hayashi A, Sun HS et al (2007) PDZ protein interactions underlying NMDA receptor-mediated excitotoxicity and neuroprotection by PSD-95 inhibitors. J Neurosci 27:9901–9915
Daniels PJ, Bittel D, Smirnova IV et al (2002) Mammalian metal response element-binding transcription factor-1 functions as a zinc sensor in yeast, but not as a sensor of cadmium or oxidative stress. Nucleic Acids Res 30:3130–3140
Denninger JW, Marletta MA (1999) Guanylate cyclase and the NO/cGMP signaling pathway. Biochim Biophys Acta 1411:334–350
Devinney MJ, Malaiyandi LM, Vergun O et al (2009) A comparison of $Zn2^+$- and Ca^{2+}-triggered depolarization of liver mitochondria reveals no evidence of Zn^{2+}-induced permeability transition. Cell Calcium 45:447–455
Dietz RM, Weiss JH, Shuttleworth CW (2009) Contributions of Ca^{2+} and Zn^{2+} to spreading depression-like events and neuronal injury. J Neurochem 109(Suppl 1):145–152
Dineley KE, Votyakova TV, Reynolds IJ (2003) Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. J Neurochem 85:563–570
Dineley KE, Richards LL, Votyakova TV et al (2005) Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria. Mitochondrion 5:55–65
Dirnagl U, Simon RP, Hallenbeck JM (2003) Ischemic tolerance and endogenous neuroprotection. Trends Neurosci 26:248–254
Du S, McLaughlin B, Pal S et al (2002) In vitro neurotoxicity of methylisothiazolinone, a commonly used industrial and household biocide, proceeds via a zinc and extracellular signal-regulated kinase mitogen-activated protein kinase-dependent pathway. J Neurosci 22:7408–7416
Erickson JR, Joiner ML, Guan X et al (2008) A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell 133:462–474

Espey MG, Thomas DD, Miranda KM et al (2002) Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. Proc Natl Acad Sci USA 99:11127–11132

Forman HJ, Fukuto JM, Torres M (2004) Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. Am J Physiol Cell Physiol 287: C246–C256

Frederickson CJ, Howell GA, Haigh MD et al (1988) Zinc-containing fiber systems in the cochlear nuclei of the rat and mouse. Hear Res 36:203–211

Frederickson CJ, Suh SW, Silva D et al (2000) Importance of zinc in the central nervous system: the zinc-containing neuron. J Nutr 130:1471s–1483s

Garnier P, Ying W, Swanson RA (2003) Ischemic preconditioning by caspase cleavage of poly (ADP-ribose) polymerase-1. J Neurosci 23:7967–7973

Geiser J, Venken KJ, De Lisle RC et al (2012) A mouse model of acrodermatitis enteropathica: loss of intestine zinc transporter ZIP4 (Slc39a4) disrupts the stem cell niche and intestine integrity. PLoS Genet 8:e1002766

Giedroc DP, Chen X, Apuy JL (2001) Metal response element (MRE)-binding transcription factor-1 (MTF-1): structure, function, and regulation. Antioxid Redox Signal 3:577–596

Grauert A, Engel DA, Ruiz AJ (2014) Endogenous zinc depresses GABAergic transmission via T-type Ca^{2+} channels and broadens the time-window for integration of glutamatergic inputs in dentate granule cells. J Physiol 592:67–86

Grunblatt E, Mandel S, Youdim MB (2000) Neuroprotective strategies in Parkinson's disease using the models of 6-hydroxydopamine and MPTP. Ann N Y Acad Sci 899:262–273

Guan D, Armstrong WE, Foehring RC (2013) Kv2 channels regulate firing rate in pyramidal neurons from rat sensorimotor cortex. J Physiol 591:4807–4825

Haase H, Maret W (2003) Intracellular zinc fluctuations modulate protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Exp Cell Res 291:289–298

Hara H, Adachi T (2002) Contribution of hepatocyte nuclear factor-4 to down-regulation of CYP2D6 gene expression by nitric oxide. Mol Pharmacol 61:194–200

Hara H, Aizenman E (2004) A molecular technique for detecting the liberation of intracellular zinc in cultured neurons. J Neurosci Methods 137:175–180

Hara H, Ohta M, Adachi T (2006) Apomorphine protects against 6-hydroxydopamine-induced neuronal cell death through activation of the Nrf2-ARE pathway. J Neurosci Res 84:860–866

Haug FM, Blackstad TW, Simonsen AH et al (1971) Timm's sulfide silver reaction for zinc during experimental anterograde degeneration of hippocampal mossy fibers. J Comp Neurol 142:23–31

He BJ, Joiner ML, Singh MV et al (2011) Oxidation of CaMKII determines the cardiotoxic effects of aldosterone. Nat Med 17:1610–1618

Hill MD, Martin RH, Mikulis D et al (2012) Safety and efficacy of NA-1 in patients with iatrogenic stroke after endovascular aneurysm repair (ENACT): a phase 2, randomised, double-blind, placebo-controlled trial. Lancet Neurol 11:942–950

Hirzel K, Muller U, Latal AT et al (2006) Hyperekplexia phenotype of glycine receptor alpha1 subunit mutant mice identifies Zn(2+) as an essential endogenous modulator of glycinergic neurotransmission. Neuron 52:679–690

Ho Y, Samarasinghe R, Knoch ME et al (2008) Selective inhibition of mitogen-activated protein kinase phosphatases by zinc accounts for extracellular signal-regulated kinase 1/2-dependent oxidative neuronal cell death. Mol Pharmacol 74:1141–1151

Hoshi T, Heinemann S (2001) Regulation of cell function by methionine oxidation and reduction. J Physiol 531:1–11

Howell GA, Welch MG, Frederickson CJ (1984) Stimulation-induced uptake and release of zinc in hippocampal slices. Nature (Lond) 308:736–738

Hua F, Ma J, Ha T et al (2007) Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion. J Neuroimmunol 190:101–111

Huang PL, Lo EH (1998) Genetic analysis of NOS isoforms using nNOS and eNOS knockout animals. Prog Brain Res 118:13–25

Huang Z, Huang PL, Ma J et al (1996) Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J Cereb Blood Flow Metab 16:981–987

Hwang JJ, Lee SJ, Kim TY et al (2008) Zinc and 4-hydroxy-2-nonenal mediate lysosomal membrane permeabilization induced by H_2O_2 in cultured hippocampal neurons. J Neurosci 28:3114–3122

Iadecola C, Zhang F, Casey R et al (1997) Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. J Neurosci 17:9157–9164

Ichijo H, Nishida E, Irie K et al (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275:90–94

Ikeda T, Kimura K, Morioka S et al (1980) Inhibitory effects of Zn^{2+} on muscle glycolysis and their reversal by histidine. J Nutr Sci Vitaminol (Tokyo) 26:357–366

Inoue K, Branigan D, Xiong ZG (2010) Zinc-induced neurotoxicity mediated by transient receptor potential melastatin 7 channels. J Biol Chem 285:7430–7439

Itoh K, Tong KI, Yamamoto M (2004) Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic Biol Med 36:1208–1213

Jaffrey SR, Erdjument-Bromage H, Ferris CD et al (2001) Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat Cell Biol 3:193–197

Jiang D, Sullivan PG, Sensi SL et al (2001) Zn(2+) induces permeability transition pore opening and release of pro-apoptotic peptides from neuronal mitochondria. J Biol Chem 276:47524–47529

Kahles T, Brandes RP (2012) NADPH oxidases as therapeutic targets in ischemic stroke. Cell Mol Life Sci 69:2345–2363

Kauppinen TM, Higashi Y, Suh SW et al (2008) Zinc triggers microglial activation. J Neurosci 28:5827–5835

Keller JN, Kindy MS, Holtsberg FW et al (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. J Neurosci 18:687–697

Kim YH, Kim EY, Gwag BJ et al (1999) Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals. Neuroscience 89:175–182

Kim GW, Kondo T, Noshita N et al (2002) Manganese superoxide dismutase deficiency exacerbates cerebral infarction after focal cerebral ischemia/reperfusion in mice: implications for the production and role of superoxide radicals. Stroke 33:809–815

Kim JB, Sig Choi J, Yu YM et al (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci 26:6413–6421

Knapp LT, Klann E (2000) Superoxide-induced stimulation of protein kinase C via thiol modification and modulation of zinc content. J Biol Chem 275:24136–24145

Knoch ME, Hartnett KA, Hara H et al (2008) Microglia induce neurotoxicity via intraneuronal Zn (2+) release and a K(+) current surge. Glia 56:89–96

Koh JY, Suh SW, Gwag BJ et al (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 272:1013–1016

Kornau HC, Schenker LT, Kennedy MB et al (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science 269:1737–1740

Krezel A, Maret W (2007a) Different redox states of metallothionein/thionein in biological tissue. Biochem J 402:551–558

Krezel A, Maret W (2007b) Dual nanomolar and picomolar Zn(II) binding properties of metallothionein. J Am Chem Soc 129:10911–10921

Krezel A, Hao Q, Maret W (2007) The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling. Arch Biochem Biophys 463:188–200

Krishna SS, Majumdar I, Grishin NV (2003) Structural classification of zinc fingers: survey and summary. Nucleic Acids Res 31:532–550
Kroemer G, Reed JC (2000) Mitochondrial control of cell death. Nat Med 6:513–519
Kroncke KD, Klotz LO (2009) Zinc fingers as biologic redox switches? Antioxid Redox Signal 11:1015–1027
Kroncke KD, Fehsel K, Schmidt T et al (1994) Nitric oxide destroys zinc-sulfur clusters inducing zinc release from metallothionein and inhibition of the zinc finger-type yeast transcription activator LAC9. Biochem Biophys Res Commun 200:1105–1110
Krotkiewska B, Banas T (1992) Interaction of Zn^{2+} and Cu^{2+} ions with glyceraldehyde-3-phosphate dehydrogenase from bovine heart and rabbit muscle. Int J Biochem 24:1501–1505
Langmade SJ, Ravindra R, Daniels PJ et al (2000) The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J Biol Chem 275:34803–34809
Lazo JS, Kuo SM, Woo ES et al (1998) The protein thiol metallothionein as an antioxidant and protectant against antineoplastic drugs. Chem Biol Interact 111–112:255–262
Leao AA (1947) Further observations on the spreading depression of activity in the cerebral cortex. J Neurophysiol 10:409–414
Lee SJ, Koh JY (2010) Roles of zinc and metallothionein-3 in oxidative stress-induced lysosomal dysfunction, cell death, and autophagy in neurons and astrocytes. Mol Brain 3:30
Lee JY, Cole TB, Palmiter RD et al (2000) Accumulation of zinc in degenerating hippocampal neurons of ZnT3-null mice after seizures: evidence against synaptic vesicle origin. J Neurosci 20:Rc79
Lee JY, Kim JH, Palmiter RD et al (2003) Zinc released from metallothionein-III may contribute to hippocampal CA1 and thalamic neuronal death following acute brain injury. Exp Neurol 184:337–347
Lee JY, Kim YJ, Kim TY et al (2008) Essential role for zinc-triggered p75NTR activation in preconditioning neuroprotection. J Neurosci 28:10919–10927
Lee SJ, Cho KS, Koh JY (2009) Oxidative injury triggers autophagy in astrocytes: the role of endogenous zinc. Glia 57:1351–1361
Lee SJ, Park MH, Kim HJ et al (2010) Metallothionein-3 regulates lysosomal function in cultured astrocytes under both normal and oxidative conditions. Glia 58:1186–1196
Leo AA (1944) Pial circulation and spreading depression of activity in the cerebral cortex. J Neurophysiol 7:391–396
Leung YM, Kang Y, Gao X et al (2003) Syntaxin 1A binds to the cytoplasmic C terminus of Kv2.1 to regulate channel gating and trafficking. J Biol Chem 278:17532–17538
Li M, Rosenberg HC, Chiu TH (1994) Zinc inhibition of GABA-stimulated Cl^- influx in rat brain regions is unaffected by acute or chronic benzodiazepine. Pharmacol Biochem Behav 49:477–482
Li Y, Maher P, Schubert D (1997) A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. Neuron 19:453–463
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
Lynch JW, Jacques P, Pierce KD et al (1998) Zinc potentiation of the glycine receptor chloride channel is mediated by allosteric pathways. J Neurochem 71:2159–2168
Malaiyandi LM, Dineley KE, Reynolds IJ (2004) Divergent consequences arise from metallothionein overexpression in astrocytes: zinc buffering and oxidant-induced zinc release. Glia 45:346–353
Malin SA, Nerbonne JM (2002) Delayed rectifier K^+ currents, IK, are encoded by Kv2 alpha-subunits and regulate tonic firing in mammalian sympathetic neurons. J Neurosci 22:10094–10105
Maret W (1994) Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. Proc Natl Acad Sci USA 91:237–241
Maret W (2000) The function of zinc metallothionein: a link between cellular zinc and redox state. J Nutr 130:1455s–1458s

Maret W (2013) Inhibitory zinc sites in enzymes. Biometals 26:197–204
Maret W, Li Y (2009) Coordination dynamics of zinc in proteins. Chem Rev 109:4682–4707
Maret W, Vallee BL (1998) Thiolate ligands in metallothionein confer redox activity on zinc clusters. Proc Natl Acad Sci USA 95:3478–3482
Maret W, Jacob C, Vallee BL et al (1999) Inhibitory sites in enzymes: zinc removal and reactivation by thionein. Proc Natl Acad Sci USA 96:1936–1940
Marin P, Israel M, Glowinski J et al (2000) Routes of zinc entry in mouse cortical neurons: role in zinc-induced neurotoxicity. Eur J Neurosci 12:8–18
Marsh BJ, Williams-Karnesky RL, Stenzel-Poore MP (2009) Toll-like receptor signaling in endogenous neuroprotection and stroke. Neuroscience 158:1007–1020
Matsushita K, Kitagawa K, Matsuyama T et al (1996) Effect of systemic zinc administration on delayed neuronal death in the gerbil hippocampus. Brain Res 743:362–365
Matthews JM, Sunde M (2002) Zinc fingers–folds for many occasions. IUBMB Life 54:351–355
Matthews JR, Botting CH, Panico M et al (1996) Inhibition of NF-kappaB DNA binding by nitric oxide. Nucleic Acids Res 24:2236–2242
McCord MC, Aizenman E (2013) Convergent Ca^{2+} and Zn^{2+} signaling regulates apoptotic Kv2.1 K^+ currents. Proc Natl Acad Sci USA 110:13988–13993
McLaughlin B, Pal S, Tran MP et al (2001) p38 activation is required upstream of potassium current enhancement and caspase cleavage in thiol oxidant-induced neuronal apoptosis. J Neurosci 21:3303–3311
McLaughlin B, Hartnett KA, Erhardt JA et al (2003) Caspase 3 activation is essential for neuroprotection in preconditioning. Proc Natl Acad Sci USA 100:715–720
Misonou H, Mohapatra DP, Park EW et al (2004) Regulation of ion channel localization and phosphorylation by neuronal activity. Nat Neurosci 7:711–718
Miyawaki A, Griesbeck O, Heim R et al (1999) Dynamic and quantitative Ca^{2+} measurements using improved cameleons. Proc Natl Acad Sci USA 96:2135–2140
Mohapatra DP, Park KS, Trimmer JS (2007) Dynamic regulation of the voltage-gated Kv2.1 potassium channel by multisite phosphorylation. Biochem Soc Trans 35:1064–1068
Moro MA, Almeida A, Bolanos JP et al (2005) Mitochondrial respiratory chain and free radical generation in stroke. Free Radic Biol Med 39:1291–1304
Murakami K, Kondo T, Kawase M et al (1998) Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. J Neurosci 18:205–213
Murakoshi H, Trimmer JS (1999) Identification of the Kv2.1 K^+ channel as a major component of the delayed rectifier K^+ current in rat hippocampal neurons. J Neurosci 19:1728–1735
Murakoshi H, Shi G, Scannevin RH et al (1997) Phosphorylation of the Kv2.1 K^+ channel alters voltage-dependent activation. Mol Pharmacol 52:821–828
Nikitovic D, Holmgren A, Spyrou G (1998) Inhibition of AP-1 DNA binding by nitric oxide involving conserved cysteine residues in Jun and Fos. Biochem Biophys Res Commun 242:109–112
Noh KM, Koh JY (2000) Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci 20:Rc111
O'Neill MJ, Murray TK, McCarty DR et al (2000) ARL 17477, a selective nitric oxide synthase inhibitor, with neuroprotective effects in animal models of global and focal cerebral ischaemia. Brain Res 871:234–244
Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488–2492
Pal S, Hartnett KA, Nerbonne JM et al (2003) Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels. J Neurosci 23:4798–4802
Pal SK, Takimoto K, Aizenman E et al (2006) Apoptotic surface delivery of K^+ channels. Cell Death Differ 13:661–667
Paoletti P, Ascher P, Neyton J (1997) High-affinity zinc inhibition of NMDA NR1-NR2A receptors. J Neurosci 17:5711–5725

Pearce LL, Gandley RE, Han W et al (2000) Role of metallothionein in nitric oxide signaling as revealed by a green fluorescent fusion protein. Proc Natl Acad Sci USA 97:477–482

Perez-Clausell J, Frederickson CJ, Danscher G (1989) Amygdaloid efferents through the stria terminalis in the rat give origin to zinc-containing boutons. J Comp Neurol 290:201–212

Perez-Rosello T, Anderson CT, Schopfer FJ et al (2013) Synaptic Zn^{2+} inhibits neurotransmitter release by promoting endocannabinoid synthesis. J Neurosci 33:9259–9272

Perry VH, Nicoll JA, Holmes C (2010) Microglia in neurodegenerative disease. Nat Rev Neurol 6:193–201

Peters S, Koh J, Choi DW (1987) Zinc selectively blocks the action of *N*-methyl-D-aspartate on cortical neurons. Science 236:589–593

Peters O, Back T, Lindauer U et al (1998) Increased formation of reactive oxygen species after permanent and reversible middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 18:196–205

Piantadosi CA, Zhang J (1996) Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. Stroke 27:327–331

Qian J, Xu K, Yoo J et al (2011) Knockout of Zn transporters Zip-1 and Zip-3 attenuates seizure-induced CA1 neurodegeneration. J Neurosci 31:97–104

Quaife CJ, Findley SD, Erickson JC et al (1994) Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia. Biochemistry 33:7250–7259

Ravati A, Ahlemeyer B, Becker A et al (2000) Preconditioning-induced neuroprotection is mediated by reactive oxygen species. Brain Res 866:23–32

Redman PT, He K, Hartnett KA et al (2007) Apoptotic surge of potassium currents is mediated by p38 phosphorylation of Kv2.1. Proc Natl Acad Sci USA 104:3568–3573

Redman PT, Hartnett KA, Aras MA et al (2009) Regulation of apoptotic potassium currents by coordinated zinc-dependent signalling. J Physiol 587:4393–4404

Reinecke F, Levanets O, Olivier Y et al (2006) Metallothionein isoform 2A expression is inducible and protects against ROS-mediated cell death in rotenone-treated HeLa cells. Biochem J 395:405–415

Romero-Isart N, Vasak M (2002) Advances in the structure and chemistry of metallothioneins. J Inorg Biochem 88:388–396

Ruiz A, Walker MC, Fabian-Fine R et al (2004) Endogenous zinc inhibits GABA(A) receptors in a hippocampal pathway. J Neurophysiol 91:1091–1096

Saadi RA, He K, Hartnett KA et al (2012) SNARE-dependent upregulation of potassium chloride co-transporter 2 activity after metabotropic zinc receptor activation in rat cortical neurons in vitro. Neuroscience 210:38–46

Saitoh M, Nishitoh H, Fujii M et al (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 17:2596–2606

Samdani AF, Dawson TM, Dawson VL (1997) Nitric oxide synthase in models of focal ischemia. Stroke 28:1283–1288

Sattler R, Xiong Z, Lu WY et al (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science 284:1845–1848

Sensi SL, Canzoniero LM, Yu SP et al (1997) Measurement of intracellular free zinc in living cortical neurons: routes of entry. J Neurosci 17:9554–9564

Sensi SL, Yin HZ, Carriedo SG et al (1999) Preferential Zn^{2+} influx through Ca^{2+}-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. Proc Natl Acad Sci USA 96:2414–2419

Sensi SL, Yin HZ, Weiss JH (2000) AMPA/kainate receptor-triggered Zn^{2+} entry into cortical neurons induces mitochondrial Zn^{2+} uptake and persistent mitochondrial dysfunction. Eur J Neurosci 12:3813–3818

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

Shi Y, Berg JM (1995) Specific DNA–RNA hybrid binding by zinc finger proteins. Science 268:282–284
Shi Y, Berg JM (1996) DNA unwinding induced by zinc finger protein binding. Biochemistry 35:3845–3848
Shichita T, Hasegawa E, Kimura A et al (2012) Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. Nat Med 18:911–917
Shih AY, Johnson DA, Wong G et al (2003) Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci 23:3394–3406
Shimizu T, Wolfe LS (1990) Arachidonic acid cascade and signal transduction. J Neurochem 55:1–15
Singer-Lahat D, Sheinin A, Chikvashvili D et al (2007) K^+ channel facilitation of exocytosis by dynamic interaction with syntaxin. J Neurosci 27:1651–1658
Skulachev VP, Chistyakov VV, Jasaitis AA et al (1967) Inhibition of the respiratory chain by zinc ions. Biochem Biophys Res Commun 26:1–6
Smith MA, Richey Harris PL, Sayre LM et al (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci 17:2653–2657
Soriano FX, Martel MA, Papadia S et al (2008) Specific targeting of pro-death NMDA receptor signals with differing reliance on the NR2B PDZ ligand. J Neurosci 28:10696–10710
Spickett CM, Pitt AR, Morrice N et al (2006) Proteomic analysis of phosphorylation, oxidation and nitrosylation in signal transduction. Biochim Biophys Acta 1764:1823–1841
St. Croix CM, Wasserloos KJ, Dineley KE et al (2002) Nitric oxide-induced changes in intracellular zinc homeostasis are mediated by metallothionein/thionein. Am J Physiol Lung Cell Mol Physiol 282:L185–L192
Stamler JS, Lamas S, Fang FC (2001) Nitrosylation, the prototypic redox-based signaling mechanism. Cell 106:675–683
Stetler RA, Zhang F, Liu C et al (2009) Ischemic tolerance as an active and intrinsic neuroprotective mechanism. Handb Clin Neurol 92:171–195
Sullivan JM, Traynelis SF, Chen HS et al (1994) Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor. Neuron 13:929–936
Tabuchi A, Oh E, Taoka A et al (1996) Rapid attenuation of AP-1 transcriptional factors associated with nitric oxide (NO)-mediated neuronal cell death. J Biol Chem 271:31061–31067
Takahashi MA, Asada K (1983) Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. Arch Biochem Biophys 226:558–566
Takeda A (2001) Zinc homeostasis and functions of zinc in the brain. Biometals 14:343–351
Tanaka H, Yokota H, Jover T et al (2004) Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. J Neurosci 24:2750–2759
Tang LH, Aizenman E (1993) The modulation of *N*-methyl-D-aspartate receptors by redox and alkylating reagents in rat cortical neurones in vitro. J Physiol 465:303–323
Tanner JJ, Parsons ZD, Cummings AH et al (2011) Redox regulation of protein tyrosine phosphatases: structural and chemical aspects. Antioxid Redox Signal 15:77–97
Thompson RB, Peterson D, Mahoney W et al (2002) Fluorescent zinc indicators for neurobiology. J Neurosci Methods 118:63–75
Thompson JW, Narayanan SV, Perez-Pinzon MA (2012) Redox signaling pathways involved in neuronal ischemic preconditioning. Curr Neuropharmacol 10:354–369
Tiran Z, Peretz A, Attali B et al (2003) Phosphorylation-dependent regulation of Kv2.1 channel activity at tyrosine 124 by Src and by protein-tyrosine phosphatase epsilon. J Biol Chem 278:17509–17514
Tiran Z, Peretz A, Sines T et al (2006) Tyrosine phosphatases epsilon and alpha perform specific and overlapping functions in regulation of voltage-gated potassium channels in Schwann cells. Mol Biol Cell 17:4330–4342

Tonder N, Johansen FF, Frederickson CJ et al (1990) Possible role of zinc in the selective degeneration of dentate hilar neurons after cerebral ischemia in the adult rat. Neurosci Lett 109:247–252

Tsuji S, Kobayashi H, Uchida Y et al (1992) Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease. EMBO J 11:4843–4850

Uchida Y, Takio K, Titani K et al (1991) The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. Neuron 7:337–347

Walder CE, Green SP, Darbonne WC et al (1997) Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. Stroke 28:2252–2258

Warner DS, Sheng H, Batinic-Haberle I (2004) Oxidants, antioxidants and the ischemic brain. J Exp Biol 207:3221–3231

Weinstein JR, Koerner IP, Moller T (2010) Microglia in ischemic brain injury. Future Neurol 5:227–246

Weiss JH, Hartley DM, Koh JY et al (1993) AMPA receptor activation potentiates zinc neurotoxicity. Neuron 10:43–49

Wenzel HJ, Cole TB, Born DE et al (1997) Ultrastructural localization of zinc transporter-3 (ZnT-3) to synaptic vesicle membranes within mossy fiber boutons in the hippocampus of mouse and monkey. Proc Natl Acad Sci USA 94:12676–12681

Windelborn JA, Lipton P (2008) Lysosomal release of cathepsins causes ischemic damage in the rat hippocampal slice and depends on NMDA-mediated calcium influx, arachidonic acid metabolism, and free radical production. J Neurochem 106:56–69

Winterbourn CC, Hampton MB (2008) Thiol chemistry and specificity in redox signaling. Free Radic Biol Med 45:549–561

Wu X, Hernandez-Enriquez B, Banas M et al (2013) Molecular mechanisms underlying the apoptotic effect of KCNB1 K^+ channel oxidation. J Biol Chem 288:4128–4134

Wudarczyk J, Debska G, Lenartowicz E (1999) Zinc as an inducer of the membrane permeability transition in rat liver mitochondria. Arch Biochem Biophys 363:1–8

Yamasaki S, Sakata-Sogawa K, Hasegawa A et al (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645

Yamashita S, Miyagi C, Fukada T et al (2004) Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature (Lond) 429:298–302

Yang QW, Wang JZ, Li JC et al (2010) High-mobility group protein box-1 and its relevance to cerebral ischemia. J Cereb Blood Flow Metab 30:243–254

Yu SP, Yeh CH, Sensi SL et al (1997) Mediation of neuronal apoptosis by enhancement of outward potassium current. Science 278:114–117

Yu SP, Farhangrazi ZS, Ying HS et al (1998) Enhancement of outward potassium current may participate in beta-amyloid peptide-induced cortical neuronal death. Neurobiol Dis 5:81–88

Zhang B, Georgiev O, Hagmann M et al (2003) Activity of metal-responsive transcription factor 1 by toxic heavy metals and H_2O_2 in vitro is modulated by metallothionein. Mol Cell Biol 23:8471–8485

Zhang Y, Wang H, Li J et al (2004) Peroxynitrite-induced neuronal apoptosis is mediated by intracellular zinc release and 12-lipoxygenase activation. J Neurosci 24:10616–10627

Zhang Y, Wang H, Li J et al (2006) Intracellular zinc release and ERK phosphorylation are required upstream of 12-lipoxygenase activation in peroxynitrite toxicity to mature rat oligodendrocytes. J Biol Chem 281:9460–9470

Zhang J, Takahashi HK, Liu K et al (2011) Anti-high mobility group box-1 monoclonal antibody protects the blood–brain barrier from ischemia-induced disruption in rats. Stroke 42:1420–1428

Chapter 5
Zinc Signaling by "Zinc Wave"

Keigo Nishida and Satoru Yamasaki

Abstract Zinc (Zn) is an essential heavy metal for all organisms. Zn homeostasis is maintained in mammalian cells through the activity of Zn transporters and Zn-permeable channels and through metallothionein expression levels. Zn is important in nucleic acid metabolism, cell replication, and tissue growth and repair. Zn deficiency is associated with a wide range of pathological conditions, such as impaired immunity, growth retardation, disorders in brain development, and delayed wound healing. Zn binds and affects the activity of several signaling molecules and of transcription factors that have a Zn-binding motif. However, whether Zn itself, as does calcium, acts as an intracellular signaling molecule has been a point of speculation. Recently, we and other groups have demonstrated that Zn does indeed act as an intracellular signaling molecule, converting extracellular stimuli to intracellular signals and controlling various cell functions. This chapter summarizes our current understanding of Zn signaling, especially with regard to the *Zn wave* and the role of Zn signaling in immune cells, and discusses how these processes contribute to allergic responses.

Keywords Immune response • Mast cell • Second messenger • Signal transduction • Zinc • Zinc transporter • Zinc wave

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5.1 Introduction

Zinc (Zn), an essential trace element, is an important structural component in a great number of proteins, including intracellular signaling enzymes and transcription factors (Prasad 1995; Vallee and Auld 1993). The wide-ranging effects of Zn in the immune and nervous systems have been demonstrated both in vivo and in vitro, and these effects mainly depend on Zn concentration (Frederickson et al. 2005; Rink and Gabriel 2000). Many researchers have reported that Zn depletion decreases immune function. Natural killer cell-mediated cytotoxic activity, antibody-mediated responses, and host defenses against pathogens and tumors are all reduced in Zn-deficient mice (Fernandes et al. 1979; Fraker et al. 1982; Keen and Gershwin 1990).

Zn plays an essential constitutive role in the conformation and activity of many enzymes, transcription factors, signaling molecules, and other components involved in cellular processes. However, high concentrations of Zn can be cytotoxic, and can induce apoptosis in T and B cells (Ibs and Rink 2003; Telford and Fraker 1995). Zn concentration and distribution within and between cells are controlled by Zn transporters, such as those in the Slc39/ZIP and Slc30/ZnT families, which increase and decrease intracellular Zn levels, respectively (Fukada and Kambe 2011; Kambe et al. 2004; Lichten and Cousins 2009), and Zn is buffered by Zn-binding molecules (Palmiter 2004; Vallee 1995).

We recently showed that Fc epsilon receptor I (FcεRI) stimulation induces an increase in intracellular free Zn, and we named this phenomenon the "Zn wave" (Yamasaki et al. 2007). The Zn wave occurs within several minutes after FcεRI stimulation and originates from the endoplasmic reticulum (ER). Thus, extracellular stimuli can affect intracellular Zn levels through Zn transporters and Zn-permeable channels, and Zn acts as an intracellular signaling molecule (Taylor et al. 2012; Yamasaki et al. 2012). We have proposed that intracellular Zn signaling can be classified into at least two categories: late Zn signaling, which depends on transcriptional changes in Zn-transporter expression, and early Zn signaling, which involves the Zn wave, in which Zn is directly released from the cell organelle by an extracellular stimulus such as FcεRI (Fukada et al. 2011; Hirano et al. 2008; Murakami and Hirano 2008; Nishida et al. 2011).

Many studies have shown that Zn is important in the immune system and that imbalances in Zn homeostasis lead to various disorders, but several questions remain as to how Zn homeostasis and signaling are regulated in mast and other immune cells, and whether Zn transporters are involved in immune cell function. In this chapter, we briefly describe aspects of the Zn-wave phenomenon and discuss the role of Zn signaling in allergic responses.

5.2 Cellular Zn Homeostasis by Zn Transporters and Metallothioneins

Computational analysis suggests that approximately 10 % of the genes in the human genome may contain Zn-binding motifs (Andreini et al. 2006), reflecting the physiological relevance of Zn and Zn-binding proteins for life in general. In fact, Zn has wide-ranging effects on cellular functions (Vallee and Falchuk 1993), and imbalances in Zn homeostasis can impair growth and cause hair loss, thickening and hyperkeratinization of the epidermis, and testicular atrophy in humans and animal models (Prasad 1991). The concentration and distribution of both intracellular and extracellular Zn are controlled via buffering by Zn-binding molecules, such as metallothioneins, and by Zn transporters, including Slc39/ZIP and Slc30/ZnT family members, which increase or decrease the intracellular Zn levels, respectively (Fig. 5.1).

Metallothioneins (MTs), small cysteine-rich proteins that bind Zn and other metal ions, are thought to be responsible for regulating intracellular Zn concentrations and detoxifying nonessential heavy metals. When intracellular free Zn reaches a threshold concentration, activation of the Zn sensor MTF-1 induces the expression of MTs, which sequester Zn ions (Andrews 2001). Thus, MTs serve as a biochemical device that controls free Zn concentrations by sequestering or releasing Zn in response to oxidative signaling and other biochemical events.

5.3 Zn Signaling

Cytosolic and cellular concentrations of free Zn change dynamically in many cells in response to various stimuli. Three major sources for cytosolic free Zn have been identified: uptake from the extracellular environment, reversible storage in vesicles,

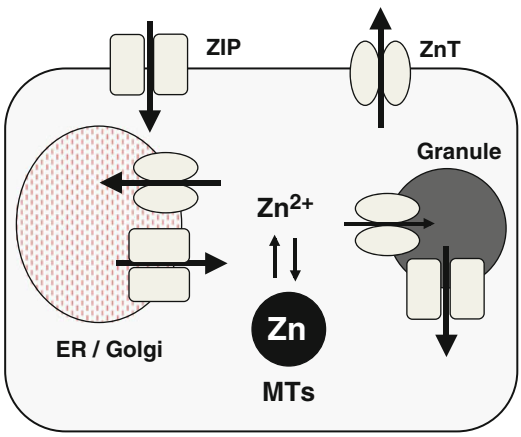

Fig. 5.1 Subcellular localization of Zn transporters and metallothioneins. An illustration of the subcellular localization and potential functions of ZIP- and ZnT family members, based on currently available information. *Arrows* indicate the predicted direction of Zn mobilization. *MTs* metallothionein1/2, *ER* endoplasmic reticulum

and oxidative release from storage proteins. Thus, Zn is increasingly recognized as a potential signaling molecule (Frederickson and Bush 2001; Haase and Rink 2009; Hirano et al. 2008; Sensi et al. 2009).

5.3.1 Zn Acts as a Neurotransmitter

Zn is highly concentrated in synaptic vesicles and is released with glutamate in an activity-dependent manner (Assaf and Chung 1984). Recently, Zn imaging techniques that allow the real-time analysis of Zn concentration and distribution using fluorescent sensor molecules have been widely applied (Kikuchi 2010). Microfluorescence imaging of Zn secretions in rodents showed that exocytotic presynaptic stimulation prompts the release of Zn from hippocampal mossy fiber terminal vesicles into the surrounding milieu (Li et al. 2001; Qian and Noebels 2005; Ueno et al. 2002), where it is taken up into the cytoplasm of neighboring cells through gated Zn channels. A rapid influx of Zn through calcium ion-permeable AMPA/kainate (Ca-A/K) channels triggers the generation of reactive oxygen species (ROS) and is potentially neurotoxic (Weiss and Sensi 2000).

In the foregoing scenario, Zn activity is similar to that of neurotransmitters, which are stored in membrane-enclosed synaptic vesicles, are released by exocytosis, and activate postsynaptic cells through transmitter-gated ion channels (Colvin et al. 2003; Hershfinkel et al. 2001; Xie and Smart 1994). Because Zn modulates both the current response (mediated by excitatory and inhibitory neurotransmitter receptors) and the efficacy of transporter-driven neurotransmitter re-uptake (Smart et al. 2004), synaptically released Zn has been proposed to function as an important regulator of synaptic transmission and plasticity (Lu et al. 2000; Vogt et al. 2000).

ZnT3/Slc30a3, which is highly expressed on synaptic vesicle membranes, is essential for Zn uptake into the vesicles. Although *ZnT3*-KO mice show a loss of stainable Zn in synapses (Cole et al. 1999), they are behaviorally normal except for an enhanced susceptibility to kainite-induced seizures (Lopantsev et al. 2003). In the Tg2576 transgenic mouse model for Alzheimer's disease (AD), Zn release during synaptic transmission induces cerebral β-amyloid (Aβ) deposits. When these mice are crossed with the *ZnT3*-KO mice, which lack synaptic Zn, the cerebral Aβ deposition is nearly abolished in the Tg2576/*ZnT3*$^{-/-}$ progeny (Lee et al. 2002). A recent study by Tamaki et al. provides evidence that Zn is co-secreted with insulin in a ZnT8-dependent manner, and that the secreted Zn not only affects neighboring endocrine cells, but also plays an important role in hepatic insulin clearance by inhibiting clathrin-dependent insulin endocytosis (Tamaki et al. 2013). This finding suggests that Zn release is involved in various pathologies, such as AD and diabetes.

Recent studies have shown that Zn is an endogenous agonist for GPR39, an orphan receptor that is structurally and functionally related to G protein-coupled receptors. The concept of GPR39 as a Zn-sensing receptor in the brain is consistent with the role of Zn as a neurotransmitter (Besser et al. 2009; Yasuda et al. 2007).

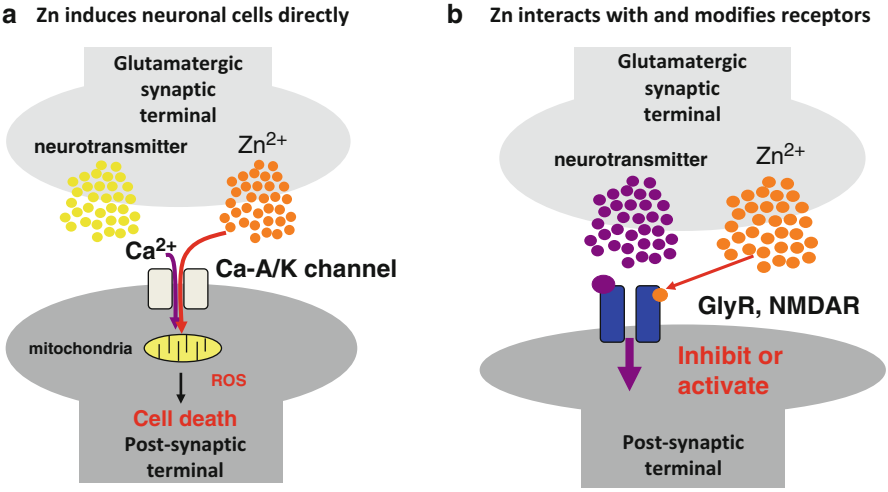

Fig. 5.2 Zn acts as a neurotransmitter. Zn behaves as a neurotransmitter in neuronal cell communication. **a** Zn is released from vesicles into the surrounding milieu upon exocytotic presynaptic stimulation, and is then taken up into the cytoplasm of neighboring cells through gated Zn channels. The rapid influx of Zn through calcium (Ca) ion-permeable AMPA/kainate (Ca-A/K) channels triggers the generation of reactive oxygen species (ROS) and is potentially neurotoxic. **b** Zn released from glutamatergic synaptic vesicles can bind to the Zn-binding sites of GlyR and NMDAR on postsynaptic neurons to modulate neurotransmission

Intercellular Zn communication mediated through the Zn-sensing receptor GPR39 is also involved in regulating epithelial cell repair (Sharir et al. 2010) and endocrine pancreatic function (Holst et al. 2009). Also supporting the role of Zn as a neurotransmitter, Hosie et al. identified two Zn-binding sites and characterized a third in the GABA receptor using site-directed mutagenesis (Hosie et al. 2003). Hirzel et al. produced knock-in mice carrying a constructed Zn-binding site (a D80A mutation) in the glycine receptor alpha-1-subunit gene (*Glra1*) and demonstrated that Zn modulates neurotransmission (Hirzel et al. 2006). Zn is also known to inhibit *N*-methyl-D-aspartate receptor (NMDAR) activity through a dual mechanism: a voltage-dependent channel block, and a voltage-independent reduction in the probability of the channel opening (Christine and Choi 1990; Legendre and Westbrook 1990; Mayer and Vyklicky 1989). Thus, Zn appears to act as a neurotransmitter in addition to its other roles in the neural system (Fig. 5.2).

5.3.2 Zn Acts as a Second Messenger

cAMP was the first intracellular second messenger to be discovered (Berthet et al. 1957), and calcium was the second. At present, only a limited number of intracellular signaling effectors are known, including cAMP, calcium, NO, lipid

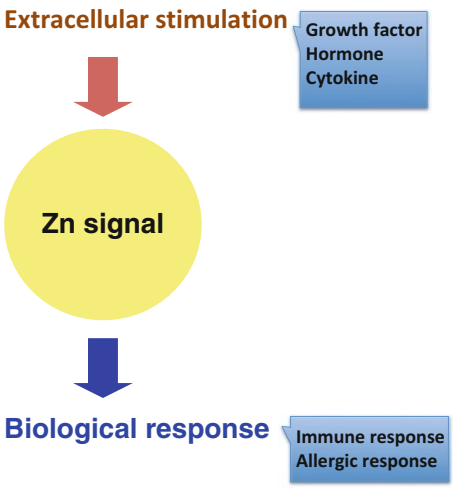

Fig. 5.3 Zn as an intracellular second messenger candidate. Second messengers are molecules that receive signals from stimuli such as hormones, growth factors, and cytokines at the cell surface and relay these signals to target molecules in the cytosol or nucleus. This action, in turn, induces intracellular events that can regulate various biological responses. Zn functions are consistent with those of second messenger molecules

mediators, and G proteins (Gomperts et al. 2002). Interestingly, Zn has also been recognized to have an essential role as a second messenger. Zn itself affects a variety of signaling molecules, including PKC, Ca/calmodulin-dependent protein kinase II (CaMKII), Erk1/2, cAMP-dependent protein kinase (PKA), protein tyrosine phosphatase, and caspase-3 (Brautigan et al. 1981; Haase and Maret 2005; Hubbard et al. 1991; Lengyel et al. 2000; Murakami et al. 1987; Park and Koh 1999; Perry et al. 1997). In addition, Zn activates ion channels such as the transient receptor potential ankyrin 1 (TRPA1) (Andersson et al. 2009; Hu et al. 2009), ATP-sensitive K^+ (Prost et al. 2004), and large-conductance Ca-activated K^+ (Hou et al. 2010) channels. Together, these findings suggest that Zn may function as an intracellular signaling molecule or second messenger if extracellular stimuli, such as cytokines or growth factors, cause the intracellular Zn status to change, whether dependently or independently of transcriptional changes in MTs or Zn transporters (Fig. 5.3).

It is important to determine whether Zn transporters are involved in Zn signaling. We reported that the ZIP6/SLC39A6 is required for the epithelial-mesenchymal transition (EMT) in the zebrafish gastrula organizer, as it is essential for nuclear retention of the E-cadherin-repressor Snail (Yamashita et al. 2004). Because ZIP6/SLC39A6 expression in the zebrafish organizer is dependent on STAT3 activation, any extracellular stimulus that regulates STAT3 activation could change the intracellular Zn level by inducing changes in ZIP6/SLC39A6 or other Zn transporters. TLR4 decreases the intracellular free Zn level by inducing changes in Zn transporter expression in dendritic (Kitamura et al. 2006) and pulmonary endothelial cells (Thambiayya et al. 2011). These observations support the role of Zn as an intracellular second messenger, that is, as a molecule whose intracellular status is altered in response to an extracellular stimulus and that is capable of transducing the extracellular stimulus into an intracellular signaling event.

The role of Zn as a second messenger is further supported by our findings that ZIP13/Slc39a13 is required for the BMP/TGF-β-induced nuclear localization of Smad proteins (Fukada et al. 2008), that ZIP14/Slc39a14 is involved in GPCR-mediated signal transduction through cAMP basal-level regulation (Hojyo et al. 2011), and that FcεRI-stimulation-induced PKC activation is dependent on ZnT5/Slc30a5 in mast cells (Nishida et al. 2009). Thus, extracellular stimuli can, by changing the expression of Zn transporters, affect intracellular signaling pathways by changing the intracellular Zn status.

Besides Zn signaling mediated by Zn transporter expression, Zn signaling is also exemplified by the Zn wave phenomenon, which is a transcription-independent increase in intracellular Zn that occurs in mast cells relatively rapidly (several minutes) after extracellular stimulation (Yamasaki et al. 2007). Zn waves, which originate from the ER, depend on calcium influx and Erk1/2 activation in mast cells. Because the Zn wave does not involve extracellular Zn, and is induced within several minutes of FcεRI stimulation, Zn here acts as an intracellular signaling molecule. Using the Zn probe FluoZin-3, Haase et al. revealed that the intracellular Zn level is elevated in peripheral blood mononuclear cells (PBMCs) after polymethyl acrylate (PMA) stimulation (Haase et al. 2006), and in human leukocytes, especially monocytes, after physiological stimulation (Haase et al. 2008). In addition, ZIP7/SlC39A7 is reported to regulate intracellular Zn at the ER membrane (Taylor et al. 2008). It is also possible that transporters and channels other than ZIP or ZnT family members generate Zn waves in individual cell types with various types of stimulation. In any case, the precise molecular mechanisms generating the Zn wave have not been clarified.

5.3.3 Zn-Signaling Gatekeepers

Recently, three independent groups reported rapid increases in free Zn, as seen in the Zn wave, in mast cells and lymphocytes upon stimulation with antigens such as FcεRI, B-cell receptors (BCRs), and T-cell receptors (TCRs) (Taniguchi et al. 2013; Yamasaki et al. 2012; Yu et al. 2011). Interestingly, these studies reported that the dynamic changes in cytosolic free Zn were regulated by Zn-permeable channels and Zn transporters.

In this section, we briefly describe gatekeepers of Zn signaling, their regulation, and their molecular targets.

5.3.3.1 LTCC-Mediated Zn Signaling in Mast Cells

L-type calcium channels (LTCCs) conduct Zn and can act as Zn-permeable channels on the plasma membrane of neurons and pancreatic β cells (Atar et al. 1995; Gyulkhandanyan et al. 2006; Sensi et al. 1997). However, whether LTCCs can function in releasing Zn from intracellular organs has not been clarified. It was

recently revealed that LTCCs have the potential to act as a gatekeeper for the Zn wave that occurs when FcεRI induces intracellular Zn signaling in mast cells (Yamasaki et al. 2012).

It is well established that LTCCs function as voltage-gated calcium channels on the plasma membrane. LTCC complexes include α_1-, β-, and α_2/δ-subunits. The α_1-subunit functions as the voltage sensor, selective filter, and ion-conducting pore (Catterall 2000). The α_1-subunit on the cell surface is thought to require an association with the β-subunit, which masks one or more ER retention signals (Bichet et al. 2000; Cornet et al. 2002).

In bone marrow-derived mast cells (BMMCs), the dominantly expressed α_1-subunit (α_{1D}) is *cacna1d*, the α_1-subunit for the LTCC. The expression of LTCC β-subunits, which are required for the localization of α_1-subunits to the plasma membrane, is very low in mast cells. Furthermore, mast cells express high levels of ZnT-1/Slc30a1, which interacts with β-subunits on the plasma membrane, reducing their availability to bind α_1 and inhibiting α_1-subunit trafficking to the plasma membrane (Levy et al. 2009). Therefore, α_{1D} in mast cells localizes to the intracellular area; it co-localizes partially with the ER marker calnexin but not with F-actin, which accumulates beneath the plasma membrane. These observations indicate that α_{1D} localizes preferentially to intracellular organelle membranes, such as the ER membrane, rather than to the plasma membrane. This intracellular localization of α_{1D} in BMMCs suggests that it plays a different role in BMMCs than in other cells, in which it is located on the plasma membrane and acts as a calcium channel.

Consistent with this idea, treating mast cells with verapamil, an LTCC inhibitor, reduces their Zn waves compared to control cells, without disturbing cell survival, FcεRI expression, or the FcεRI-mediated calcium elevation. Diltiazem, another type of LTCC antagonist, also inhibits Zn waves without disturbing the calcium elevation. Not only does verapamil have no effect on the FcεRI-mediated calcium elevation in BMMCs, but calcium-mediated signaling, such as the normal nuclear translocation of NFAT2 in response to calcium elevation, is also unaffected (Yamasaki et al. 2012).

On the other hand, treatment with the LTCC agonist (*s*)-(–)-BayK8644 without antigen stimulation elevates the intracellular Zn level but not the calcium level in mast cells (Yamasaki et al. 2012). These findings indicate that LTCC function in mast cells differs from its function as a calcium channel in other cell types, such as neurons and pancreatic β cells. LTCC may have little effect on the intracellular calcium regulation in mast cells because the main mode of calcium influx in these cells, as in lymphocytes, is store-operated calcium (SOC) entry (Vig and Kinet 2009). LTCC agonists induce an increase in intracellular Zn even in the absence of calcium, and this increase is inhibited by verapamil. FcεRI induces similar Zn increases in BMMCs in the presence or absence of an LTCC agonist, indicating that the mechanisms responsible for the FcεRI-induced Zn wave and the LTCC agonist-induced Zn elevation are probably similar.

Although the FcεRI-induced Zn wave is significantly reduced in α_{1D}-knockdown BMMCs, the FcεRI-induced calcium elevation is intact in these cells, as is the case

with verapamil-treated cells. Moreover, the ectopic expression of wild-type α_{1D} rescues the inhibitory effect of α_{1D} siRNA knockdown on the Zn wave. These results suggest that the LTCC α_{1D} subunit is a gatekeeper for the Zn wave.

This finding raises the question of how LTCC regulates the release of Zn from the ER into the cytoplasm. The α_1-subunit of LTCC contains a voltage-sensor domain, and channel activity is elevated after membrane depolarization. The plasma-membrane potential in BMMCs is hyperpolarized after FcεRI stimulation (Shumilina et al. 2008; Vennekens et al. 2007). However, inhibiting the FcεRI-mediated plasma-membrane hyperpolarization by high KCl treatment does not impair the induction of the Zn wave. Treatment with bongkrekic acid, which inhibits ADP/ATP transport, inhibits FcεRI-mediated intracellular membrane depolarization but does not inhibit the induction of the Zn wave. These observations suggest that regulation of the membrane potential might not affect the Zn wave generation.

Phosphorylation of the pore-forming α_1-subunit has an additional effect on channel activity; in fact, cAMP-mediated channel activity is reduced by site-directed mutagenesis of the PKA consensus sites of α_{1D} (Ramadan et al. 2009). However, a PKA inhibitor did not inhibit the Zn wave. Therefore, PKA might not assist in regulating the Zn wave, at least not in mast cells. This event may be controlled by as yet unidentified regulatory proteins and mechanisms on the ER membrane.

These results show that the LTCC pore-forming α_{1D}-subunit, when expressed on the ER membrane, functions as a gatekeeper for the Zn wave in mast cells, and that this LTCC-mediated Zn wave may function as an intracellular Zn signal that can positively modify signal transduction to produce inflammatory cytokines (Fig. 5.4).

5.3.3.2 ZIP9-Mediated Zn Signaling in B Cells

Taniguchi et al., using the DT40 chicken B-cell line as a model, found that the Zn transporter ZIP9/SLC39A9 induces an increase in intracellular Zn. In contrast to the Zn wave, which originates from the ER, the source of intracellular free Zn in these chicken ZIP9-knockout DT40 (cZip9KO) cells is thought to be the Golgi bodies. Consistent with this scenario, ZIP9 is thought to function in releasing Zn from the Golgi bodies to the cytosol (Taniguchi et al. 2013).

The BCR-signaling pathway is critical for many cellular events, including cell growth, cell proliferation, and apoptosis (Dal Porto et al. 2004; Harwood and Batista 2008, 2010). BCR activation transduces the signal to several cascades, including the PI-3K/Akt, PLCγ2/PKC, and Ras/Raf/ERK cascades (Brazil and Hemmings 2001; Hashimoto et al. 1998; Kurosaki 2011). These cascades are important for the differentiation of antibody-producing cells and memory B cells. ZIP9-mediated Zn signaling affects BCR-mediated Akt and ERK phosphorylations. In fact, BCR-induced Akt and ERK phosphorylations are significantly decreased, along with the enzymatic activity of protein tyrosine phosphatase (PTPase), in cZip9KO cells. Consistent with this observation, overexpressing hZIP9 decreases

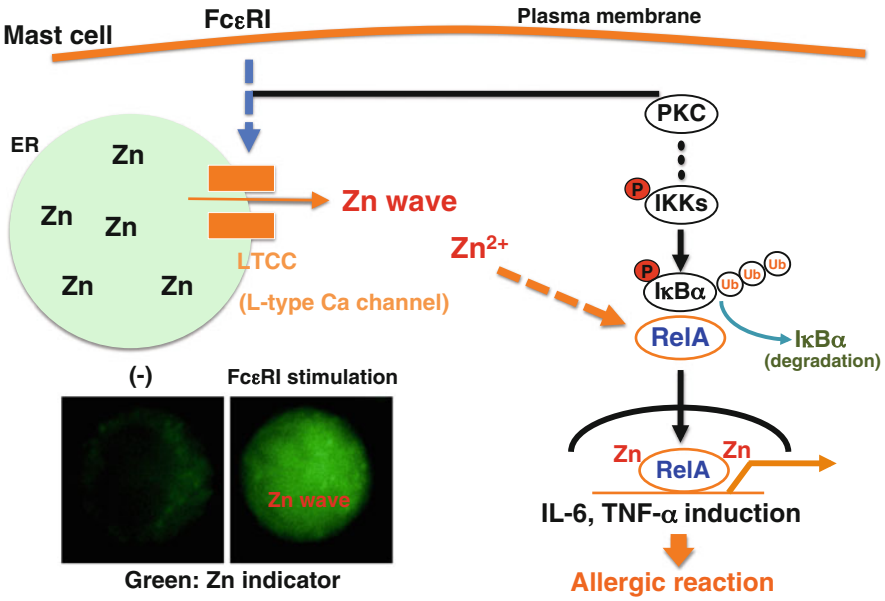

Fig. 5.4 The Zn wave in FcεRI-mediated mast cell activation. In a phenomenon we named the *Zinc wave*, FcεRI stimulation induces the rapid elevation of intracellular Zn in mast cells by releasing Zn from the ER at the perinuclear region. The LTCCα_{1D} subunit expressed on the ER membrane acts as the Zn-wave gatekeeper in mast cells. LTCC-mediated Zn waves positively regulate the NF-κB DNA-binding activity and are involved in regulating cytokine production

the PTPase activity in Zn-treated cZip9KO cells (Taniguchi et al. 2013). At present, it is not known how ZIP9-mediated Zn signaling is regulated by BCR stimulation. ZIP7 is reported to be activated by its phosphorylation by protein kinase CK2 in a human breast cancer cell line (Taylor et al. 2012). This finding raises the possibility that CK2 can phosphorylate ZIP9 and regulate ZIP9-mediated Zn signaling.

This study concluded that ZIP9 on the Golgi body membrane regulates cytosolic Zn, enhancing the Akt and ERK phosphorylations in B cells (Fig. 5.5). Thus, Zn signaling in B cells may occur via a mechanism similar to that of the Zn wave in mast cells.

5.3.3.3 ZIP6-Mediated Zn Signaling in T Cells

Yu et al. found Zn signaling in T cells; TCR stimulation triggers an increase in cytoplasmic Zn concentration within 1 min (Yu et al. 2011). This increase is dependent on extracellular Zn concentration, suggesting that TCR stimulation induces an influx of extracellular Zn into the T cell. Moreover, this influx of Zn is inhibited by silencing the Zn transporter ZIP6, which is expressed on the cytoplasmic membrane.

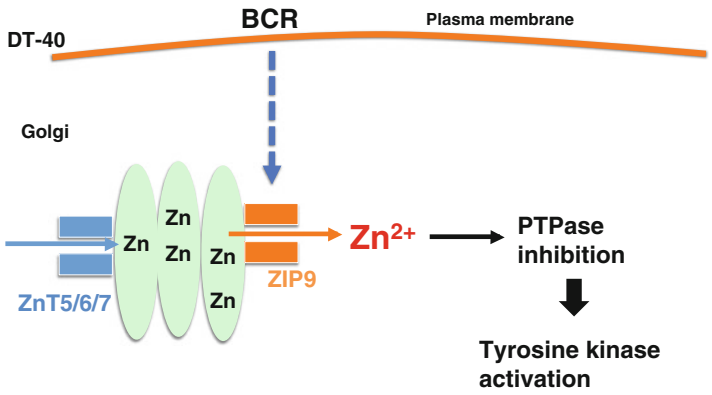

Fig. 5.5 Proposed action sites of intracellular Zn release by ZIP9 in the activation of B-cell receptor signaling in DT40 cells. An illustration of the proposed mechanism by which Zn induces the inhibition of protein tyrosine phosphatase (PTPase) by ZIP9 in DT40 cells, leading to the activation of B-cell receptor (*BCR*) signaling. Intracellular Zn is incorporated into Golgi bodies by ZnT5/6/7, and ZIP9 induces its release from the Golgi back into the cytosol. This Zn inhibits PTPase activity and induces tyrosine kinase activation probably indirectly by regulating upstream components of the signal transduction

Early TCR signaling events include the tyrosine phosphorylation of several signaling molecules. The Src protein kinase Lck is primarily responsible for the early phosphorylation of tyrosines within the ITAM motifs of CD3ζ and ZAP70 (Palacios and Weiss 2004). Extracellular Zn influences ZAP70 phosphorylation and inhibits negative regulatory feedback loops, accounting at least in part for the increase in ZAP70 phosphorylation. SHP-1, which dephosphorylates tyrosines within the ITAM motifs of ZAP70 and other signaling molecules after being recruited to Lck (Altan-Bonnet and Germain 2005), is a prime candidate target for the ZIP6-mediated Zn signaling. In fact, an increase in Zn influx reduces SHP-1 recruitment to the TCR activation complex, augments ZAP70 phosphorylation, and sustains calcium influx. Thus, Yu et al. proposed that the influx of Zn after TCR stimulation leads to a local increase in cytoplasmic Zn that modifies early TCR signaling events (Fig. 5.6).

5.4 Zn and Zn Signaling in Allergies

Many studies have reported that Zn depletion decreases immune function, indicating that Zn acts as a positive regulator in immune responses. However, the precise roles of Zn and the molecular mechanism(s) of its function in allergic responses have not been clarified. Here, we describe the effect of Zn and Zn homeostasis on biological events, especially those of mast cell-mediated allergy responses, and outline our current understanding of the physiological role of the Zn wave.

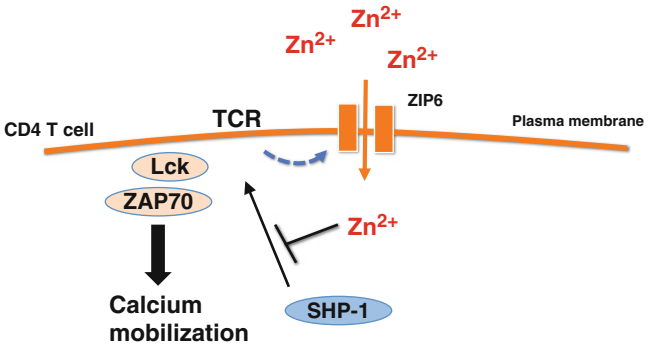

Fig. 5.6 Zn functions as an ionic signaling molecule after T-cell activation. Cytoplasmic Zn concentrations increase within 1 min after T-cell receptor (*TCR*) triggering as a result of Zn influx via the transporter ZIP6. This increase, which is most pronounced in the immediate subsynaptic area, enhances TCR signaling, at least partly by inhibiting the recruitment of SHP-1

5.4.1 The role of Zn in Mast Cell-Mediated Allergic Responses

Allergy-related cells, such as mast cells, eosinophils, and basophils, are involved in allergic reactions such as anaphylaxis, asthma, and atopic dermatitis (Galli et al. 2008; Kawakami et al. 2009; Metz et al. 2007). Mast cell activation leads to the secretion of two classes of mediators. The first consists of preformed mediators that are stored in granules and can be quickly secreted in activated cells. The second class of mediators, the cytokines and chemokines, must be newly synthesized and are secreted more slowly. These released molecules play pivotal roles in the inflammatory reactions observed in patients with allergies.

Intracellular Zn levels and distribution can be analyzed using the Zn probe Zinquin, which allows the imaging of distinct pools of Zn in allergy-related cells. Granules in mast cells, for instance, fluoresce intensely with Zinquin (Ho et al. 2004). Airway epithelial cells are also rich in Zn (Truong-Tran et al. 2000). Furthermore, Zn deficiency is reported to increase allergic eosinophilic inflammation, whereas dietary Zn supplementation attenuates its intensity (Richter et al. 2003a). Interestingly, Zn deficiency is a risk factor for asthma development (Riccioni and D'Orazio 2005; Zalewski et al. 2005). These reports suggest that Zn is involved in the development of allergic disease. In addition, high levels of *ZIP2* are observed in the leukocytes of asthmatic infants (Xu et al. 2009). However, the precise role of Zn and Zn transporters in allergy-related cells is poorly understood.

We showed that Zn is required for both degranulation and cytokine production in mast cells. The Zn chelator TPEN [*N*,*N*,*N*,*N*-tetrakis (2-pyridylmethyl) ethylenediamine] inhibits histamine release, cytokine production, and lipid mediator secretion. The inhibitory effects of TPEN are rescued by Zn supplement, and chelators of other metals do not affect mast cell function (Kabu et al. 2006). These observations indicate that Zn is crucial for degranulation and cytokine production in mast cells. Similarly, it has been reported that Zn depletion by TPEN or by the

clinically used heavy metal chelator DMPS (Torres-Alanis et al. 1995) inhibits the mRNA expression of eotaxin and other chemokines in human lung cell lines (Richter et al. 2003b). All together, these reports suggest that Zn chelators and their derivatives are a promising source of anti-allergy drugs that may act differently from histamine antagonists and other currently available allergy treatments.

It is not clear exactly how Zn chelators inhibit mast cell function. We reported that the degranulation of mast cells can be divided into two processes. First, FcεRI stimulation triggers microtubule polymerization and granule translocation to the plasma membrane in a calcium-independent manner. Fyn/Gab2/RhoA signaling, but not Lyn/SLP-76 signaling, is critical in this calcium-independent microtubule-dependent pathway (Nishida et al. 2005). Second, the granules fuse with the plasma membrane in a well-characterized calcium-dependent manner. To clarify the mechanisms behind the effect of Zn chelators, we first examined whether TPEN interfered with either of these two processes. Although TPEN had little effect on calcium mobilization or the early FcεRI-induced tyrosine phosphorylation of various signaling molecules, it suppressed the FcεRI-induced granule translocation. Given that granule translocation depends on cytoskeletal proteins such as tubulin and actin (Goode et al. 2000), and that microtubules are critical for granule translocation and vesicle transport (Nishida et al. 2005; Smith et al. 2003), we speculated that TPEN might affect microtubule assembly. However, such an effect by TPEN on FcεRI-induced microtubule formation has not been found, suggesting the existence of Zn-regulated molecule(s) that are directly linked to microtubules and granules. Kinesin receptors, or linker-cargo proteins, have been identified as key molecules for microtubule-dependent vesicle trafficking (Schnapp 2003); thus, the TPEN target might have a kinesin-interacting region through which it links kinesin to vesicles. In addition, TPEN suppresses FcεRI-mediated cytokine production and the transcription of interleukin (IL)-6 and tumor necrosis factor (TNF)-α mRNAs. PKC is activated upon FcεRI stimulation and is involved in cytokine production through NF-κB activation (Klemm et al. 2006; Nechushtan et al. 2000), and TPEN inhibits the FcεRI-mediated plasma membrane translocation of PKC (Kabu et al. 2006). These results suggest PKC as one of the TPEN targets in regulating cytokine production. This hypothesis is supported by findings from other groups showing that the Zn-binding domain of PKC is required for PKC translocation to the plasma membrane after stimulation (Oancea et al. 1998).

We demonstrated that ZnT5 is crucial in mast cell activation and mast cell-mediated allergic reactions. Znt5 is expressed at high levels in mast cells, and its transcription is enhanced by FcεRI stimulation, suggesting that Znt5 is involved in mast cell-mediated allergic reactions. *ZnT5-KO* mice have defects in mast cell-mediated, delayed-type allergic reactions such as contact hypersensitivity, but not in immediate-type reactions such as anaphylaxis (Nishida et al. 2009). Consistent with this in vivo analysis, ZnT5 is required for FcεRI-mediated cytokine production, but not for degranulation, in mast cells. In *ZnT5-KO* mast cells, the FcεRI-induced IL-6 and TNF-α mRNAs are reduced. Finally, we showed that ZnT5 is required for the FcεRI-induced translocation of PKC to the plasma membrane and for the nuclear translocation of NF-κB. Thus, ZnT5 is selectively required for mast cell-mediated, delayed-type allergic responses, and is a novel player in PKC/NF-κB

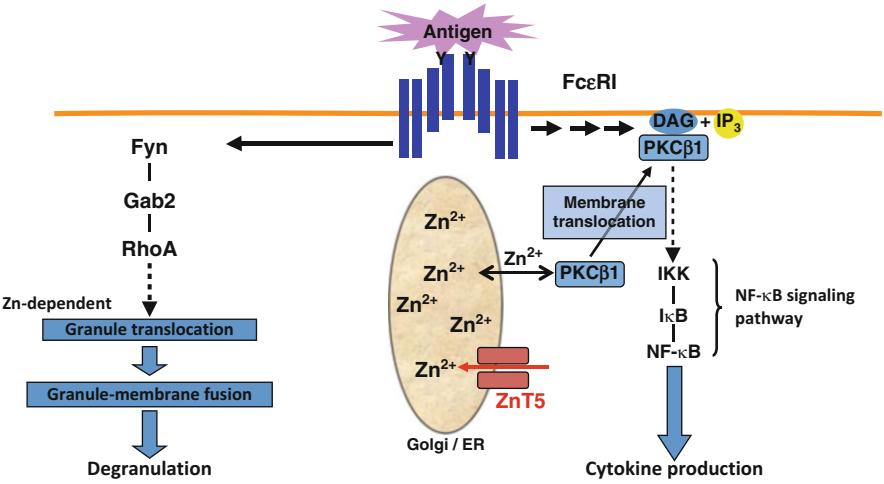

Fig. 5.7 Zn and Zn transporters are involved in FcεRI-mediated mast cell activation. Zn is required in multiple steps of FcεRI-induced mastcell activation, including degranulation and cytokine production. Zn levels depend on FcεRI-induced granule translocation, regulated by the Fyn/Gab2/RhoA-mediated signaling pathway. Zn and ZnT5 are also required for PKC translocation to the plasma membrane and NF-κBs subsequent nuclear translocation, leading to the production of cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α

signaling. How ZnT5 regulates PKC translocation to the plasma membrane is still unknown. PKC contains a Zn-binding motif, and Zn is essential for maintaining the structure of PKC (Corbalan-Garcia and Gomez-Fernandez 2006). Mutational analysis showed that the Zn-binding domain of PKC is essential for the plasma membrane translocation of PKC. Furthermore, in experiments utilizing *ZnT5-KO* DT40 cells, Suzuki and colleagues showed that ZnT5 expressed on the ER–Golgi membrane is required for the enzymatic activity of the Zn-dependent alkaline phosphatases (ALPs), which are processed from apoALPs to holoALPs in the ER–Golgi (Suzuki et al. 2005a, b). Together, these findings indicate that ZnT5 may be involved in supplying Zn to the Zn finger-like domain in PKC and ALP.

All these findings suggest that Zn and Zn transporters are involved in mast cell-mediated allergic responses by regulating degranulation and cytokine production, and that Zn transporters modulate the PKC/NF-κB signaling pathway, which regulates the gene expression levels of cytokine and chemokines (Fig. 5.7).

5.4.2 *Role of the Zn Wave in Mast Cell-Mediated Allergic Responses*

As already mentioned, we found that the pore-forming α_{1D}-subunit of LTCC on the ER membrane is involved in generating the Zn wave in mast cells. Using siRNA or an LTCC antagonist, we found that the Zn wave can regulate cytokine gene

induction. LTCC antagonist-treated mast cells cannot increase the intracellular concentration of free Zn, either through a Zn wave or through the mRNA induction and protein synthesis of IL-6 and TNF-α. Furthermore, both FcεRI-induced Zn waves and cytokine gene induction are inhibited in mast cells treated with siRNA against the LTCC α_{1D}-subunit. These results suggest that the Zn wave is required for the FcεRI-induced cytokine production in mast cells.

It is not certain how the Zn wave acts to induce cytokine genes. We found that the LTCC-mediated intracellular Zn signal upregulates NF-κB DNA-binding activity and transactivation of inflammatory cytokines. This NF-κB-mediated transactivation can be divided into three steps: NF-κB first dissociates from IκB after IκB phosphorylation and degradation, then translocates from the cytosol to the nucleus, and finally binds to its target sequences. We found that the frequency of NF-κB p65 nuclear translocation is reduced in LTCC antagonist-treated cells, even though upstream regulators are unaffected. Treatment with the exportin inhibitor LMB enhances the amount of NF-κB in nuclei in both LTCC antagonist-treated and LMB-treated cells, suggesting that the Zn wave is not involved in NF-κB nuclear translocation. However, our evidence indicates that the Zn wave is required for NF-κB DNA-binding activity. In further support of this scenario, LTCC antagonist treatment reduces NF-κB DNA-binding activity, whereas supplementing cell lysates with Zn enhances it. These findings suggest that Zn wave-induced increases in intracellular Zn positively regulate NF-κB DNA-binding activity (Fig. 5.4).

Finally, we evaluated the role of the Zn wave in allergic reactions in vivo using an LTCC antagonist. Mast cells are effector cells for allergic responses in vivo, and mast cell-derived cytokines, which are induced by the FcεRI-mediated activation of the PKC/Bcl10/Malt1/NF-κB signaling pathway, are involved in delayed-type allergic responses such as contact hypersensitivity (CHS) (Klemm et al. 2006; Nishida et al. 2009). Mast cell-derived TNF is required for a maximum CHS response; it induces leukocyte infiltration at the inflammation site (Biedermann et al. 2000), enhances the elongation of cutaneous nerves (Kakurai et al. 2006), and enhances the dendritic cell migration to draining lymph nodes (Suto et al. 2006). In this study, we revealed that treating mice with the LTCC antagonist verapamil inhibits CHS, which is a delayed-type immune response, without affecting passive cutaneous anaphylaxis (PCA), which is an immediate-type response. Consistent with these results, we showed that verapamil treatment inhibits the FcεRI-mediated activation of NF-κB DNA-binding activity and cytokine gene induction, but not calcium elevation or degranulation in BMMCs. Thus, the inhibitory effect of verapamil on CHS might depend at least partly on the reduction of mast cell-derived cytokine production and be independent of histamine and other mediators.

In future studies, knocking out the LTCCα_{1D}-subunit in mice will further clarify Zn wave in vivo roles in allergic and other immune responses.

5.5 Perspective

We have discussed new insights into the relevance of Zn, its channels, and its transporters in immune cell responses, particularly focusing on the role of Zn as a signaling molecule. Our studies and those of other researchers indicate that Zn signals affect a variety of immune-signaling pathways to produce biological outputs. Thus, we propose that Zn functions as a signaling molecule (Fukada et al. 2011; Hirano et al. 2008; Murakami and Hirano 2008; Nishida et al. 2011). Many questions about the role of Zn in signaling remain to be answered. We do not yet know the other targets, the biological significance in vivo, or the regulatory mechanisms of Zn signaling, particularly in the Zn wave. As with calcium, it is likely that Zn is an important intracellular signaling molecule in various systems including immune system. We hope that recognition of the importance of Zn signaling and Zn biology will open new avenues for future research.

References

Altan-Bonnet G, Germain RN (2005) Modeling T cell antigen discrimination based on feedback control of digital ERK responses. PLoS Biol 3:e356

Andersson DA, Gentry C, Moss S, Bevan S (2009) Clioquinol and pyrithione activate TRPA1 by increasing intracellular Zn^{2+}. Proc Natl Acad Sci USA 106:8374–8379

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196–201

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

Assaf SY, Chung SH (1984) Release of endogenous Zn^{2+} from brain tissue during activity. Nature (Lond) 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

Berthet J, Rall TW, Sutherland EW (1957) The relationship of epinephrine and glucagon to liver phosphorylase. IV. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. J Biol Chem 224:463–475

Besser L, Chorin E, Sekler I, Silverman WF, Atkin S, Russell JT, Hershfinkel M (2009) Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus. J Neurosci 29:2890–2901

Bichet D, Cornet V, Geib S, Carlier E, Volsen S, Hoshi T, Mori Y, De Waard M (2000) The I-II loop of the Ca^{2+} channel alpha1 subunit contains an endoplasmic reticulum retention signal antagonized by the beta subunit. Neuron 25:177–190

Biedermann T, Kneilling M, Mailhammer R, Maier K, Sander CA, et al. (2000) Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. J Exp Med 192: 1441–1452

Brautigan DL, Bornstein P, Gallis B (1981) Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn. J Biol Chem 256:6519–6522

Brazil DP, Hemmings BA (2001) Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci 26:657–664

Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. Annu Rev Cell Dev Biol 16:521–555
Christine CW, Choi DW (1990) Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. J Neurosci 10:108–116
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
Colvin RA, Fontaine CP, Laskowski M, Thomas D (2003) $Zn2^{+}$ transporters and Zn^{2+} homeostasis in neurons. Eur J Pharmacol 479:171–185
Corbalan-Garcia S, Gomez-Fernandez JC (2006) Protein kinase C regulatory domains: the art of decoding many different signals in membranes. Biochim Biophys Acta 1761:633–654
Cornet V, Bichet D, Sandoz G, Marty I, Brocard J, Bourinet E, Mori Y, Villaz M, De Waard M (2002) Multiple determinants in voltage-dependent P/Q calcium channels control their retention in the endoplasmic reticulum. Eur J Neurosci 16:883–895
Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J (2004) B cell antigen receptor signaling 101. Mol Immunol 41:599–613
Fernandes G, Nair M, Onoe K, Tanaka T, Floyd R, Good RA (1979) Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice. Proc Natl Acad Sci USA 76:457–461
Fraker PJ, Caruso R, Kierszenbaum F (1982) Alteration of the immune and nutritional status of mice by synergy between zinc deficiency and infection with *Trypanosoma cruzi*. J Nutr 112:1224–1229
Frederickson CJ, Bush AI (2001) Synaptically released zinc: physiological functions and pathological effects. Biometals 14:353–366
Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6:449–462
Fukada T, Kambe T (2011) Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics 3:662–674
Fukada T, Civic N, Furuichi T, Shimoda S, Mishima K, Higashiyama H, Idaira Y, Asada Y, Kitamura H, Yamasaki S et al (2008) The zinc transporter SLC39A13/ZIP13 is required for connective tissue development; its involvement in BMP/TGF-beta signaling pathways. PLoS One 3:e3642
Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signaling in health and diseases: zinc signaling. J Biol Inorg Chem 16:1123–1134
Galli SJ, Tsai M, Piliponsky AM (2008) The development of allergic inflammation. Nature (Lond) 454:445–454
Gomperts BD, Tatham PER, Kramer IM (2002) Signal transduction. Academic, San Diego
Goode BL, Drubin DG, Barnes G (2000) Functional cooperation between the microtubule and actin cytoskeletons. Curr Opin Cell Biol 12:63–71
Gyulkhandanyan AV, Lee SC, Bikopoulos G, Dai F, Wheeler MB (2006) The Zn^{2+}-transporting pathways in pancreatic beta-cells: a role for the L-type voltage-gated Ca^{2+} channel. J Biol Chem 281:9361–9372
Haase H, Maret W (2005) Protein tyrosine phosphatases as targets of the combined insulinomimetic effects of zinc and oxidants. Biometals 18:333–338
Haase H, Rink L (2009) Functional significance of zinc-related signaling pathways in immune cells. Annu Rev Nutr 29:133–152
Haase H, Hebel S, Engelhardt G, Rink L (2006) Flow cytometric measurement of labile zinc in peripheral blood mononuclear cells. Anal Biochem 352:222–230
Haase H, Ober-Blobaum JL, Engelhardt G, Hebel S, Heit A, Heine H, Rink L (2008) Zinc signals are essential for lipopolysaccharide-induced signal transduction in monocytes. J Immunol 181:6491–6502
Harwood NE, Batista FD (2008) New insights into the early molecular events underlying B cell activation. Immunity 28:609–619
Harwood NE, Batista FD (2010) Early events in B cell activation. Annu Rev Immunol 28:185–210

Hashimoto A, Okada H, Jiang A, Kurosaki M, Greenberg S, Clark EA, Kurosaki T (1998) Involvement of guanosine triphosphatases and phospholipase C-gamma2 in extracellular signal-regulated kinase, c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinase activation by the B cell antigen receptor. J Exp Med 188:1287–1295

Hershfinkel M, Moran A, Grossman N, Sekler I (2001) A zinc-sensing receptor triggers the release of intracellular Ca^{2+} and regulates ion transport. Proc Natl Acad Sci USA 98:11749–11754

Hirano T, Murakami M, Fukada T, Nishida K, Yamasaki S, Suzuki T (2008) Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. Adv Immunol 97:149–176

Hirzel K, Muller U, Latal AT, Hulsmann S, Grudzinska J, Seeliger MW, Betz H, Laube B (2006) Hyperekplexia phenotype of glycine receptor alpha1 subunit mutant mice identifies Zn(2+) as an essential endogenous modulator of glycinergic neurotransmission. Neuron 52:679–690

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

Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin BH, Koseki H, Hirano T (2011) The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. PLoS One 6:e18059

Holst B, Egerod KL, Jin C, Petersen PS, Ostergaard MV, Hald J, Sprinkel AM, Storling J, Mandrup-Poulsen T, Holst JJ et al (2009) G protein-coupled receptor 39 deficiency is associated with pancreatic islet dysfunction. Endocrinology 150:2577–2585

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

Hou S, Vigeland LE, Zhang G, Xu R, Li M, Heinemann SH, Hoshi T (2010) Zn^{2+} activates large conductance Ca^{2+}-activated K^+ channel via an intracellular domain. J Biol Chem 285:6434–6442

Hu H, Bandell M, Petrus MJ, Zhu MX, Patapoutian A (2009) Zinc activates damage-sensing TRPA1 ion channels. Nat Chem Biol 5:183–190

Hubbard SR, Bishop WR, Kirschmeier P, George SJ, Cramer SP, Hendrickson WA (1991) Identification and characterization of zinc binding sites in protein kinase C. Science 254:1776–1779

Ibs KH, Rink L (2003) Zinc-altered immune function. J Nutr 133:1452S–1456S

Kabu K, Yamasaki S, Kamimura D, Ito Y, Hasegawa A, Sato E, Kitamura H, Nishida K, Hirano T (2006) Zinc is required for Fc-epsilon-RI-mediated mast cell activation. J Immunol 177:1296–1305

Kakurai M, Monteforte R, Suto H, Tsai M, Nakae S, Galli SJ (2006) Mast cell-derived tumor necrosis factor can promote nerve fiber elongation in the skin during contact hypersensitivity in mice. Am J Pathol 169:1713–1721

Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M (2004) Overview of mammalian zinc transporters. Cell Mol Life Sci 61:49–68

Kawakami T, Ando T, Kimura M, Wilson BS, Kawakami Y (2009) Mast cells in atopic dermatitis. Curr Opin Immunol 21:666–678

Keen CL, Gershwin ME (1990) Zinc deficiency and immune function. Annu Rev Nutr 10:415–431

Kikuchi K (2010) Design, synthesis and biological application of chemical probes for bio-imaging. Chem Soc Rev 39:2048–2053

Kitamura H, Morikawa H, Kamon H, Iguchi M, Hojyo S, Fukada T, Yamashita S, Kaisho T, Akira S, Murakami M, Hirano T (2006) Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nat Immunol 7:971–977

Klemm S, Gutermuth J, Hultner L, Sparwasser T, Behrendt H, Peschel C, Mak TW, Jakob T, Ruland J (2006) The Bcl10-Malt1 complex segregates Fc epsilon RI-mediated nuclear factor kappa B activation and cytokine production from mast cell degranulation. J Exp Med 203:337–347

Kurosaki T (2011) Regulation of BCR signaling. Mol Immunol 48:1287–1291

Lee JY, Cole TB, Palmiter RD, Suh SW, Koh JY (2002) 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

Legendre P, Westbrook GL (1990) The inhibition of single *N*-methyl-D-aspartate-activated channels by zinc ions on cultured rat neurones. J Physiol 429:429–449

Lengyel I, Fieuw-Makaroff S, Hall AL, Sim AT, Rostas JA, Dunkley PR (2000) Modulation of the phosphorylation and activity of calcium/calmodulin-dependent protein kinase II by zinc. J Neurochem 75:594–605

Levy S, Beharier O, Etzion Y, Mor M, Buzaglo L, Shaltiel L, Gheber LA, Kahn J, Muslin AJ, Katz A et al (2009) Molecular basis for zinc transporter 1 action as an endogenous inhibitor of L-type calcium channels. J Biol Chem 284:32434–32443

Li Y, Hough CJ, Suh SW, Sarvey JM, Frederickson CJ (2001) Rapid translocation of Zn(2+) from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation. J Neurophysiol 86:2597–2604

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr 29:153–176

Lopantsev V, Wenzel HJ, Cole TB, Palmiter RD, Schwartzkroin PA (2003) Lack of vesicular zinc in mossy fibers does not affect synaptic excitability of CA3 pyramidal cells in zinc transporter 3 knockout mice. Neuroscience 116:237–248

Lu YM, Taverna FA, Tu R, Ackerley CA, Wang YT, Roder J (2000) Endogenous Zn(2+) is required for the induction of long-term potentiation at rat hippocampal mossy fiber-CA3 synapses. Synapse 38:187–197

Mayer ML, Vyklicky L Jr (1989) The action of zinc on synaptic transmission and neuronal excitability in cultures of mouse hippocampus. J Physiol 415:351–365

Metz M, Grimbaldeston MA, Nakae S, Piliponsky AM, Tsai M, Galli SJ (2007) Mast cells in the promotion and limitation of chronic inflammation. Immunol Rev 217:304–328

Murakami M, Hirano T (2008) Intracellular zinc homeostasis and zinc signaling. Cancer Sci 99:1515–1522

Murakami K, Whiteley MK, Routtenberg A (1987) Regulation of protein kinase C activity by cooperative interaction of Zn^{2+} and Ca^{2+}. J Biol Chem 262:13902–13906

Nechushtan H, Leitges M, Cohen C, Kay G, Razin E (2000) Inhibition of degranulation and interleukin-6 production in mast cells derived from mice deficient in protein kinase C-beta. Blood 95:1752–1757

Nishida K, Yamasaki S, Ito Y, Kabu K, Hattori K, Tezuka T, Nishizumi H, Kitamura D, Goitsuka R, Geha RS et al (2005) FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. J Cell Biol 170:115–126

Nishida K, Hasegawa A, Nakae S, Oboki K, Saito H, Yamasaki S, Hirano T (2009) Zinc transporter Znt5/Slc30a5 is required for the mast cell-mediated delayed-type allergic reaction but not the immediate-type reaction. J Exp Med 206:1351–1364

Nishida K, Fukada T, Yamasaki S, Murakami M, Hirano T (2011) Zinc in allergy, autoimmune, and hard and connective tissue diseases. IOS Press, Netherlands

Oancea E, Teruel MN, Quest AF, Meyer T (1998) Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells. J Cell Biol 140:485–498

Palacios EH, Weiss A (2004) Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene 23:7990–8000

Palmiter RD (2004) Protection against zinc toxicity by metallothionein and zinc transporter 1. Proc Natl Acad Sci USA 101:4918–4923

Park JA, Koh JY (1999) Induction of an immediate early gene EGR-1 by zinc through extracellular signal-regulated kinase activation in cortical culture: its role in zinc-induced neuronal death. J Neurochem 73:450–456

Perry DK, Smyth MJ, Stennicke HR, Salvesen GS, Duriez P, Poirier GG, Hannun YA (1997) Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. J Biol Chem 272:18530–18533

Prasad AS (1991) Discovery of human zinc deficiency and studies in an experimental human model. Am J Clin Nutr 53:403–412

Prasad AS (1995) Zinc: an overview. Nutrition 11:93–99

Prost AL, Bloc A, Hussy N, Derand R, Vivaudou M (2004) Zinc is both an intracellular and extracellular regulator of KATP channel function. J Physiol 559:157–167

Qian J, Noebels JL (2005) Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse. J Physiol 566:747–758

Ramadan O, Qu Y, Wadgaonkar R, Baroudi G, Karnabi E, Chahine M, Boutjdir M (2009) Phosphorylation of the consensus sites of protein kinase A on alpha1D L-type calcium channel. J Biol Chem 284:5042–5049

Riccioni G, D'Orazio N (2005) The role of selenium, zinc and antioxidant vitamin supplementation in the treatment of bronchial asthma: adjuvant therapy or not? Expert Opin Invest Drugs 14:1145–1155

Richter M, Bonneau R, Girard MA, Beaulieu C, Larivee P (2003a) Zinc status modulates bronchopulmonary eosinophil infiltration in a murine model of allergic inflammation. Chest 123:446S

Richter M, Cantin AM, Beaulieu C, Cloutier A, Larivee P (2003b) Zinc chelators inhibit eotaxin, RANTES, and MCP-1 production in stimulated human airway epithelium and fibroblasts. Am J Physiol Lung Cell Mol Physiol 285:L719–L729

Rink L, Gabriel P (2000) Zinc and the immune system. Proc Nutr Soc 59:541–552

Schnapp BJ (2003) Trafficking of signaling modules by kinesin motors. J Cell Sci 116:2125–2135

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, Paoletti P, Bush AI, Sekler I (2009) Zinc in the physiology and pathology of the CNS. Nat Rev Neurosci 10:780–791

Sharir H, Zinger A, Nevo A, Sekler I, Hershfinkel M (2010) Zinc released from injured cells is acting via the Zn^{2+}-sensing receptor, ZnR, to trigger signaling leading to epithelial repair. J Biol Chem 285:26097–26106

Shumilina E, Lam RS, Wolbing F, Matzner N, Zemtsova IM, Sobiesiak M, Mahmud H, Sausbier U, Biedermann T, Ruth P et al (2008) Blunted IgE-mediated activation of mast cells in mice lacking the Ca^{2+}-activated K^+ channel KCa3.1. J Immunol 180:8040–8047

Smart TG, Hosie AM, Miller PS (2004) Zn^{2+} ions: modulators of excitatory and inhibitory synaptic activity. Neuroscientist 10:432–442

Smith AJ, Pfeiffer JR, Zhang J, Martinez AM, Griffiths GM, Wilson BS (2003) Microtubule-dependent transport of secretory vesicles in RBL-2H3 cells. Traffic 4:302–312

Suto H, Nakae S, Kakurai M, Sedgwick JD, Tsai M, Galli SJ (2006) Mast cell-associated TNF promotes dendritic cell migration. J Immunol 176:4102–4112

Suzuki T, Ishihara K, Migaki H, Matsuura W, Kohda A, Okumura K, Nagao M, Yamaguchi-Iwai Y, Kambe T (2005a) 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

Suzuki T, Ishihara K, Migaki H, Nagao M, Yamaguchi-Iwai Y, Kambe T (2005b) Two different zinc transport complexes of cation diffusion facilitator proteins localized in the secretory pathway operate to activate alkaline phosphatases in vertebrate cells. J Biol Chem 280:30956–30962

Tamaki M, Fujitani Y, Hara A, Uchida T, Tamura Y, Takeno K, Kawaguchi M, Watanabe T, Ogihara T, Fukunaka A et al (2013) The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. J Clin Invest 123:4513–4524

Taniguchi M, Fukunaka A, Hagihara M, Watanabe K, Kamino S, Kambe T, Enomoto S, Hiromura M (2013) Essential role of the zinc transporter ZIP9/SLC39A9 in regulating the activations of Akt and Erk in B-cell receptor signaling pathway in DT40 cells. PLoS One 8:e58022

Taylor KM, Vichova P, Jordan N, Hiscox S, Hendley R, Nicholson RI (2008) ZIP7-mediated intracellular zinc transport contributes to aberrant growth factor signaling in antihormone-resistant breast cancer cells. Endocrinology 149:4912–4920

Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P (2012) Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal 5:ra11

Telford WG, Fraker PJ (1995) Preferential induction of apoptosis in mouse CD4 + CD8+ alpha beta TCRloCD3 epsilon lo thymocytes by zinc. J Cell Physiol 164:259–270

Thambiayya K, Wasserloos KJ, Huang Z, Kagan VE, St. Croix CM, Pitt BR (2011) LPS-induced decrease in intracellular labile zinc, $[Zn]_i$, contributes to apoptosis in cultured sheep pulmonary artery endothelial cells. Am J Physiol Lung Cell Mol Physiol 300:L624–L632

Torres-Alanis O, Garza-Ocanas L, Pineyro-Lopez A (1995) Evaluation of urinary mercury excretion after administration of 2,3-dimercapto-1-propane sulfonic acid to occupationally exposed men. J Toxicol Clin Toxicol 33:717–720

Truong-Tran AQ, Ruffin RE, Zalewski PD (2000) Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines. Am J Physiol Lung Cell Mol Physiol 279:L1172–L1183

Ueno S, Tsukamoto M, Hirano T, Kikuchi K, Yamada MK, Nishiyama N, Nagano T, Matsuki N, Ikegaya Y (2002) Mossy fiber Zn^{2+} spillover modulates heterosynaptic *N*-methyl-D-aspartate receptor activity in hippocampal CA3 circuits. J Cell Biol 158:215–220

Vallee BL (1995) The function of metallothionein. Neurochem Int 27:23–33

Vallee BL, Auld DS (1993) Cocatalytic zinc motifs in enzyme catalysis. Proc Natl Acad Sci USA 90:2715–2718

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

Vennekens R, Olausson J, Meissner M, Bloch W, Mathar I, Philipp SE, Schmitz F, Weissgerber P, Nilius B, Flockerzi V, Freichel M (2007) Increased IgE-dependent mast cell activation and anaphylactic responses in mice lacking the calcium-activated nonselective cation channel TRPM4. Nat Immunol 8:312–320

Vig M, Kinet JP (2009) Calcium signaling in immune cells. Nat Immunol 10:21–27

Vogt K, Mellor J, Tong G, Nicoll R (2000) The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron 26:187–196

Weiss JH, Sensi SL (2000) Ca^{2+}-Zn^{2+} permeable AMPA or kainate receptors: possible key factors in selective neurodegeneration. Trends Neurosci 23:365–371

Xie X, Smart TG (1994) Modulation of long-term potentiation in rat hippocampal pyramidal neurons by zinc. Pflugers Arch 427:481–486

Xu TF, Wang XL, Yang JZ, Hu XY, Wu WF, Guo L, Kang LD, Zhang LY (2009) Overexpression of Zip-2 mRNA in the leukocytes of asthmatic infants. Pediatr Pulmonol 44:763–767

Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, Kurosaki T, Yamashita S, Tokunaga M, Nishida K, Hirano T (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645

Yamasaki S, Hasegawa A, Hojyo S, Ohashi W, Fukada T, Nishida K, Hirano T (2012) A novel role of the L-type calcium channel alpha1D subunit as a gatekeeper for intracellular zinc signaling: zinc wave. PLoS One 7:e39654

Yamashita S, Miyagi C, Fukada T, Kagara N, Che YS, Hirano T (2004) Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature (Lond) 429:298–302

Yasuda S, Miyazaki T, Munechika K, Yamashita M, Ikeda Y, Kamizono A (2007) Isolation of Zn^{2+} as an endogenous agonist of GPR39 from fetal bovine serum. J Recept Signal Transduct Res 27:235–246

Yu M, Lee WW, Tomar D, Pryshchep S, Czesnikiewicz-Guzik M, Lamar DL, Li G, Singh K, Tian L, Weyand CM, Goronzy JJ (2011) Regulation of T cell receptor signaling by activation-induced zinc influx. J Exp Med 208:775–785

Zalewski PD, Truong-Tran AQ, Grosser D, Jayaram L, Murgia C, Ruffin RE (2005) Zinc metabolism in airway epithelium and airway inflammation: basic mechanisms and clinical targets. A review. Pharmacol Ther 105:127–149

Chapter 6
The Zinc-Sensing Receptor, ZnR/GPR39: Signaling and Significance

Michal Hershfinkel

Abstract The structural role of Zn^{2+} in stabilizing numerous enzymes and zinc fingers of transcription factors has been well established for several decades. Therefore, the major effects of this metal ion on growth, development, and cognitive function were not surprising. The importance of cellular homeostasis of Zn^{2+} is underlined by the identification of more than 20 proteins involved in transport of Zn^{2+} into the cytoplasm and various organelles. In the past decade, evidence for a signaling role of Zn^{2+} is emerging, suggesting this ion acts as a first and second messenger. Thus, transient changes in extracellular and intracellular Zn^{2+} were monitored and Zn^{2+}-dependent regulation of multiple signaling pathways was shown. We have identified the function of ZnR, a Gq protein-coupled receptor that triggers the release of Ca^{2+} via the IP3 pathway. This ZnR-dependent signaling regulates cellular pathways involved in survival and proliferation, such as the MAP and PI3 kinase pathways, as well as modulation of ion transport systems such as the Na^+/H^+ exchanger or the K^+/Cl^- co-transporter. The GPR39 was then shown to mediate extracellular Zn^{2+}-dependent ZnR signaling. Here we discuss the major role of ZnR/GPR39 as the link between extracellular Zn^{2+} and intracellular signaling pathways regulating the physiological function of epithelial and neuronal cells. A major future challenge will be to identify the role of ZnR/GPR39 in diseases linked to Zn^{2+} dyshomeostasis.

Keywords GPCR • Zinc signaling • Zn-sensing receptor • ZnR/GPR39

6.1 Introduction

Zn^{2+} is an essential micronutrient that is required for proper development and function of the digestive, immune, nervous, secretory, and integumentary systems (Prasad 1995, 2008; Sandstead et al. 2000; Kambe et al. 2008; Kelleher et al. 2011; Roohani et al. 2013). The role of Zn^{2+} as a cofactor or structural element in numerous proteins, enzymes, and transcription factors has been established for

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several decades (Vallee and Falchuk 1993; Maret 2001b). More recent work has shown that changes in extracellular Zn^{2+} as well as intracellular Zn^{2+} affect cellular signaling (Hershfinkel 2006; Fukada et al. 2011; Maret 2001a). Changes in extracellular Zn^{2+} may result by its release from vesicles containing high concentrations of this ion that are present in the pancreas, brain, mammary gland, salivary glands, and digestive system (Frederickson et al. 1987, 1992; Frederickson and Danscher 1990; Ishii et al. 1999; Danscher and Stoltenberg 2003; McCormick et al. 2010). Zn^{2+} is packed into these vesicles by specific Zn^{2+} transporters from the ZnT family that transport this ion from the cytoplasm (Liuzzi and Cousins 2004; Eide 2006; Sekler et al. 2007). Changes in extracellular Zn^{2+} may also occur following the release of this ion from injured cells. For example, following injury of keratinocytic cells, endogenous Zn^{2+} is released and may act as a paracrine agonist to a Zn^{2+}-sensing receptor ZnR, leading to activation of intracellular signaling pathways associated with wound healing (Sharir et al. 2010). These transient changes are quickly reversed either by re-uptake of Zn^{2+} via an extensive transporter system consisting of Zip and ZnT proteins or by the buffering of free Zn^{2+} by proteins such as metallothioneins (Vasak 2005; Krezel and Maret 2006; Sekler et al. 2007; Fukada et al. 2011). The dramatic changes in the extracellular concentration of Zn^{2+} may be recruited for cellular signaling; thus, a mechanism for sensing the changes is required. Regulation of key cellular functions based on these changes may be implicated by the fact that Zn^{2+} has a major role in the physiological functions of virtually all organ systems. Indeed, at the cellular level Zn^{2+} triggers proliferation of cells, particularly epithelial cells. Its deficiency is associated with cell death and growth retardation, as well as impaired wound healing, and it is considered an important factor in various forms of diarrhea (Prasad 1995, 2008; MacDonald 2000; Walker and Black 2010; Sandstead 2012). Among the most studied roles of Zn^{2+} is its role as an essential micronutrient for cognitive functions: its deficiency results in neurological disorders and in neuronal death (Sandstead et al. 2000; Takeda 2001; Takeda et al. 2003; Sensi et al. 2011). Indeed, Zn^{2+} dyshomeostasis is implicated in neuronal loss during Alzheimer's disease, ischemia, and stroke, as well as epilepsy (Choi and Koh 1998; Weiss et al. 2000; Bush 2003; Stoltenberg et al. 2007; Sensi et al. 2011).

Numerous studies have shown that extracellular Zn^{2+} indirectly activates cell signaling via allosteric modulation. For example, Zn^{2+} interacts with all major neuronal membrane transporters: the dopamine transporter, NMDA, glycine and the GABA ionotropic receptors (Paoletti et al. 1997; Lynch et al. 1998; Han and Wu 1999; Hosie et al. 2003; Herin and Aizenman 2004). Regulation of the purinergic receptors and store-operated channel (SOC), which are found on most cell types and tissues, links between Zn^{2+} and the important second messenger, Ca^{2+} (Wildman et al. 1999; Acuna-Castillo et al. 2000; Gore et al. 2004). A role for intracellular and extracellular Zn^{2+} was suggested as a trigger of intracellular pathways that induce cell proliferation and survival. For example, extracellular Zn^{2+} upregulated the PI3 kinase pathway, leading to activation of AKT in fibroblasts (Kim et al. 2000). Another major pathway linked to cell proliferation is the mitogen-activated kinase (MAPK) pathway that was also activated by extracellular Zn^{2+}, which in turn,

induced expression of p21(CiP/WAF1) and cyclin D1 (Oh et al. 2002). It was suggested that intracellular Zn^{2+} induces transactivation of epidermal growth factor receptor (EGFR) by Src in airway epithelial cells (Wu et al. 2002, 2003, 2005). Although binding sites were found on intracellular kinases and membrane receptors, a direct and distinct mechanism linking extracellular Zn^{2+} to cellular signaling was unknown for many years. We hypothesized that such a mechanism exists and subsequently described the interaction of extracellular Zn^{2+} with a specific target, an extracellular Zn^{2+}-sensing receptor that we have functionally termed ZnR (Hershfinkel et al. 2001, 2007). We then showed that ZnR is the major mediator of Zn^{2+}-dependent intracellular signaling, as is discussed in the current review.

6.2 The ZnR/GPR39 Is Mediating Zn^{2+}-Dependent Signaling

6.2.1 Functional Identification of a Putative Zn^{2+}-Sensing Receptor, the ZnR

Because of the crosstalk between the ions and the central role of Ca^{2+} in regulating many of the signaling pathways known to be activated by Zn^{2+}, we asked if Zn^{2+}-dependent signaling triggers changes in intracellular Ca^{2+}. Indeed, application of extracellular Zn^{2+} triggered a rise in intracellular Ca^{2+} released from thapsigargin-sensitive stores via the IP3 pathway (Hershfinkel et al. 2001; Maret 2001a, b) (Fig. 6.1). Focusing on physiologically relevant tissues, we and others have shown Zn^{2+}-dependent Ca^{2+} release in colonocytes, keratinocytes, pancreatic cells, prostate cancer cells, and salivary gland cells, all epithelial cells with high proliferation rates (Azriel-Tamir et al. 2004; Sharir and Hershfinkel 2005; Dubi et al. 2008; Holst et al. 2009). Zn^{2+}-dependent activation of this pathway was also shown in neurons (Besser et al. 2009). Inhibitors of Gαq (Takasaki et al. 2004; Taniguchi et al. 2004), inositol 1,4,5-trisphosphate (IP3) receptor, and phospholipase C (PLC) attenuated this Zn^{2+}-dependent Ca^{2+} rise, indicating that the Ca^{2+} release is mediated by activation of a Gαq-coupled receptor (Hershfinkel et al. 2001; Azriel-Tamir et al. 2004). The Zn^{2+}-dependent Ca^{2+} rise was not attenuated by an inhibitor of tyrosine kinase, suggesting that the response it is not mediated by this family of receptors. The specificity of the receptor to Zn^{2+} was also shown, as the Ca^{2+} response was not triggered by other heavy metal ions tested, such as Mn^{2+}, Cu^{2+}, and Fe^{2+} (Hershfinkel et al. 2001). This finding was in agreement with another study, which also showed that cation-sensing receptors are not activated by Zn^{2+} (Riccardi 1999). The other well-described cation receptor is the Ca^{2+}-sensing receptor (CaSR) (Brown 2000; Brennan et al. 2013); despite the link between these ions, the Zn^{2+}-dependent Ca^{2+} increase was distinct from the activity of the CaSR as overexpression of CaSR did not produce an increase in Ca^{2+} following application of extracellular Zn^{2+} (Hershfinkel et al. 2001). Thus, our

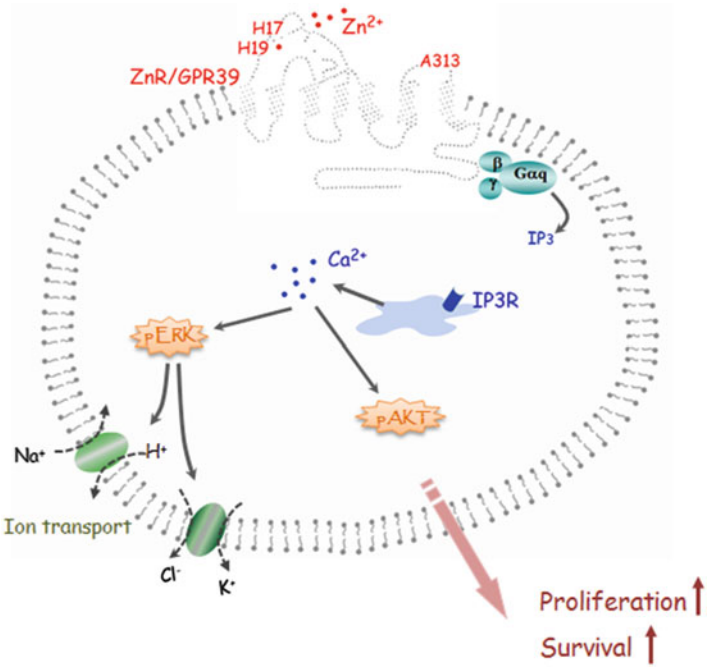

Fig. 6.1 A scheme for ZnR/GPR39 signaling. ZnR/GPR39, found on the cellular membrane, binds Zn^{2+} via the indicated residues. Asp313 serves as pH sensor on ZnR/GPR39. Shown are the cellular pathways activated by ZnR/GPR39 and the physiological roles of the receptor

experiments revealed a Gq-coupled, Zn^{2+}-sensing receptor, ZnR, linking changes in extracellular Zn^{2+} and downstream signal transduction pathways. Following the Ca^{2+} rise triggered by Zn^{2+}, enhanced activation of the mitogen-activated protein (MAP) kinase pathway and the PI3 kinase pathways was observed. These pathways are closely linked to enhanced cell survival and proliferation (Chappell et al. 2011), and thus the ZnR may be the mediator of the well-established role of Zn^{2+} (MacDonald 2000). In neurons, ZnR activation was also shown to activate the calcium calmodulin (CAM) kinase II pathway, an essential pathway related to cognitive function (Rongo 2002). Finally, ZnR was shown to have a paracrine role triggering signaling in neighboring cells via inducing release of ATP from salivary gland ductal cells, HSY (Sharir and Hershfinkel 2005). Using a coculture of ZnR-expressing HSY cells and vascular smooth muscle cells (VSMCs), which do not express a functional ZnR, it was shown that application of Zn^{2+} induced a Ca^{2+} rise in both HSY cells and VSMC. The Ca^{2+} rise in the VSMC was inhibited by the ATP scavenger apyrase. Such a paracrine effect of ZnR extends the effect of the receptor by enhancing the Ca^{2+} signals also in neighboring cells that do not express a ZnR. For example, this phenomenon may enhance wound-healing effects of Zn^{2+} by promoting the proliferation and migration of both fibroblasts and keratinocytes (Huang et al. 1999), although fibroblasts do not have a functional ZnR (Hershfinkel et al. 2001).

A hallmark of Gq protein-coupled receptor (GPCR) activity is a desensitization process that occurs after a brief exposure to their ligands in which the receptor undergoes internalization and degradation (McDonald and Lefkowitz 2001; Kohout and Lefkowitz 2003). There are striking differences in the degree of functional desensitization among GPCRs, mainly reflecting the ratio between the recycling versus degradation of the receptor. The degree of desensitization thus regulates the cellular signaling triggered by these receptors (Mohan et al. 2012). The fact that Zn^{2+} is not scavenged as are most ligands of GPCRs suggested that protection of cells from excessive Ca^{2+} signaling must occur via desensitization of the ZnR. Indeed, brief exposure of the epithelial and neuronal cells to subtoxic concentrations of Zn^{2+} led to profound and prolonged desensitization of the ZnR (Azriel-Tamir et al. 2004; Dubi et al. 2008; Besser et al. 2009). Remarkably, Zn^{2+} in the presence of citrate, an extracellular chelator of Zn^{2+}, also induced desensitization of the ZnR signaling (Dubi et al. 2008). This desensitization effectively inhibited Zn^{2+}-dependent signaling, suggesting that the ZnR is the major regulator of cellular Zn^{2+} signaling.

6.2.2 GPR39 Is the Molecular Moiety of ZnR

Although the functional and physiological relevance of the ZnR was established, the molecular moiety that mediates its function was unknown. The activity of the G protein-coupled receptor 39 (GPR39) was suggested to be modulated by zinc, but the physiological relevance of this ion was not addressed (Holst et al. 2004; Elling et al. 2006). GPR39 was an orphan receptor related to the gherlin/neurotensin receptor subfamily (McKee et al. 1997). GPR39 is expressed in all vertebrates; it is expressed in a range of tissues including adipose tissue, thyroid, and heart, but it is mostly expressed in the pancreas, GI tract, liver, and kidney (Jackson et al. 2006; Egerod et al. 2007). The receptor is composed of 435 amino acids, encoded via two exons (Popovics and Stewart 2011). A splice variant has been identified that is encoded by the first exon only and is assumed to be nonfunctional (Egerod et al. 2007). Although obestatin was originally suggested to activate GPR39 and have an opposing effect to ghrelin regulating food intake (Zhang et al. 2005), subsequent studies could not verify this activity (Lauwers et al. 2006; Holst et al. 2007). It was then established that Zn^{2+} activates GPR39 signaling, and this ion was also singled out as the trigger of GPR39 signaling within serum (Holst et al. 2007; Yasuda et al. 2007). GPR39 signaling is mediated through Gαq, which stimulates IP3 and downstream release of Ca^{2+}, but it was also shown to activate the cAMP response element that mediated transcription via MAPK activation. A major role for ZnR/GPR39 in enhancing epithelial cell proliferation and survival was indeed shown, as is discussed next. Interestingly, GPR39 activity was suggested to be neuroprotective in a hippocampal cell line (Dittmer et al. 2008), but the role of Zn^{2+} in this system was not described. GPR39 was suggested to have high constitutive activity as measured by the levels of IP_3 (Dittmer et al. 2008), which may be

insufficient to trigger downstream Ca^{2+} signaling or MAP and PI3 kinase phosphorylation, as activation of these pathways was not observed in cells endogenously expressing the receptor as well as in overexpressing HEK-293 cells (Cohen et al. 2012a, b). Another explanation, to the constitutive activity as well as the response to obestatin, may be that the activation of signaling likely resulted from residual Zn^{2+} often found in physiological solutions (Kay 2004; Maret 2012; Wilson et al. 2012).

Although cellular studies addressed the signaling triggered by the ZnR/GPR39, in vivo studies regarding the physiological role of GPR39 were done using GPR39 knockout (KO) mice (Moechars et al. 2006). These mice were characterized by accelerated gastric emptying and with a higher volume of gastric fluid secretion, which were not related to higher acid secretion; however, in this work a link to Zn^{2+} or its deficiency was not shown (Moechars et al. 2006). The relevance of this receptor to systems where Zn^{2+} deficiency plays a major role led us to hypothesize that GPR39 is the functionally described ZnR. We therefore established an siRNA silencing paradigm in which we silenced the expression of GPR39 and determined the signaling pathway activated by Zn^{2+} (Besser et al. 2009). In several experiments we have shown that ZnR activity is mediated by GPR39 in keratinocytes, colonocytes, salivary gland ductal cells, and prostate cancer cells as well as in neurons (Besser et al. 2009; Sharir et al. 2010; Cohen et al. 2012a, b; Asraf et al. 2014). In agreement, we monitored loss of ZnR-dependent signaling in native colon tissue and hippocampal CA3 neurons (Chorin et al. 2011; Cohen et al. 2012a, b). Thus, we conclude that ZnR and GPR39 are one and the same receptor, termed ZnR/GPR39.

6.2.3 Regulation of ZnR/GPR39

The Zn^{2+}-binding site on GPR39 consists of three residues: His 17, His19, and Asp313 (Storjohann et al. 2008). The Asp residue was suggested to act as a tethered inverse agonist that is diverted upon binding of Zn^{2+} to trigger signaling. The physiologically relevant PK_a of the residues acting as the Zn^{2+}-binding site raised the hypothesis that ZnR/GPR39 may be regulated by changes in pH (Cohen et al. 2012a, b). Epithelial cells that express ZnR/GPR39 may face changes in extracellular pH within the digestive system lumen or in the epidermis during physiological activity, and also in the brain, most likely during pathological conditions (Perdikis et al. 1998; Sharma et al. 2005; Holzer 2009, 2011; Sandoval et al. 2011). Indeed, the colonocytic ZnR/GPR39-dependent Ca^{2+} responses are maximal at pH 7.4 but are completely abolished at pH 6.5. ZnR/GPR39-dependent activation of MAP or PI3 kinases was abolished at an acidic extracellular pH of 6.5 (Cohen et al. 2012a, b). Surprisingly, the histidine residues forming the Zn^{2+}-binding site, His17 or His19, or other extracellular-facing histidines, did not affect the pH dependence of ZnR/GPR39. In contrast, replacing Asp313 to alanine

resulted in loss of the pH dependence of the ZnR/GPR39 response, as similar Ca^{2+} responses were triggered by Zn^{2+} at pH 7.4 or 6.5. Importantly, although substitution of Asp313 to Ala abolished the pH dependence, substitution to His or Glu residues restored pH sensitivity of the receptor. Thus, Asp313 is an essential residue of the pH sensor of ZnR/GPR39 that is tuned to sense physiologically relevant changes in extracellular pH regulating its activity (Cohen et al. 2012a, b).

The important role of heterodimerization of GPCRs that diversifies the physiological response of these receptors to their ligands (Gomes et al. 2004; Albizu et al. 2006) raised the question whether ZnR/GPR39 also interacts with other GPCRs. Notably, only two ion-sensing receptors were described thus far, the Ca^{2+} sensing receptor, CaSR (Brown 2000; Chattopadhyay et al. 1997) and the ZnR/GPR39. Both the ZnR/GPR39 and the CaSR exhibit similarity in the signaling pathway, which they activate via a Gq-dependent pathway. However the CaSR is related to GPCR family-C receptors and the ZnR/GPR39 is a family-A member. Importantly, Zn^{2+}-dependent activity does not require Ca^{2+} per se and is not directly mediated by the CaSR, as was shown using a dominant negative as well as siRNA. Moreover, the CaSR is not activated by Zn^{2+} although other divalent ions may activate this receptor. Nevertheless, extracellular Ca^{2+} alters the apparent cooperativity and affinity of ZnR/GPR39 to Zn^{2+} (Sharir and Hershfinkel 2005). Similar to the effect of Ca^{2+}, spermine, a CaSR ligand, synergistically increased the cellular response when applied with Zn^{2+}, although it did not activate the ZnR/GPR39 itself. In contrast, silencing of the CaSR downregulated the response to Zn^{2+}, suggesting that direct interaction between the receptors regulates the signaling triggered by ZnR/GPR39. Direct interaction between the CaSR and ZnR/GPR39 was indeed monitored using coimmunoprecipitation (Asraf et al. 2014). Changes in CaSR surface expression occur following exposure to its ligand (Grant et al. 2011). Thus, changes in CaSR localization induced by spermine or Ca^{2+} may have affected surface expression of ZnR/GPR39, thereby enhancing the Zn^{2+}-dependent response.

6.3 Physiological Aspects of ZnR/GPR39 Signaling

6.3.1 The Digestive System

Zinc is essential for digestive system function, and its deficiency is manifested in severe diarrhea and pathogenesis of ulcerative colon disease (Luk et al. 2002; Maret and Sandstead 2006; Walker and Black 2010). The World Health Organization (WHO) suggests zinc supplementation for effective treatment of diarrhea, and Zn^{2+} has been also suggested to reduce the severity and prolong the relapse of episodes of inflammatory ulcerative disease, such as Crohn's disease and colitis (Krasovec and Frenk 1996; Sturniolo et al. 2001; Luk et al. 2002; El-Tawil 2003; Walker and Black 2010; Alam et al. 2011). The general role of Zn^{2+} in preserving the digestive

epithelium in human diseases is underscored by the following: (1) Zn^{2+} supplementation dramatically reduces diarrhea-related symptoms and mortality (Scrimgeour and Lukaski 2008; Walker and Black 2010), and (2) Zn^{2+} reduces the permeability of the gut and enhances remission in ulcerative diseases (Sturniolo et al. 2001). Zn^{2+} plays a major role in enhancing proliferation and survival of colon epithelial (colonocytes) cell cultures (Hershfinkel 2006; Cohen et al. 2012a, b) as well as differentiation of colonocytes and barrier formation (Glover et al. 2003, 2004; Finamore et al. 2008; Geiser et al. 2012). Zinc deficiency, dietary or genetically induced by loss of the transporter that is responsible for absorption of Zn^{2+}, impairs barrier function and increases permeability and cell death, and its supplementation can reverse these processes (Hoque and Binder 2006; Finamore et al. 2008; Geiser et al. 2013).

In colonocytes, ZnR/GPR39 mediates Zn^{2+}-dependent activation of the MAP kinase and the PI3 kinase pathways that are essential for cell proliferation and survival (Azriel-Tamir et al. 2004). Silencing of ZnR/GPR39 in vivo, GPR39 KO, or in vitro using siRNA, as well as functional downregulation of its activity via desensitization by Zn^{2+}, is followed by inhibition of the Zn^{2+}-dependent Ca^{2+} rise and phosphorylation of ERK1/2, indicating that the ZnR is a principal link between extracellular Zn^{2+} and ERK1/2. Activation of ZnR/GPR39 signaling pathway was shown to upregulate the Na^{+}/H^{+} exchanger (NHE) and enhance the recovery from acidic pH in colon cells and in native tissue (Azriel-Tamir et al. 2004; Cohen et al. 2012a, b). It should be noted that colonocytes are chronically exposed to short-chain fatty acids, which induce acid load on these cells (Busche et al. 1997; Topping and Clifton 2001; Tang et al. 2011). Thus, ZnR-dependent upregulation of NHE may be an effective trigger to enhance pHi recovery in these cells.

Among the short-chain fatty acids found in the colon, butyrate, produced by bacterial fermentation in the colon, is found at high concentrations. Butyrate is essential for colonocyte nutrition but imposes an acidic stress that may lead to apoptosis of colonocytes and has been implicated in ulcerative colon diseases (Bordonaro et al. 2008; Scharlau et al. 2009; Yu et al. 2010; Zhang et al. 2010). Acidification of the colonocytes by application of extracellular Zn^{2+} before butyrate treatment significantly enhanced recovery of the colonocytes from the acid load produced by butyrate (Cohen et al. 2012a, b). Furthermore, short exposure of colonocytes to concentrations of Zn^{2+} sufficient to activate but not desensitize the ZnR/GPR39, or to induce changes in intracellular Zn^{2+}, attenuated butyrate-induced cell death (Cohen et al. 2012a, b). This effect was not mediated by regulation of intracellular pH, because the inhibition of the Na^{+}/H^{+} exchanger did not alter colon cell survival. Moreover, molecular silencing of ZnR/GPR39 or desensitization of the receptor reversed the protective effect of Zn^{2+}. Similarly, inhibition of the MAP and PI3 kinase pathways that are activated by Zn^{2+} via ZnR/GPR39 (Azriel-Tamir et al. 2004) also reversed the protective effect of Zn^{2+}. Thus, a clear role for ZnR/GPR39 is enhancing cell survival via upregulation of intracellular signaling. A candidate for mediating these effects of ZnR/GPR39 is the pro-survival glycoprotein, clusterin (also known as apolipoprotein J) (Pajak and Orzechowski 2006; Shannan et al. 2006; Djeu and Wei 2009; Mazzarelli

et al. 2009), which is upregulated by intracellular Ca^{2+} signaling such as is triggered by the ZnR/GPR39. Activation of the ZnR/GPR39 signaling pathway synergistically with butyrate treatment led to enhancement of the expression of the secreted isoform of clusterin (sCLU). Interestingly, ZnR/GPR39-dependent upregulation of sCLU expression required activation of the PI3 kinase pathway, further suggesting a survival role for the ZnR/GPR39-dependent pathway (Cohen et al. 2012a, b). The level of expression, moreover, was enhanced further by application of Zn^{2+} and butyrate, suggesting a synergistic mechanism. Using siRNA silencing of clusterin, the role of this pathway was shown to be essential for the Zn^{2+}-dependent prevention of butyrate-induced cell death (Cohen et al. 2012a, b). As butyrate was shown to induce colon cancer cell apoptosis, this may suggest a role for ZnR/GPR39 in the etiology of colon cancer that is yet to be addressed. A role for GPR39 in enhancing human esophageal squamous cell carcinoma was suggested, but this was not related to activation of the receptor by Zn^{2+} (Xie et al. 2011).

6.3.2 The Prostate

The highest concentration of Zn^{2+} in soft tissues is monitored in the prostate where millimolar concentrations were documented. Notably, dramatic changes in extracellular Zn^{2+}, resulting in a tenfold decrease in Zn^{2+} and its binding protein citrate (Costello et al. 1999; Huang et al. 2006), are observed during tumorigenesis in this organ. We have shown that an androgen-independent prostate cancer cell line, PC-3, mediates Zn^{2+}-dependent intracellular Ca^{2+} signals via ZnR/GPR39. The intracellular Ca^{2+} release mediated by ZnR/GPR39 subsequently triggers activation of MAP and PI3 kinases shown to enhance prostate cell proliferation and survival (Papatsoris et al. 2007; Agoulnik et al. 2011; Bartholomeusz and Gonzalez-Angulo 2012). The PI3K pathway is particularly prominent in prostate cancer, where constitutively activated PI3K has been correlated with severity of the tumor (Arcaro and Guerreiro 2007). Surprisingly, ZnR/GPR39 expression was shown to upregulate not only activation but also the total AKT expression level, which is associated with a more malignant phenotype of adrenal carcinomas (Fassnacht et al. 2005). This mechanism also upregulated the phosphorylation of this kinase, as basal phosphorylated AKT level measured in control cells treated with EDTA was higher than in the siGPR39 cells.

Intracellular Ca^{2+} changes may also regulate the calcium-binding proteins S100A that enable cell migration and invasion and are specifically linked to enhanced prostate cancer growth (Hermani et al. 2005, 2006; Grebhardt et al. 2014). Indeed, extracellular Zn^{2+} treatment, at concentrations that activate ZnR/GPR39, increased the expression of S100A4 protein in PC3 cells. Similarly, a decrease in S100A4 expression was monitored in siGPR39 cells. The S100A4 enhanced PC3 cell proliferation and invasiveness via induction of the metalloprotease MMP-9 (Joiner et al. 2012).

Thus, ZnR/GPR39 is a mediator of Zn^{2+}-dependent activation of signaling pathways that are all major pathways enhancing prostate cancer malignancy. An apparent paradox may be suggested by the fact the ZnR/GPR39 signaling is largely associated with enhanced cell growth and survival whereas a profound reduction in Zn^{2+} is seen during prostate cancer. Interestingly ZnR/GPR39 is completely desensitized by high Zn^{2+} concentrations such as found in the normal prostate (Costello and Franklin 2000; Franklin et al. 2005; Singh et al. 2006; Franklin and Costello 2007, even in the presence of citrate. Such desensitization renders ZnR/GPR39 quiescent in the nonneoplastic prostate. In contrast, reduced Zn^{2+} concentrations in the neoplastic prostate may allow expression of ZnR/GPR39, and thus Zn^{2+}-dependent signaling may be activated, leading to enhanced cell growth. Release of Zn^{2+} from cells following tissue destruction, such as seen for the skin cells, may activate ZnR/GPR39 signaling and enhance cell proliferation and survival. Thus, we suggest that ZnR/GPR39 may be a major target for reducing prostate cancer cells growth.

6.3.3 Keratinocytes of the Skin

High concentrations of Zn^{2+} accumulate in the intracellular and extracellular matrix of the skin, these are particularly high following injury (Andrews and Gallagher-Allred 1999; Nitzan et al. 2004; Lansdown et al. 2007). Severe zinc deficiency in a genetic disorder, acrodermatitis enteropathica, resulting from dysfunction of the zinc transporter Zip4, is manifested by skin lesions (Andrews 2008). A similar phenotype was recently described in a case of neonatal Zn^{2+} deficiency caused by a mutation in the ZnT-2 transporter in the mammary gland of the lactating mother (Lasry et al. 2012). Thus, dietary or genetic Zn^{2+} deficiency is manifested by severe skin lesions and impaired wound healing that can be reversed by Zn^{2+} supplementation (Jensen et al. 2008; Takahashi et al. 2008). For many years it has been accepted that topical addition of zinc, in ointments or bandages, stimulates wound healing and the re-epithelialization process (Lansdown 1996; Barceloux 1999; Schwartz et al. 2005; Lansdown et al. 2007). Although an antimicrobial effect was suggested as a role for Zn^{2+} during injury, subsequent studies identified Zn^{2+}-dependent signaling that is linked to enhanced proliferation and migration also in keratinocytes. Extracellular Zn^{2+}, at the concentrations found in the epidermis and released during injury, triggers Ca^{2+} release from thapsigargin-sensitive stores (Sharir et al. 2010). The response to Zn^{2+} is mediated through a Gαq-coupled receptor, ZnR/GPR39, which activates PLC and the IP_3 pathway and the downstream MAP kinase. Analysis of the Zn^{2+}-dependent Ca^{2+} response indicated that the keratinocytic-ZnR has high affinity and selectivity for Zn^{2+}. ZnR/GPR39-dependent signaling induces upregulation of the activity of the Na^+/H^+ exchanger NHE1 and thereby enhances the recovery of intracellular pH from acid load. The enhanced activity of NHE1 also forms a local extracellular microenvironment with acidic pH that strengthens the epithelial permeability barrier (Hachem et al. 2005;

Stuwe et al. 2007; Stock et al. 2008). Thus, ZnR/GPR39 activation may regulate the formation of the permeability barrier and thereby enhance the antiinflammatory effects of Zn^{2+}. The physiological significance of the keratinocytic-ZnR/GPR39 activity is further demonstrated by the fact that ZnR/GPR39 is essential for keratinocyte proliferation and migration, as the activation of ZnR/GPR39 enhanced the rate of scratch closure and silencing of the receptor or its signaling pathway reversed the effects of Zn^{2+} (Sharir et al. 2010). Importantly, Zn/GPR39, inhibition of the Zn^{2+}-induced NHE1 upregulation, also resulted in attenuated migration and scratch closure. Interestingly, although in colonocytes ZnR/GPR39 regulation of NHE did not affect cell survival, albeit it enhanced the recovery from acid load, in keratinocytes NHE activation enhanced recovery from acid load and enhanced cell proliferation. Altogether, it has been shown that ZnR/GPR39 is mediating the beneficial effects of Zn^{2+} on wound healing, and thus generation of agonists that will activate, but will not desensitize, this receptor may provide novel and effective drugs to accelerate wound healing.

6.3.4 The Pancreas

Zn^{2+} is packed into the insulin-containing vesicles by a specific Zn^{2+} transporter (ZnT8) and is released together with this hormone (Chimienti et al. 2004, 2006; Seve et al. 2004; Wenzlau et al. 2007; Gyulkhandanyan et al. 2008). It was first suggested that Zn^{2+} has a structural stabilizing function to the insulin hexamers; however, its concentration within the vesicles is much higher than required for this function and may suggest signaling activity mediated by this ion. Furthermore, Zn^{2+} release from pancreatic cells was monitored following glucose stimulation (Gee et al. 2002; Qian et al. 2003). Indeed, ZnR/GPR39 expression was identified in pancreatic β cells and within the duct epithelium (Holst et al. 2009). GPR39 expression was decreased in cells differentiated toward the exocrine phenotype but increased in the cells programmed to the endocrine phenotype (Tremblay et al. 2009); this suggests that similar to the autocrine effect of insulin itself, binding to insulin receptors on β cells, Zn^{2+} co-released with the hormone also has a specific target on the cells. The role of ZnR/GPR39 on the ductal cells has not been addressed, yet previous studies on salivary ductal epithelium indicated that ZnR/GPR39 activation in these cells triggers release of ATP (Sharir and Hershfinkel 2005), likely inducing a paracrine response in neighboring cells. Smooth muscle cells that do not express a functional ZnR/GPR39, for example, may be a target for the ATP release following ZnR/GPR39 activation in ductal epithelial cells; this signal may regulate muscle contraction and release of secretagogues through the duct.

In the initial studies describing GPR39 KO mice, no effect on metabolic function was observed. Further studies of older animals found impaired glucose tolerance and altered glucose-induced insulin secretion from isolated islets from GPR39 KO mice (Moechars et al. 2006). Interestingly, the phenotype of the GPR39 KO mice

was revealed under conditions of "increased demand" such as age-dependent or diet-induced insulin resistance.

Although the morphology of the pancreatic islets in GPR39 KO mice was normal, the expression of key regulators of islet development was decreased. The transcription factor HNF-1α was also downregulated, and in agreement with other studies, this showed a gender difference in the phenotype, leading to more severe glucose intolerance in female mice (Holst et al. 2009). Among the pathways regulated by the ZnR/GPR39, the NFAT was shown to be effectively increased in HEK293 cells transfected with GPR39. This pathway was shown to be an important regulator for the expression of genes that control β-cell differentiation, proliferation, and adaptive islet responses in vivo. Thus, a possible explanation for the effects of Zn^{2+} would be via ZnR/GPR39-dependent regulation of this pathway and thereby serve as a therapeutic target for diabetes (Popovics and Stewart 2011).

6.3.5 The Brain

One of the most notable pools of free Zn^{2+} ions is found in neurons (Frederickson and Moncrieff 1994; Frederickson 2003b). The development of fluorescent tools enabled the study of this specific pool of Zn^{2+} (Burdette et al. 2001, 2003; Frederickson 2003a; Pan et al. 2011). Extensive research indicated that Zn^{2+} bound to intracellular proteins can be liberated into the cytoplasm during oxidative or nitrosative neuronal injury, leading to cell death (Aizenman et al. 2000; Zhang et al. 2004; Zhang et al. 2006; Redman et al. 2009; McLaughlin et al. 2001), but the role of the second pool of synaptic free Zn^{2+} was less understood. Synaptic Zn^{2+} is transported into vesicles by the Zn^{2+} transporter 3 (ZnT3) and is released into the synaptic cleft during neuronal activity in a Ca^{2+}-dependent manner (Qian and Noebels 2005, 2006; Frederickson et al. 2006). Using ZnT3 KO mice, it was shown that the synaptic Zn^{2+} regulates neuronal excitability and can strongly influence seizure activity (Vogt et al. 2000; Smart et al. 2004; Sensi et al. 2011). Similarly, removal of synaptic Zn^{2+} by dietary means or chemical chelation also led to enhanced susceptibility to epileptic seizures (Cole et al. 2000; Takeda et al. 2003; Blasco-Ibáñez et al. 2004; Takeda et al. 2005). Interestingly Zn^{2+} deficiency was also linked to some forms of human epilepsy (Goldberg and Sheehy 1982; Ganesh and Janakiraman 2008; Seven et al. 2013).

Synaptic Zn^{2+} is tightly packed in the mossy fiber terminals of the hippocampus, facing the CA3 neurons (Frederickson and Danscher 1990; Frederickson et al. 1992). ZnR/GPR39 expression in these postsynaptic cells induces metabotropic Ca^{2+} signaling following Zn^{2+} release (Besser et al. 2009). Blockade of synaptic transmission by tetrodotoxin or CdCl inhibited the ZnR-mediated Ca^{2+} rises. The Zn^{2+}-dependent Ca^{2+} signaling then induced phosphorylation of extracellular-regulated kinase (ERK1/2) and Ca^{2+}/calmodulin kinase II (CAMK). Importantly, although the metabotropic response may be triggered by other released factors, application of a nonpermeable Zn^{2+} chelator, CaEDTA, largely abolished

the response. Moreover, the response was also diminished in ZnT3 knockdown mice lacking synaptic Zn^{2+} (Besser et al. 2009).

KCC2 is the major outward transporter of chloride in neurons, necessary and sufficient for creating a chloride equilibrium potential that is negative to the resting membrane voltage (Lu et al. 1999; Lee et al. 2005), thereby rendering $GABA_A$- and glycine-mediated synaptic potentials inhibitory (Farrant and Kaila 2007; Viitanen et al. 2010). Increases in KCC2 activity may thus enhance the inhibitory actions of GABA and glycine (Zhu et al. 2005, 2008; Huberfeld et al. 2007; Khirug et al. 2010). To address a physiological role for ZnR/GPR39 activation that may link it to the effects of Zn^{2+} in seizure, it was suggested that ZnR/GPR39 may regulate the K^+/Cl^- cotransporter, KCC2, that is strongly associated with inhibitory activity (Stein et al. 2004; Lee et al. 2005; Blaesse et al. 2006; Khirug et al. 2010). Activation of ZnR/GPR39 by synaptic Zn^{2+} enhanced KCC2 activity and surface expression, thereby inducing a hyperpolarizing shift in $GABA_A$ reversal potential (Chorin et al. 2011). Direct phosphorylation of KCC2 regulates its activity (Strange et al. 2000; Rinehart et al. 2009; Kahle et al. 2010) and may also lead to enhanced KCC2 surface expression (Lee et al. 2007, 2010; Wake et al. 2007; Watanabe et al. 2009). The upregulation of KCC2 activity by ZnR/GPR39 was mediated by phosphorylation of ERK1/2 and induced higher surface expression of the cotransporter (Chorin et al. 2011). The insertion of KCC2 into the plasma membrane was dependent on the SNARE proteins that are associated with exocytotic processes (Saadi et al. 2012). By identifying a physiological role for ZnR/GPR39 in regulating the neuronal Cl^- gradient via KCC2 activity, the relevance of this pathway to the anticonvulsive actions of Zn^{2+} was established. Indeed, the fact that synaptic Zn^{2+} can profoundly influence inhibitory drive is concordant with the enhanced susceptibility to kainate-triggered seizures present in ZnT3 KO mice (Buhl et al. 1996; Cole et al. 2000; Elsas et al. 2009). The release of vesicular Zn^{2+} during application of kainate (Frederickson et al. 1988a) could trigger the activity of ZnR/GPR39 and enhance KCC2 activity, thus limiting kainate-triggered epileptic activity by rendering the $GABA_A$ receptor inhibitory drive more effective.

Another brain region that contains a large pool of synaptic Zn^{2+} is the dorsal cochlear nucleus (DCN), which is part of the auditory brainstem (Frederickson et al. 1988a, b). Similar to the hippocampus, ZnR/GPR39-dependent Ca^{2+} signaling was also monitored in this brain region (Perez-Rosello et al. 2013). Following its activation, ZnR/GPR39 induced retrograde endocannabinoid signaling; this, in turn, affected the probability of release from glutamatergic terminals. Although a single action potential could induce synaptic Zn^{2+} release (Qian and Noebels 2005; Pan et al. 2011), the release of endocannabinoids required a train of presynaptic action potentials, and a similar train was also required to trigger ZnR/GPR39 activation in the hippocampus (Besser et al. 2009; Perez-Rosello et al. 2013). These results support a physiologically interesting role for the synaptic Zn^{2+} and the ZnR/GPR39 that acts as a sensor for increased release of excitatory neurotransmitter, activates negative feedback, and enhances the inhibitory drive.

6.4 Conclusions

The identification of the ZnR as a functional GPCR that mediates Zn^{2+}-dependent signaling in epithelial and neuronal cells provided the basis for understanding of many physiological roles of this ion. The subsequent link to GPR39 now establishes the required molecular machinery to address the mechanistic aspects of ZnR and Zn^{2+} signaling. Although Zn^{2+} was described as an important structural element for many years, the identification of free Zn^{2+} ions in many systems suggested that this ion has a signaling role as well. Identification of ZnR/GPR39 as a distinct target for Zn^{2+} implicates this ion as an important ligand mediating physiological signaling. The importance of Zn^{2+} signaling in numerous diseases and the major role of the ZnR/GPR39 in mediating the effects of this ion, as described in this review, presents this receptor as a key candidate for novel therapeutic approaches. Moreover, ZnR is a member of the Gq protein-coupled receptor family (GPCR), which is at present a major focus of the pharmaceutical industry (Custodi et al. 2012; Wootten et al. 2013). Using high-throughput systems for identification of novel and specific agonists may elucidate a whole new battery of therapeutic tools to efficiently modulate cellular signaling linked to disease.

References

Acuna-Castillo C, Morales B, Huidobro-Toro JP (2000) Zinc and copper modulate differentially the P2X4 receptor. J Neurochem 74(4):1529–1537

Agoulnik IU, Hodgson MC, Bowden WA, Ittmann MM (2011) INPP4B: the new kid on the PI3K block. Oncotarget 2(4):321–328

Aizenman E, Stout AK, Hartnett KA, Dineley KE, McLaughlin B, Reynolds IJ (2000) Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. J Neurochem. 75(5):1878–1888

Alam DS, Yunus M, El Arifeen S, Chowdury HR, Larson CP, Sack DA, Baqui AH, Black RE (2011) Zinc treatment for 5 or 10 days is equally efficacious in preventing diarrhea in the subsequent 3 months among Bangladeshi children. J Nutr 141(2):312–315

Albizu L, Balestre MN, Breton C, Pin JP, Manning M, Mouillac B, Barberis C, Durroux T (2006) Probing the existence of G protein-coupled receptor dimers by positive and negative ligand-dependent cooperative binding. Mol Pharmacol 70(5):1783–1791

Andrews GK (2008) Regulation and function of Zip4, the acrodermatitis enteropathica gene. Biochem Soc Trans 36(pt 6):1242–1246

Andrews M, Gallagher-Allred C (1999) The role of zinc in wound healing. Adv Wound Care 12 (3):137–138

Arcaro A, Guerreiro AS (2007) The phosphoinositide 3-kinase pathway in human cancer: genetic alterations and therapeutic implications. Curr Genomics 8(5):271–306

Asraf H, Salomon S, Nevo A, Sekler I, Mayer D, Hershfinkel M (2014) The ZnR/GPR39 interacts with the CaSR to enhance signaling in prostate and salivary epithelia. J Cell Physiol 229:868–877

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(50):51804–51816

Barceloux DG (1999) Zinc. J Toxicol Clin Toxicol 37(2):279–292
Bartholomeusz C, Gonzalez-Angulo AM (2012) Targeting the PI3K signaling pathway in cancer therapy. Expert Opin Ther Targets 16(1):121–130
Besser L, Chorin E, Sekler I, Silverman WF, Atkin S, Russell JT, Hershfinkel M (2009) Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus. J Neurosci 29(9):2890–2901
Blaesse P, Guillemin I, Schindler J, Schweizer M, Delpire E, Khiroug L, Friauf E, Nothwang HG (2006) Oligomerization of KCC2 correlates with development of inhibitory neurotransmission. J Neurosci 26(41):10407–10419
Blasco-Ibáñez JM, Poza-Aznar J, Crespo C, Marqués-Marí AI, Gracia-Llanes FJ, Martínez-Guijarro FJ (2004) Chelation of synaptic zinc induces overexcitation in the hilar mossy cells of the rat hippocampus. Neurosci Lett. 355(1-2):101–104
Bordonaro M, Lazarova DL, Sartorelli AC (2008) Butyrate and Wnt signaling: a possible solution to the puzzle of dietary fiber and colon cancer risk? Cell Cycle 7(9):1178–1183
Brennan SC, Thiem U, Roth S, Aggarwal A, Fetahu IS, Tennakoon S, Gomes AR, Brandi ML, Bruggeman F, Mentaverri R, Riccardi D, Kallay E (2013) Calcium sensing receptor signalling in physiology and cancer. Biochim Biophys Acta 1833:1732–1744
Brown EM (2000) The extracellular Ca^{2+} sensing receptor: central mediator of systemic calcium homeostasis. Annu Rev Nutr 20:507–533
Buhl EH, Otis TS, Mody I (1996) Zinc-induced collapse of augmented inhibition by GABA in a temporal lobe epilepsy model. Science 271(5247):369–373
Burdette SC, Walkup GK, Spingler B, Tsien RY, Lippard SJ (2001) Fluorescent sensors for Zn(2+) based on a fluorescein platform: synthesis, properties and intracellular distribution. J Am Chem Soc 123(32):7831–7841
Burdette SC, Frederickson CJ, Bu W, Lippard SJ (2003) ZP4, an improved neuronal Zn^{2+} sensor of the Zinpyr family. J Am Chem Soc 125(7):1778–1787
Busche R, Bartels J, Genz AK, von Engelhardt W (1997) Effect of SCFA on intracellular pH and intracellular pH regulation of guinea-pig caecal and colonic enterocytes and of HT29-19a monolayers. Comp Biochem Physiol A Physiol 118(2):395–398
Bush AI (2003) Copper, zinc, and the metallobiology of Alzheimer disease. Alzheimer Dis Assoc Disord 17(3):147–150
Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, Basecke J, Stivala F, Donia M, Fagone P, Malaponte G, Mazzarino MC, Nicoletti F, Libra M, Maksimovic-Ivanic D, Mijatovic S, Montalto G, Cervello M, Laidler P, Milella M, Tafuri A, Bonati A, Evangelisti C, Cocco L, Martelli AM, McCubrey JA (2011) Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget 2 (3):135–164
Chattopadhyay N, Vassilev PM, Brown EM. (1997) Calcium-sensing receptor: roles in and beyond systemic calcium homeostasis. Biol Chem. 378(8):759–68
Chimienti F, Devergnas S, Favier A, Seve M (2004) Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. Diabetes 53 (9):2330–2337
Chimienti F, Devergnas S, Pattou F, Schuit F, Garcia-Cuenca R, Vandewalle B, Kerr-Conte J, Van Lommel L, Grunwald D, Favier A, Seve M (2006) In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. J Cell Sci 119 (pt 20):4199–4206
Choi DW, Koh JY (1998) Zinc and brain injury. Annu Rev Neurosci 21:347–375
Chorin E, Vinograd O, Fleidervish I, Gilad D, Herrmann S, Sekler I, Aizenman E, Hershfinkel M (2011) Upregulation of KCC2 activity by zinc-mediated neurotransmission via the mZnR/GPR39 receptor. J Neurosci 31(36):12916–12926
Cohen L, Asraf H, Sekler I, Hershfinkel M (2012a) Extracellular pH regulates zinc signaling via an Asp residue of the zinc-sensing receptor (ZnR/GPR39). J Biol Chem 287(40):33339–33350

Cohen L, Azriel-Tamir H, Arotsker N, Sekler I, Hershfinkel M (2012b) Zinc sensing receptor signaling, mediated by GPR39, reduces butyrate-induced cell death in HT29 colonocytes via upregulation of clusterin. PLoS One 7(4):e35482
Cole TB, Robbins CA, Wenzel HJ, Schwartzkroin PA, Palmiter RD (2000) Seizures and neuronal damage in mice lacking vesicular zinc. Epilepsy Res 39(2):153–169
Costello LC, Franklin RB (2000) The intermediary metabolism of the prostate: a key to understanding the pathogenesis and progression of prostate malignancy. Oncology 59(4):269–282
Costello LC, Liu Y, Zou J, Franklin RB (1999) Evidence for a zinc uptake transporter in human prostate cancer cells which is regulated by prolactin and testosterone. J Biol Chem 274 (25):17499–17504
Custodi C, Nuti R, Oprea TI, Macchiarulo A (2012) Fitting the complexity of GPCRs modulation into simple hypotheses of ligand design. J Mol Graph Model 38:70–81
Danscher G, Stoltenberg M (2003) Zinc-enriched neurons. J Neurochem 85(suppl 2):10
Dittmer S, Sahin M, Pantlen A, Saxena A, Toutzaris D, Pina AL, Geerts A, Golz S, Methner A (2008) The constitutively active orphan G-protein-coupled receptor GPR39 protects from cell death by increasing secretion of pigment epithelium-derived growth factor. J Biol Chem 283 (11):7074–7081
Djeu JY, Wei S (2009) Clusterin and chemoresistance. Adv Cancer Res 105:77–92
Dubi N, Gheber L, Fishman D, Sekler I, Hershfinkel M (2008) Extracellular zinc and zinc-citrate, acting through a putative zinc-sensing receptor, regulate growth and survival of prostate cancer cells. Carcinogenesis (Oxf) 29(9):1692–1700
Egerod KL, Holst B, Petersen PS, Hansen JB, Mulder J, Hokfelt T, Schwartz TW (2007) GPR39 splice variants versus antisense gene LYPD1: expression and regulation in gastrointestinal tract, endocrine pancreas, liver, and white adipose tissue. Mol Endocrinol 21(7):1685–1698
Eide DJ (2006) Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta 1763 (7):711–722
Elling CE, Frimurer TM, Gerlach LO, Jorgensen R, Holst B, Schwartz TW (2006) Metal ion site engineering indicates a global toggle switch model for seven-transmembrane receptor activation. J Biol Chem 281(25):17337–17346
Elsas SM, Hazany S, Gregory WL, Mody I (2009) Hippocampal zinc infusion delays the development of afterdischarges and seizures in a kindling model of epilepsy. Epilepsia 50 (4):870–879
El-Tawil AM (2003) Zinc deficiency in men with Crohn's disease may contribute to poor sperm function and male infertility. Andrologia 35(6):337–341
Farrant M, Kaila K (2007) The cellular, molecular and ionic basis of GABA(A) receptor signalling. Prog Brain Res 160:59–87
Fassnacht M, Weismann D, Ebert S, Adam P, Zink M, Beuschlein F, Hahner S, Allolio B (2005) AKT is highly phosphorylated in pheochromocytomas but not in benign adrenocortical tumors. J Clin Endocrinol Metab 90(7):4366–4370
Finamore A, Massimi M, Conti Devirgiliis L, Mengheri E (2008) Zinc deficiency induces membrane barrier damage and increases neutrophil transmigration in Caco-2 cells. J Nutr 138(9):1664–1670
Franklin RB, Costello LC (2007) Zinc as an anti-tumor agent in prostate cancer and in other cancers. Arch Biochem Biophys 463(2):211–217
Franklin RB, Milon B, Feng P, Costello LC (2005) Zinc and zinc transporters in normal prostate and the pathogenesis of prostate cancer. Front Biosci 10:2230–2239
Frederickson C (2003a) Imaging zinc: old and new tools. Sci STKE 2003(182):pe18
Frederickson CJ (2003b) Synaptically released zinc: neuromodulator and toxin. J Neurochem 85 (suppl 2):10
Frederickson CJ, Danscher G (1990) Zinc-containing neurons in hippocampus and related CNS structures. Prog Brain Res 83:71–84
Frederickson CJ, Moncrieff DW (1994) Zinc-containing neurons. Biol Signals 3(3):127–139

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(5):579–583

Frederickson CJ, Hernandez MD, Goik SA, Morton JD, McGinty JF (1988a) Loss of zinc staining from hippocampal mossy fibers during kainic acid induced seizures: a histofluorescence study. Brain Res 446(2):383–386

Frederickson CJ, Howell GA, Haigh MD, Danscher G (1988b) Zinc-containing fiber systems in the cochlear nuclei of the rat and mouse. Hear Res 36(2-3):203–211

Frederickson CJ, Rampy BA, Reamy Rampy S, Howell GA (1992) Distribution of histochemically reactive zinc in the forebrain of the rat. J Chem Neuroanat 5(6):521–530

Frederickson CJ, Giblin LJ 3rd, Balaji RV, Masalha R, Frederickson CJ, Zeng Y, Lopez EV, Koh JY, Chorin U, Besser L, Hershfinkel M, Li Y, Thompson RB, Krezel A (2006) Synaptic release of zinc from brain slices: factors governing release, imaging, and accurate calculation of concentration. J Neurosci Methods 154(1-2):19–29

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signaling in health and diseases: zinc signaling. J Biol Inorg Chem 16(7):1123–1134

Ganesh R, Janakiraman L (2008) Serum zinc levels in children with simple febrile seizure. Clin Pediatr (Phila) 47(2):164–166

Gee KR, Zhou ZL, Qian WJ, Kennedy R (2002) Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator. J Am Chem Soc 124(5):776–778

Geiser J, Venken KJ, De Lisle RC, Andrews GK (2012) A mouse model of acrodermatitis enteropathica: loss of intestine zinc transporter ZIP4 (Slc39a4) disrupts the stem cell niche and intestine integrity. PLoS Genet 8(6):e1002766

Geiser J, De Lisle RC, Finkelstein D, Adlard PA, Bush AI, Andrews GK (2013) Clioquinol synergistically augments rescue by zinc supplementation in a mouse model of acrodermatitis enteropathica. PLoS One 8(8):e72543

Glover CN, Bury NR, Hogstrand C (2003) Zinc uptake across the apical membrane of freshwater rainbow trout intestine is mediated by high affinity, low affinity, and histidine-facilitated pathways. Biochim Biophys Acta 1614(2):211–219

Glover CN, Bury NR, Hogstrand C (2004) Intestinal zinc uptake in freshwater rainbow trout: evidence for apical pathways associated with potassium efflux and modified by calcium. Biochim Biophys Acta 1663(1-2):214–221

Goldberg HJ, Sheehy EM (1982) Fifth day fits: an acute zinc deficiency syndrome? Arch Dis Child. 57(8):633–635

Gomes I, Gupta A, Filipovska J, Szeto HH, Pintar JE, Devi LA (2004) A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. Proc Natl Acad Sci USA 101(14):5135–5139

Gore A, Moran A, Hershfinkel M, Sekler I (2004) Inhibitory mechanism of store-operated Ca^{2+} channels by zinc. J Biol Chem 279(12):11106–11111

Grant MP, Stepanchick A, Cavanaugh A, Breitwieser GE (2011) Agonist-driven maturation and plasma membrane insertion of calcium-sensing receptors dynamically control signal amplitude. Sci Signal 4(200):ra78

Grebhardt S, Muller-Decker K, Bestvater F, Hershfinkel M, Mayer D (2014) Impact of S100A8/A9 expression on prostate cancer progression in vitro and in vivo. J Cell Physiol 229(5):661–671

Gyulkhandanyan AV, Lu H, Lee SC, Bhattacharjee A, Wijesekara N, Fox JE, MacDonald PE, Chimienti F, Dai FF, Wheeler MB (2008) Investigation of transport mechanisms and regulation of intracellular Zn^{2+} in pancreatic alpha-cells. J Biol Chem 283(15):10184–10197

Hachem JP, Behne M, Aronchik I, Demerjian M, Feingold KR, Elias PM, Mauro TM (2005) Extracellular pH controls NHE1 expression in epidermis and keratinocytes: implications for barrier repair. J Invest Dermatol 125(4):790–797

Han Y, Wu SM (1999) Modulation of glycine receptors in retinal ganglion cells by zinc. Proc Natl Acad Sci USA 96(6):3234–3238

Herin GA, Aizenman E (2004) Amino terminal domain regulation of NMDA receptor function. Eur J Pharmacol 500(1-3):101–111

Hermani A, Hess J, De Servi B, Medunjanin S, Grobholz R, Trojan L, Angel P, Mayer D (2005) Calcium-binding proteins S100A8 and S100A9 as novel diagnostic markers in human prostate cancer. Clin Cancer Res 11(14):5146–5152

Hermani A, De Servi B, Medunjanin S, Tessier PA, Mayer D (2006) S100A8 and S100A9 activate MAP kinase and NF-kappaB signaling pathways and trigger translocation of RAGE in human prostate cancer cells. Exp Cell Res 312(2):184–197

Hershfinkel M (2006) Zinc, a dynamic signaling molecule. In: Tamas M, Klomp L, Martinoia E (eds) Molecular biology of metal homeostasis and detoxification: from microbes to man, Springer, Germany

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(20):11749–11754

Hershfinkel M, Silverman WF, Sekler I (2007) The zinc sensing receptor, a link between zinc and cell signaling. Mol Med 13(7-8):331–336

Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW (2004) Common structural basis for constitutive activity of the ghrelin receptor family. J Biol Chem 279(51):53806–53817

Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG, Schwartz TW (2007) GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology 148(1):13–20

Holst B, Egerod KL, Jin C, Petersen PS, Ostergaard MV, Hald J, Sprinkel AM, Storling J, Mandrup-Poulsen T, Holst JJ, Thams P, Orskov C, Wierup N, Sundler F, Madsen OD, Schwartz TW (2009) G protein-coupled receptor 39 deficiency is associated with pancreatic islet dysfunction. Endocrinology 150(6):2577–2585

Holzer P (2009) Acid-sensitive ion channels and receptors. Handb Exp Pharmacol 194:283–332

Holzer P (2011) Acid sensing by visceral afferent neurones. Acta Physiol (Oxf) 201(1):63–75

Hoque KM, Binder HJ (2006) Zinc in the treatment of acute diarrhea: current status and assessment. Gastroenterology 130(7):2201–2205

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(4):362–369

Huang JS, Mukherjee JJ, Chung T, Crilly KS, Kiss Z (1999) Extracellular calcium stimulates DNA synthesis in synergism with zinc, insulin and insulin-like growth factor I in fibroblasts. Eur J Biochem 266(3):943–951

Huang L, Kirschke CP, Zhang Y (2006) Decreased intracellular zinc in human tumorigenic prostate epithelial cells: a possible role in prostate cancer progression. Cancer Cell Int 6:10

Huberfeld G, Wittner L, Clemenceau S, Baulac M, Kaila K, Miles R, Rivera C (2007) Perturbed chloride homeostasis and GABAergic signaling in human temporal lobe epilepsy. J Neurosci 27(37):9866–9873

Ishii K, Sato M, Akita M, Tomita H (1999) Localization of zinc in the rat submandibular gland and the effect of its deficiency on salivary secretion. Ann Otol Rhinol Laryngol 108(3):300–308

Jackson VR, Nothacker HP, Civelli O (2006) GPR39 receptor expression in the mouse brain. Neuroreport 17(8):813–816

Jensen SL, McCuaig C, Zembowicz A, Hurt MA (2008) Bullous lesions in acrodermatitis enteropathica delaying diagnosis of zinc deficiency: a report of two cases and review of the literature. J Cutan Pathol 35(suppl 1):1–13

Joiner ML, Koval OM, Li J, He BJ, Allamargot C, Gao Z, Luczak ED, Hall DD, Fink BD, Chen B, Yang J, Moore SA, Scholz TD, Strack S, Mohler PJ, Sivitz WI, Song LS, Anderson ME (2012) CaMKII determines mitochondrial stress responses in heart. Nature (Lond) 491(7423):269–273

Kahle KT, Rinehart J, Lifton RP (2010) Phosphoregulation of the Na-K-2Cl and K-Cl cotransporters by the WNK kinases. Biochim Biophys Acta 1802(12):1150–1158

Kambe T, Weaver BP, Andrews GK (2008) The genetics of essential metal homeostasis during development. Genesis 46(4):214–228
Kay AR (2004) Detecting and minimizing zinc contamination in physiological solutions. BMC Physiol 4:4–5
Kelleher SL, McCormick NH, Velasquez V, Lopez V (2011) Zinc in specialized secretory tissues: roles in the pancreas, prostate, and mammary gland. Adv Nutr 2(2):101–111
Khirug S, Ahmad F, Puskarjov M, Afzalov R, Kaila K, Blaesse P (2010) A single seizure episode leads to rapid functional activation of KCC2 in the neonatal rat hippocampus. J Neurosci 30 (36):12028–12035
Kim S, Jung Y, Kim D, Koh H, Chung J (2000) Extracellular zinc activates p70 S6 kinase through the phosphatidylinositol 3-kinase signaling pathway. J Biol Chem 275(34):25979–25984
Kohout TA, Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. Mol Pharmacol 63(1):9–18
Krasovec M, Frenk E (1996) Acrodermatitis enteropathica secondary to Crohn's disease. Dermatology 193(4):361–363
Krezel A, Maret W (2006) Zinc-buffering capacity of a eukaryotic cell at physiological pZn. J Biol Inorg Chem 11(8):1049–1062
Lansdown AB (1996) Zinc in the healing wound. Lancet 347(9003):706–707
Lansdown AB, Mirastschijski U, Stubbs N, Scanlon E, Agren MS (2007) Zinc in wound healing: theoretical, experimental, and clinical aspects. Wound Repair Regen 15(1):2–16
Lasry I, Seo YA, Ityel H, Shalva N, Pode-Shakked B, Glaser F, Berman B, Berezovsky I, Goncearenco A, Klar A, Levy J, Anikster Y, Kelleher SL, Assaraf YG (2012) A dominant negative heterozygous G87R mutation in the zinc transporter, ZnT-2 (SLC30A2), results in transient neonatal zinc deficiency. J Biol Chem 287(35):29348–29361
Lauwers E, Landuyt B, Arckens L, Schoofs L, Luyten W (2006) Obestatin does not activate orphan G protein-coupled receptor GPR39. Biochem Biophys Res Commun 351(1):21–25
Lee H, Chen CX, Liu YJ, Aizenman E, Kandler K (2005) KCC2 expression in immature rat cortical neurons is sufficient to switch the polarity of GABA responses. Eur J Neurosci 21 (9):2593–2599
Lee HH, Walker JA, Williams JR, Goodier RJ, Payne JA, Moss SJ (2007) Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. J Biol Chem 282(41):29777–29784
Lee HH, Jurd R, Moss SJ (2010) Tyrosine phosphorylation regulates the membrane trafficking of the potassium chloride co-transporter KCC2. Mol Cell Neurosci 45(2):173–179
Liuzzi JP, Cousins RJ (2004) Mammalian zinc transporters. Annu Rev Nutr 24:151–172
Lu J, Karadsheh M, Delpire E (1999) Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. J Neurobiol 39(4):558–568
Luk HH, Ko JK, Fung HS, Cho CH (2002) Delineation of the protective action of zinc sulfate on ulcerative colitis in rats. Eur J Pharmacol 443(1-3):197–204
Lynch JW, Jacques P, Pierce KD, Schofield PR (1998) Zinc potentiation of the glycine receptor chloride channel is mediated by allosteric pathways. J Neurochem 71(5):2159–2168
MacDonald RS (2000) The role of zinc in growth and cell proliferation. J Nutr 130 (5S (suppl)):1500S–1508S
Maret W (2001a) Crosstalk of the group IIa and IIb metals calcium and zinc in cellular signaling. Proc Natl Acad Sci USA 98(22):12325–12327
Maret W (2001b) Zinc biochemistry, physiology, and homeostasis – recent insights and current trends. Biometals 14(3–4):187–190
Maret W (2012) New perspectives of zinc coordination environments in proteins. J Inorg Biochem 111:110–116
Maret W, Sandstead HH (2006) Zinc requirements and the risks and benefits of zinc supplementation. J Trace Elem Med Biol 20(1):3–18
Mazzarelli P, Pucci S, Spagnoli LG (2009) CLU and colon cancer. The dual face of CLU: from normal to malignant phenotype. Adv Cancer Res 105:45–61

McCormick N, Velasquez V, Finney L, Vogt S, Kelleher SL (2010) X-ray fluorescence microscopy reveals accumulation and secretion of discrete intracellular zinc pools in the lactating mouse mammary gland. PLoS One 5(6):e11078

McDonald PH, Lefkowitz RJ (2001) Beta-Arrestins: new roles in regulating heptahelical receptors' functions. Cell Signal 13(10):683–689

McKee KK, Tan CP, Palyha OC, Liu J, Feighner SD, Hreniuk DL, Smith RG, Howard AD, Van der Ploeg LH (1997) Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. Genomics 46(3):426–434

McLaughlin B, Pal S, Tran MP, Parsons AA, Barone FC, Erhardt JA, Aizenman E (2001) p38 activation is required upstream of potassium current enhancement and caspase cleavage in thiol oxidant-induced neuronal apoptosis. J Neurosci. 15;21(10):3303–3311

Moechars D, Depoortere I, Moreaux B, de Smet B, Goris I, Hoskens L, Daneels G, Kass S, Ver Donck L, Peeters T, Coulie B (2006) Altered gastrointestinal and metabolic function in the GPR39-obestatin receptor-knockout mouse. Gastroenterology 131(4):1131–1141

Mohan ML, Vasudevan NT, Gupta MK, Martelli EE, Naga Prasad SV (2012) G-protein coupled receptor resensitization: appreciating the balancing act of receptor function. Curr Mol Pharmacol May 30, 5(3):350–361

Nitzan YB, Sekler I, Silverman WF (2004) Histochemical and histofluorescence tracing of chelatable zinc in the developing mouse. J Histochem Cytochem 52(4):529–539

Oh SY, Park KS, Kim JA, Choi KY (2002) Differential modulation of zinc-stimulated p21 (Cip/WAF1) and cyclin D1 induction by inhibition of PI3 kinase in HT-29 colorectal cancer cells. Exp Mol Med 34(1):27–31

Pajak B, Orzechowski A (2006) Clusterin: the missing link in the calcium-dependent resistance of cancer cells to apoptogenic stimuli. Postepy Hig Med Dosw (Online) 60:45–51

Pan E, Zhang XA, Huang Z, Krezel A, Zhao M, Tinberg CE, Lippard SJ, McNamara JO (2011) Vesicular zinc promotes presynaptic and inhibits postsynaptic long-term potentiation of mossy fiber-CA3 synapse. Neuron 71(6):1116–1126

Paoletti P, Ascher P, Neyton J (1997) High-affinity zinc inhibition of NMDA NR1-NR2A receptors. J Neurosci 17(15):5711–5725

Papatsoris AG, Karamouzis MV, Papavassiliou AG (2007) The power and promise of "rewiring" the mitogen-activated protein kinase network in prostate cancer therapeutics. Mol Cancer Ther 6(3):811–819

Perdikis DA, Davies R, Zhuravkov A, Brenner B, Etter L, Basson MD (1998) Differential effects of mucosal pH on human (Caco-2) intestinal epithelial cell motility, proliferation, and differentiation. Dig Dis Sci 43(7):1537–1546

Perez-Rosello T, Anderson CT, Schopfer FJ, Zhao Y, Gilad D, Salvatore SR, Freeman BA, Hershfinkel M, Aizenman E, Tzounopoulos T (2013) Synaptic Zn^{2+} inhibits neurotransmitter release by promoting endocannabinoid synthesis. J Neurosci 33(22):9259–9272

Popovics P, Stewart AJ (2011) GPR39: a Zn(2+)-activated G protein-coupled receptor that regulates pancreatic, gastrointestinal and neuronal functions. Cell Mol Life Sci 68(1):85–95

Prasad AS (1995) Zinc: an overview. Nutrition 11(1 suppl):93–99

Prasad AS (2008) Zinc in human health: effect of zinc on immune cells. Mol Med 14(5-6):353–357

Qian J, Noebels JL (2005) Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse. J Physiol 566(pt 3):747–758

Qian J, Noebels JL (2006) Exocytosis of vesicular zinc reveals persistent depression of neurotransmitter release during metabotropic glutamate receptor long-term depression at the hippocampal CA3-CA1 synapse. J Neurosci 26(22):6089–6095

Qian WJ, Gee KR, Kennedy RT (2003) Imaging of Zn^{2+} release from pancreatic beta-cells at the level of single exocytotic events. Anal Chem 75(14):3468–3475

Redman PT, Hartnett KA, Aras MA, Levitan ES, Aizenman E (2009) Regulation of apoptotic potassium currents by coordinated zinc-dependent signalling. J Physiol. 15;587(Pt 18):4393-4404

Riccardi D (1999) Cell surface, Ca^{2+} (cation)-sensing receptor(s): one or many? Cell Calcium 26 (3-4):77–83

Rinehart J, Maksimova YD, Tanis JE, Stone KL, Hodson CA, Zhang J, Risinger M, Pan W, Wu D, Colangelo CM, Forbush B, Joiner CH, Gulcicek EE, Gallagher PG, Lifton RP (2009) Sites of regulated phosphorylation that control K-Cl cotransporter activity. Cell 138(3):525–536

Rongo C (2002) A fresh look at the role of CaMKII in hippocampal synaptic plasticity and memory. Bioessays 24(3):223–233

Roohani N, Hurrell R, Kelishadi R, Schulin R (2013) Zinc and its importance for human health: an integrative review. J Res Med Sci 18(2):144–157

Saadi RA, He K, Hartnett KA, Kandler K, Hershfinkel M, Aizenman E (2012) SNARE-dependent upregulation of potassium chloride co-transporter 2 activity after metabotropic zinc receptor activation in rat cortical neurons in vitro. Neuroscience 210:38–46

Sandoval M, Burgos J, Sepulveda FV, Cid LP (2011) Extracellular pH in restricted domains as a gating signal for ion channels involved in transepithelial transport. Biol Pharm Bull 34(6):803–809

Sandstead HH (2012) Zinc nutrition from discovery to global health impact. Adv Nutr 3(5):718–719

Sandstead HH, Frederickson CJ, Penland JG (2000) History of zinc as related to brain function. J Nutr 130(2S(suppl)):496S–502S

Scharlau D, Borowicki A, Habermann N, Hofmann T, Klenow S, Miene C, Munjal U, Stein K, Glei M (2009) Mechanisms of primary cancer prevention by butyrate and other products formed during gut flora-mediated fermentation of dietary fibre. Mutat Res 682(1):39–53

Schwartz JR, Marsh RG, Draelos ZD (2005) Zinc and skin health: overview of physiology and pharmacology. Dermatol Surg 31(7 pt 2):837–847, discussion 847

Scrimgeour AG, Lukaski HC (2008) Zinc and diarrheal disease: current status and future perspectives. Curr Opin Clin Nutr Metab Care 11(6):711–717

Sekler I, Sensi SL, Hershfinkel M, Silverman WF (2007) Mechanism and regulation of cellular zinc transport. Mol Med 13(7-8):337–343

Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, Hershfinkel M (2011) The neurophysiology and pathology of brain zinc. J Neurosci 31(45):16076–16085

Seve M, Chimienti F, Devergnas S, Favier A (2004) In silico identification and expression of SLC30 family genes: an expressed sequence tag data mining strategy for the characterization of zinc transporters' tissue expression. BMC Genomics 5(1):32

Seven M, Basaran SY, Cengiz M, Unal S, Yuksel A.(2013) Deficiency of selenium and zinc as a causative factor for idiopathic intractable epilepsy. Epilepsy Res 104(1-2):35–9

Shannan B, Seifert M, Leskov K, Willis J, Boothman D, Tilgen W, Reichrath J (2006) Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. Cell Death Differ 13(1):12–19

Sharir H, Hershfinkel M (2005) The extracellular zinc-sensing receptor mediates intercellular communication by inducing ATP release. Biochem Biophys Res Commun 332(3):845–852

Sharir H, Zinger A, Nevo A, Sekler I, Hershfinkel M (2010) Zinc released from injured cells is acting via the Zn2 + -sensing receptor, ZnR, to trigger signaling leading to epithelial repair. J Biol Chem 285(34):26097–26106

Sharma M, Sahu K, Dube A, Gupta PK (2005) Extracellular pH influences the mode of cell death in human colon adenocarcinoma cells subjected to photodynamic treatment with chlorin p6. J Photochem Photobiol B 81(2):107–113

Singh KK, Desouki MM, Franklin RB, Costello LC (2006) Mitochondrial aconitase and citrate metabolism in malignant and nonmalignant human prostate tissues. Mol Cancer 5:14

Smart TG, Hosie AM, Miller PS. (2004) Zn2+ ions: modulators of excitatory and inhibitory synaptic activity. Neuroscientist. 10(5):432–442

Stein V, Hermans-Borgmeyer I, Jentsch TJ, Hubner CA (2004) Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. J Comp Neurol 468(1):57–64

Stock C, Cardone RA, Busco G, Krahling H, Schwab A, Reshkin SJ (2008) Protons extruded by NHE1: digestive or glue? Eur J Cell Biol 87(8-9):591–599

Stoltenberg M, Bush AI, Bach G, Smidt K, Larsen A, Rungby J, Lund S, Doering P, Danscher G (2007) Amyloid plaques arise from zinc-enriched cortical layers in APP/PS1 transgenic mice and are paradoxically enlarged with dietary zinc deficiency. Neuroscience 150(2):357–369

Storjohann L, Holst B, Schwartz TW (2008) Molecular mechanism of Zn^{2+} agonism in the extracellular domain of GPR39. FEBS Lett 582(17):2583–2588

Strange K, Singer TD, Morrison R, Delpire E (2000) Dependence of KCC2 K-Cl cotransporter activity on a conserved carboxy terminus tyrosine residue. Am J Physiol Cell Physiol 279(3): C860–C867

Sturniolo GC, Di Leo V, Ferronato A, D'Odorico A, D'Inca R (2001) Zinc supplementation tightens "leaky gut" in Crohn's disease. Inflamm Bowel Dis 7(2):94–98

Stuwe L, Muller M, Fabian A, Waning J, Mally S, Noel J, Schwab A, Stock C (2007) pH dependence of melanoma cell migration: protons extruded by NHE1 dominate protons of the bulk solution. J Physiol 585(pt 2):351–360

Takahashi H, Nakazawa M, Takahashi K, Aihara M, Minami M, Hirasawa T, Ikezawa Z (2008) Effects of zinc deficient diet on development of atopic dermatitis-like eruptions in DS-Nh mice. J Dermatol Sci 50(1):31–39

Takasaki J, Saito T, Taniguchi M, Kawasaki T, Moritani Y, Hayashi K, Kobori M (2004) A novel Galphaq/11-selective inhibitor. J Biol Chem 279(46):47438–47445

Takeda A (2001) Zinc homeostasis and functions of zinc in the brain. Biometals 14(3-4):343–351

Takeda A, Hirate M, Tamano H, Nisibaba D, Oku N (2003) Susceptibility to kainate-induced seizures under dietary zinc deficiency. J Neurochem 85(6):1575–1580

Takeda A, Yamada K, Minami A, Nagano T, Oku N (2005) Enhanced excitability of hippocampal mossy fibers and CA3 neurons under dietary zinc deficiency. Epilepsy Res. 63(2-3):77–84

Tang Y, Chen Y, Jiang H, Nie D (2011) The role of short-chain fatty acids in orchestrating two types of programmed cell death in colon cancer. Autophagy 7(2):235–237

Taniguchi M, Suzumura K, Nagai K, Kawasaki T, Takasaki J, Sekiguchi M, Moritani Y, Saito T, Hayashi K, Fujita S, Tsukamoto S, Suzuki K (2004) YM-254890 analogues, novel cyclic depsipeptides with Galpha(q/11) inhibitory activity from *Chromobacterium* sp. QS3666. Bioorg Med Chem 12(12):3125–3133

Topping DL, Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81(3):1031–1064

Tremblay F, Richard AM, Will S, Syed J, Stedman N, Perreault M, Gimeno RE (2009) Disruption of G protein-coupled receptor 39 impairs insulin secretion in vivo. Endocrinology 150 (6):2586–2595

Vallee BL, Falchuk KH (1993) The biochemical basis of zinc physiology. Physiol Rev 73(1):79–118

Vasak M (2005) Advances in metallothionein structure and functions. J Trace Elem Med Biol 19 (1):13–17

Viitanen T, Ruusuvuori E, Kaila K, Voipio J (2010) The K^+-Cl^- cotransporter KCC2 promotes GABAergic excitation in the mature rat hippocampus. J Physiol 588(pt 9):1527–1540

Vogt K, Mellor J, Tong G, Nicoll R. (2000) The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron. 26(1):187–96

Wake H, Watanabe M, Moorhouse AJ, Kanematsu T, Horibe S, Matsukawa N, Asai K, Ojika K, Hirata M, Nabekura J (2007) Early changes in KCC2 phosphorylation in response to neuronal stress result in functional downregulation. J Neurosci 27(7):1642–1650

Walker CL, Black RE (2010) Zinc for the treatment of diarrhoea: effect on diarrhoea morbidity, mortality and incidence of future episodes. Int J Epidemiol 39(suppl 1):i63–i69

Watanabe M, Wake H, Moorhouse AJ, Nabekura J (2009) Clustering of neuronal K^+-Cl^- cotransporters in lipid rafts by tyrosine phosphorylation. J Biol Chem 284(41):27980–27988

Weiss JH, Sensi SL, Koh JY (2000) Zn(2+): a novel ionic mediator of neural injury in brain disease. Trends Pharmacol Sci 21(10):395–401

Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, Hutton JC (2007) The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci USA 104(43):17040–17045

Wildman SS, King BF, Burnstock G (1999) Modulatory activity of extracellular H^+ and Zn^{2+} on ATP-responses at rP2X1 and rP2X3 receptors. Br J Pharmacol 128(2):486–492

Wilson M, Hogstrand C, Maret W (2012) Picomolar concentrations of free zinc(II) ions regulate receptor protein-tyrosine phosphatase beta activity. J Biol Chem 287(12):9322–9326

Wootten D, Christopoulos A, Sexton PM (2013) Emerging paradigms in GPCR allostery: implications for drug discovery. Nat Rev Drug Discov 12(8):630–644

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(27):24252–24257

Wu W, Wang X, Zhang W, Reed W, Samet JM, Whang YE, Ghio AJ (2003) Zinc-induced PTEN protein degradation through the proteasome pathway in human airway epithelial cells. J Biol Chem 278(30):28258–28263

Wu W, Silbajoris RA, Whang YE, Graves LM, Bromberg PA, Samet JM (2005) p38 and EGF receptor kinase-mediated activation of the phosphatidylinositol 3-kinase/Akt pathway is required for Zn^{2+}-induced cyclooxygenase-2 expression. Am J Physiol Lung Cell Mol Physiol 289(5):L883–L889

Xie F, Liu H, Zhu YH, Qin YR, Dai Y, Zeng T, Chen L, Nie C, Tang H, Li Y, Fu L, Guan XY (2011) Overexpression of GPR39 contributes to malignant development of human esophageal squamous cell carcinoma. BMC Cancer 11:86

Yasuda S, Miyazaki T, Munechika K, Yamashita M, Ikeda Y, Kamizono A (2007) Isolation of Zn^{2+} as an endogenous agonist of GPR39 from fetal bovine serum. J Recept Signal Transduct Res 27(4):235–246

Yu DC, Waby JS, Chirakkal H, Staton CA, Corfe BM (2010) Butyrate suppresses expression of neuropilin I in colorectal cell lines through inhibition of Sp1 transactivation. Mol Cancer 9 (276):276

Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ (2005) Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 310 (5750):996–999

Zhang Y, Aizenman E, DeFranco DB, Rosenberg PA (2007) Intracellular zinc release, 12-lipoxygenase activation and MAPK dependent neuronal and oligodendroglial death. Mol Med. 13(7-8):350–355

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(47):10616–10627

Zhang Y, Zhou L, Bao YL, Wu Y, Yu CL, Huang YX, Sun Y, Zheng LH, Li YX (2010) Butyrate induces cell apoptosis through activation of JNK MAP kinase pathway in human colon cancer RKO cells. Chem Biol Interact 185(3):174–181

Zhu L, Lovinger D, Delpire E (2005) Cortical neurons lacking KCC2 expression show impaired regulation of intracellular chloride. J Neurophysiol 93(3):1557–1568

Zhu L, Polley N, Mathews GC, Delpire E (2008) NKCC1 and KCC2 prevent hyperexcitability in the mouse hippocampus. Epilepsy Res 79(2–3):201–212

Chapter 7
Genetically Encoded Fluorescent Probes for Intracellular Zn^{2+} Imaging

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Abstract In this chapter we provide an overview of the various genetically encoded fluorescent Zn^{2+} sensors that have been developed over the past 5 to 10 years. We focus on sensors based on Förster resonance energy transfer (FRET), as these have so far proven to be the most useful for detecting Zn^{2+} in biological samples. Our goal is to provide a balanced discussion of the pros and cons of the various sensors and their application in intracellular imaging. Following the description of the various sensors, several recent applications of these sensors are discussed. We end the chapter by identifying remaining challenges in this field and discussing future perspectives.

Keywords Fluorescence • FRET • Imaging • Microscopy • Sensor • Zinc

7.1 Introduction

Transition metals such as zinc pose an interesting dilemma for living organisms because they are essential cofactors for numerous enzymes and proteins, but at the same time are toxic even at low concentrations in their free form (Valko et al. 2005). Mechanisms to control this delicate balance may vary for different metal ions and also between organisms. Copper homeostasis in eukaryotes has been shown to involve specific copper chaperone proteins that transfer Cu^{+} to various cellular targets without releasing it into the cytosol (Rae et al. 1999). Similar chaperones have not been identified for Zn^{2+}; instead, a general Zn^{2+}-buffering mechanism has been proposed in which the free cytosolic Zn^{2+} concentration in mammalian cells is kept constant at pM–nM levels (Cousins et al. 2006; Krezel and Maret 2006). The free concentration of Zn^{2+} is also likely to differ substantially between subcellular locations, as mM concentrations of total Zn^{2+} have been reported for pancreatic β-cell granules (Hutton et al. 1983) and inferred for secretory vesicles in neuronal (Linkous et al. 2008) and mast cells (Ho et al. 2004).

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To advance our understanding of zinc homeostasis and the putative role of Zn^{2+} in (intracellular) signal transduction, tools are required that allow direct (sub) cellular imaging of Zn^{2+} concentrations in single living cells in real time. Fluorescence is ideally suited for this purpose, because it combines high sensitivity with subcellular resolution (Kikuchi 2010). Such fluorescent sensors should have an appropriate affinity for Zn^{2+} under physiological conditions, show high selectivity for Zn^{2+} over other biologically abundant metals, and translate Zn^{2+} binding into a strong increase in fluorescence, or even better, a ratiometric change in fluorescent properties. Currently, Zn^{2+}-sensitive fluorescent dyes are still the most commonly imaging probes for monitoring Zn^{2+} in biological samples. The development of synthetic sensors continues to be an active area in chemical biology, and an impressive variety of Zn^{2+}-sensitive fluorescent dyes (such as Zinquin, rhodzin-3, and FluoZin-3) has been developed, some of which have also been applied to monitor Zn^{2+} fluctuations in living cells (Domaille et al. 2008; Nolan and Lippard 2009). However, synthetic probes come with some intrinsic limitations, notably a lack of full control over subcellular localization and the need to achieve high intracellular concentrations of the dye, which may perturb free levels of Zn^{2+}. In addition, it has proven challenging to create synthetic dyes that rival the affinity and specificity typically observed with metalloproteins, which is important to reliably determine the extremely low concentrations of Zn^{2+} and other transition metal ions (Krezel and Maret 2006; Bozym et al. 2006; Van Dongen et al. 2007).

Genetically encoded fluorescent sensors offer several advantages compared to small molecule-based probes. Small-molecule probes need to enter the cell via diffusion over the cell membrane. Although these probes can be trapped by hydrolysis of methylesters by intracellular esterases, controlling their concentration over prolonged time intervals remains challenging. Even more importantly, once inside the cell, little control over subcellular localization is possible, which is an important caveat given that Zn^{2+} concentrations can vary considerably between different organelles. Protein-based, genetically encoded probes are produced by the cell itself, which in principle allows control over their intracellular concentration, prevents leakage, and provides excellent control over intracellular localization. A second advantage of genetically encoded probes is that they allow one to take advantage of the excellent affinity and specificity displayed by natural metal-binding proteins, which can be further improved by both rational and directed evolution approaches. Although adding a fluorescent dye to a cell maybe slightly easier than relying on DNA transfection, the choice of useful commercially available Zn^{2+} dyes is limited, which presents an important restriction for those scientists who cannot synthesize these probes themselves. DNA-encoded probes, on the other hand, can be easily replicated by standard molecular biology techniques and distributed through depositories such as AddGene.

Most applications of genetically encoded fluorescent sensors have been limited to studies in immortalized cell lines using transient transfection, but genetically encoded fluorescent sensors can also be applied in primary cells using viral vectors or even entire organisms. Although at present the latter still requires a substantial effort, new developments in genetic engineering are expected to make the latter

possibility more readily accessible in the future. In this chapter we provide an overview of the various genetically encoded fluorescent Zn^{2+} sensors that have been developed during the past 5 to 10 years. We focus on sensors based on Förster resonance energy transfer (FRET), as these have so far proven to be the most useful for detecting Zn^{2+} in biological samples. Our goal is to provide a discussion of the pros and cons of the various sensors and their application in intracellular imaging. Following the description of the various sensors, several recent applications of these sensors are discussed. We end the chapter by identifying remaining challenges in this field and discussing future perspectives.

7.2 Genetically Encoded Zn^{2+} Sensors

7.2.1 Sensor Principles

Several strategies have been explored to develop Zn^{2+}-responsive fluorescent proteins. One approach is to introduce a Zn^{2+}-binding site close to the chromophore of the fluorescent protein. This principle was first reported by Barondeau et al., who created a Zn^{2+}-binding variant of green fluorescent protein (GFP) in which the tyrosine that is part of the original fluorophore was replaced by a metal-coordinating histidine (Barondeau et al. 2002). This sensor binds both Zn^{2+} and Cu^{2+} with micromolar affinity. A twofold increase in fluorescence intensity was observed upon addition of Zn^{2+}, whereas Cu^{2+} binding resulted in quenching of fluorescence. This sensor has not been used beyond the initial proof of concept study, however, most likely because its affinity for Zn^{2+} is too weak for intracellular Zn^{2+} detection and because it is not easily calibrated. Another strategy for single-domain fluorescent Zn^{2+} sensors was reported by Mizuno et al., who fused de novo designed metal ion-responsive coiled-coil peptides with circularly permutated green fluorescent protein (cpGFP) (Mizuno et al. 2007). Metal binding to histidine residues in these peptides induces a structural change from a random coil structure to an α-helical, trimeric, coiled-coil structure, which stabilizes the cpGFP domain and results in an increase in fluorescence. Although the Zn^{2+} affinity for this probe was higher than the sensor reported by Barondeau ($K_d = 570$ nM), binding of Cu^{2+} and Ni^{2+} resulted in similar increase in fluorescence.

More recently, incorporation of metal-chelating, non-natural amino acids has been explored to obtain metal-responsive fluorescent proteins. Wang and coworkers developed a circularly permuted variant of super folder GFP in which the tyrosine present in the chromophore was replaced by 8-hydroxyquinolin-alanine (HqAla), yielding a fluorescent sensor that showed a sevenfold increase in fluorescent intensity in the presence of Zn^{2+} ($\lambda_{ex} = 495$ nm; $\lambda_{em} = 537$ nm) (Liu et al. 2013b). In contrast to the two other examples just discussed, Zn^{2+} binding also results in a blue shift of both excitation and emission spectra, allowing ratiometric detection. At present the applicability of this probe is still limited, not

only by the requirement to use non-natural amino acids but also because of the relatively weak Zn^{2+} affinity ($K_d = 50–100$ μM). Besides their relatively low Zn^{2+} affinities and metal specificity, most single-domain fluorescent sensor proteins are intensity based, that is, they show an increase or decrease in fluorescence but their spectral properties do not change. This is a disadvantage for quantitative intracellular applications (Liu et al. 2013a), because fluorescence intensity is not only a function of the Zn^{2+} concentration but also depends on expression levels and can be affected by photobleaching.

The most commonly used strategy to design fluorescent sensor proteins takes advantage of the principle of Förster resonance energy transfer (FRET). FRET is a mechanism in which excitation energy is transferred from a donor to an acceptor fluorescent domain. The efficiency of this process is distance- and orientation dependent and therefore useful to detect conformational changes as a result of metal binding to a receptor domain. FRET-based Zn^{2+} sensors consist of one or more metal-binding domains flanked by a donor and acceptor fluorescent domain. The most frequently used FRET pair consists of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), although more red-shifted FRET pairs have recently also been reported (Lindenburg et al. 2013; Miranda et al. 2012). A key advantage of FRET-based sensors is that they are ratiometric, that is, the ratio of acceptor and donor emission provides a measure of the metal-binding state that is independent of the sensor concentration. In principle, FRET sensor design is also more modular, although developing FRET sensors with a large change in emission ratio can be challenging and often requires much optimization. Other important properties that determine the performance of FRET-based sensors for intracellular Zn^{2+} imaging are their Zn^{2+} affinity and specificity, their binding kinetics, and their pH sensitivity. In the next paragraph we discuss the various FRET-based Zn^{2+} sensor systems that have been developed thus far and discuss these aspects. The first family of Zn^{2+} sensors that we discuss, the CALWY-sensors, will also be used to introduce some of the general issues that are important when considering the applications of these sensors.

7.2.2 Genetically Encoded FRET-Based Sensors

7.2.2.1 CALWY Sensors

Sensors from the CALWY family consist of two small metal-binding domains, Atox1 and WD4, that each contain a metal-binding CXXC motif, fused by a long and flexible linker. In the original sensor (CALWY), the metal-binding domain was flanked by cyan and yellow fluorescent domains, hence their name (CFP-Atox1-Linker-WD4-YFP) (Fig. 7.1a) (Van Dongen et al. 2006; Van Dongen et al. 2007). ATOX 1 and WD4 are native Cu(I)-binding domains that play a role in maintaining copper homeostasis, and the sensor was initially developed to create a genetically encoded Cu^{+} sensor based on the Cu^{+}-induced dimerization of these two domains.

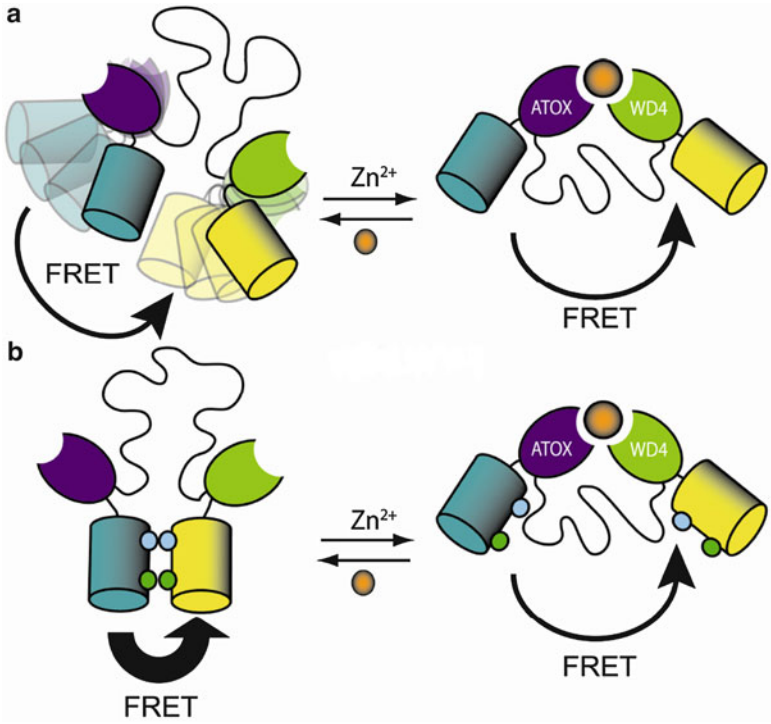

Fig. 7.1 Schematic representation of the CALWY (**a**) and eCALWY-1 (**b**) sensor designs, both consisting of two metal-binding domains, ATOX1 and WD4, connected via a flexible peptide linker and flanked by two fluorescent domains. **a** The CALWY sensor yielded a small Förster resonance energy transfer (FRET) change between the Zn^{2+}-free and Zn^{2+}-bound state. **b** In the eCALWY constructs two mutations, S208F and V224L, were introduced on both fluorescent domains, leading to high energy transfer in the Zn^{2+}-free state. Zn^{2+} binding disrupts the complex, resulting in a large decrease in FRET. (Adapted from Vinkenborg et al. 2009)

However, it was discovered that Zn^{2+} was able to form a very stable tetrahedral complex by binding the four cysteines present in the two copper-binding domains, yielding a K_d of approximately 0.23 pM at pH 7.1. Unfortunately, the change in emission ratio of the original CALWY sensor was small, showing a 15 % decrease in emission ratio upon Zn^{2+} binding. Because this poor dynamic would make the sensor less suitable for intracellular Zn^{2+} imaging, improved variants were developed, resulting in the so-called eCALWY series of Zn^{2+} sensors. First, ECFP and EYFP were replaced by cerulean and citrine, respectively, fluorescent domains with increased intensity (cerulean) and pH stability (citrine). Most importantly, the ratiometric response was improved sixfold by introduction of two mutations (S208F and V224L) on both cerulean and citrine that promote intramolecular complex formation between the two fluorescent domains in the absence of Zn^{2+}. Binding of Zn^{2+} to ATOX1 and WD4 disrupts the interaction between the fluorescent domains, resulting in a large decrease in FRET corresponding to a twofold

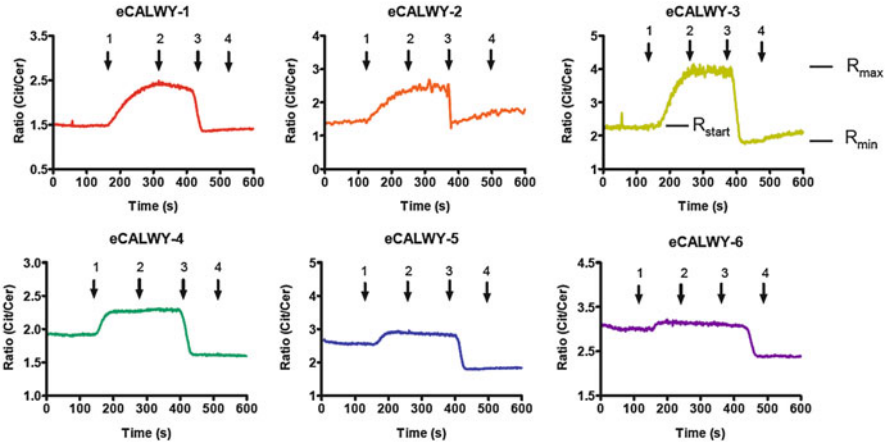

Fig. 7.2 Responses of single INS-1(832/13) cells expressing eCALWY-1–6 to addition of 50 μM TPEN (*1*), 5 μM pyrithione (*2*), 5 μM pyrithione/100 μM Zn^{2+} (*3*), and no additives (*4*). Traces show the responses of individual cells. In the response trace of eCALWY-3, the emission ratios used for R_{max}, R_{min}, and R_{start} are displayed. (Adapted from Vinkenborg et al. 2009)

change in emission ratio. As the interaction between the fluorescent domains competed with Zn^{2+} binding, the K_d for Zn^{2+} binding was attenuated by a factor of 10, resulting in a K_d of 2 pM at pH 7.1 (Fig. 7.1b) (Vinkenborg et al. 2009).

Without knowing the free cytosolic Zn^{2+} concentration in mammalian cells beforehand, eCALWY-1 was tested in HEK293 cells and INS-1(832/13) cells. Figure 7.2 shows the ratio of citrine to cerulean emission of a single cell using excitation of cerulean. Addition of the strong cell-permeable Zn^{2+} chelator TPEN results in an increase in emission ratio, consistent with dissociation of Zn^{2+} from the sensor. As expected, subsequent addition of the Zn^{2+} ionophore pyrithione and excess Zn^{2+} resulted in a decrease in emission ratio. This experiment showed that at the start of the experiment, the eCALWY-1 sensor was already fully occupied with Zn^{2+}, suggesting that the free cytosolic Zn^{2+} concentration was substantially higher than the 2 pM Zn^{2+} affinity of eCALWY-1. Therefore, a toolbox of eCALWY-based sensors was developed by systematically tuning the Zn^{2+} affinity of eCALWY-1. First, eCALWY-4 was created by introducing a single cysteine-to-serine mutation in the Zn^{2+}-binding pocket of the sensor, resulting in a 300-fold weakening of the affinity for Zn^{2+} ($K_d = 630$ pM) This mutation also abrogated Cu^{+} binding to the protein, as eCALWY-4 did not show any response up to micromolar Cu^{+} levels. Because the free cellular copper concentration has been estimated to be about 10^{-18} M (Wegner et al. 2010), Cu^{+} binding will not interfere with Zn^{2+} binding inside living cells. Further fine tuning of the Zn^{2+} affinity was achieved by shortening the flexible peptide linker between the metal-binding domains, yielding a series of Zn^{2+} sensors (eCALWY-1–6) with affinities ranging from low picomolar to low nanomolar and at least a twofold change in emission ratio upon Zn^{2+} binding at a physiological relevant pH (Table 7.1). Figure 7.2 shows the fluorescence

Table 7.1 Sensor properties of different Förster resonance energy transfer (FRET)-based Zn^{2+}

Sensor variant	Ratiometric change (in vitro)	K_d (pH 7.1)	Zn^{2+}-binding pocket
CALWY (Van Dongen et al. 2007)	15 %	0.2 pM	Cys_4
eCALWY-1 (Vinkenborg et al. 2009)	240 %	2 pM	Cys_4
eCALWY-2 (Vinkenborg et al. 2009)	270 %	9 pM	Cys_4
eCALWY-3 (Vinkenborg et al. 2009)	215 %	45 pM	Cys_4
eCALWY-4 (Vinkenborg et al. 2009)	250 %	630 pM	Cys_3
eCALWY-5 (Vinkenborg et al. 2009)	300 %	1,850 pM	Cys_3
eCALWY-6 (Vinkenborg et al. 2009)	200 %	2,900 pM	Cys_3
		0.5 μM (pH 6.0)	
redCALWY-1 (Lindenburg et al. 2013)	62 %	12.3 pM	Cys_4
redCALWY-4 (Lindenburg et al. 2013)	30 %	234 pM	Cys_3
ZifCY1 (Dittmer et al. 2009)	220 %	1.7 μM	Cys_2His_2
ZifCY2 (Dittmer et al. 2009)	400 %	160 μM	His_4
ZapCY1 (Qin et al. 2011)	130 %	2.5 pM	Cys_4
ZapCY2 (Qin et al. 2011)	70 %	811 pM	Cys_2His_2
ZapOC2 (Miranda et al. 2012)	12 % (in situ)	Nd	Cys_2His_2
ZinCh-9 (Evers et al. 2007)	360 %	213 nM (pH 8.0)	Cys_2
eZinCh-1 (Evers et al. 2007)	800 %	8.2 μM	Cys_2
		253 nM (pH 8.0)	
		250 μM (pH 6.0)	
CLY9-2His (Evers et al. 2008)	65 %	47 nM (pH 8.0)	2 His_6 tags

responses of INS-1(832/13) cells transiently expressing each of these six eCALWY variants upon addition of the membrane permeable Zn^{2+} chelator TPEN and subsequent treatment of Zn^{2+} and pyrithione. A consistent trend between sensor response and sensor affinity was observed, with the high-affinity eCALWY-1 being fully saturated whereas the sensor with the lowest affinity (eCALWY-6) was nearly empty at the start of the experiment.

For each of these sensors, the Zn^{2+} occupancy at the start of the experiment was calculated using Eq. (7.1). R_{max} and R_{min} are the steady-state ratios after TPEN and

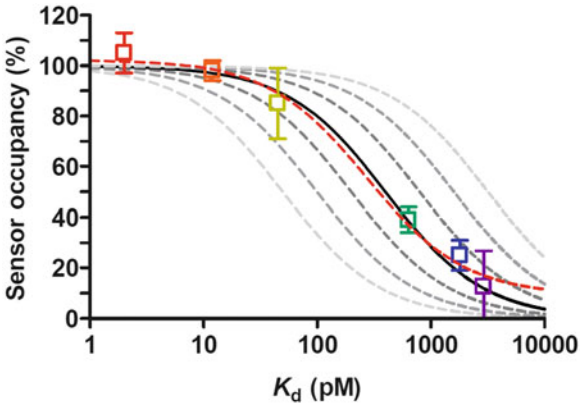

Fig. 7.3 Sensor occupancy in INS-1(832/13) cells as a function of the sensor K_d. Data points show the occupancy of the different eCALWY variants, determined from the traces of individual cells. A nonlinear least-squares fit yielded a free Zn^{2+} concentration of ~0.4 nM. The *dashed lines* depict the expected responses assuming free Zn^{2+} concentrations of 0.05, 0.1, 0.2, (0.4 *solid line*), 0.8, 1.6, and 3.2, respectively. (Adapted from Vinkenborg et al. 2009)

pyrithione/Zn^{2+} addition, respectively, and R_{start} is the ratio at the start of the experiment (Fig. 7.2).

$$\text{Occupancy} = \frac{R_{max} - R_{start}}{R_{max} - R_{min}} \cdot 100\ \% \tag{7.1}$$

Plotting the sensor occupancies for all six variants as a function of their K_d revealed that the sensor occupancies were consistent with a free Zn^{2+} concentration of about 0.4 nM (Fig. 7.3). The same results were obtained in mouse pancreatic beta cells (INS-1(832/13)) and in HEK293 cells. Although the cytosolic free Zn^{2+} concentration may also depend on cell type and conditions, subsequent work in other cell types and using these and other sensors (see following) has confirmed that the cytosolic Zn^{2+} in mammalian cells is relatively well-buffered between 100 pM and 1 nM. The observation that a single free Zn^{2+} concentration of 400 pM was sufficient to explain the occupancies of both high- and low-affinity sensors also indicates that the sensors do not significantly perturb the free Zn^{2+} concentration. In contrast to synthetic fluorescent Zn^{2+} sensors, which are added to the cells in relatively high concentrations at the time of the imaging experiment, genetically encoded fluorescent proteins are constitutively expressed and in this way become part of the cellular Zn^{2+} buffer machinery (Qin et al. 2013).

To gain further insight into the regulation of intracellular Zn^{2+} homeostasis and the possible role of Zn^{2+} as a secondary messenger, one would like to be able to monitor Zn^{2+} in different cellular compartments in the same cell at the same time or simultaneously monitor the relationship between Zn^{2+} concentration and other important signal transduction pathways such as Ca^{2+}, cAMP, and kinase activities. Therefore, spectrally distinct variants of the eCALWY sensors have recently been

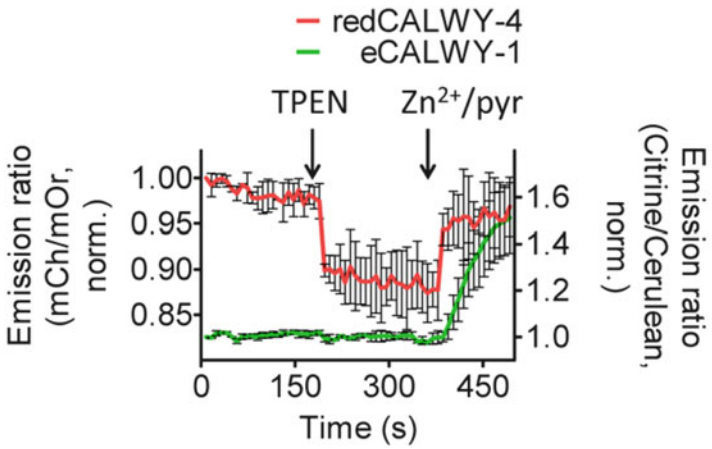

Fig. 7.4 Response of HeLa cells expressing both eCALWY-1 (*green*) and redCALWY-4 (*red*) to the addition of Zn^{2+}/pyrithione followed by excess TPEN. Traces represent the average of multiple cells after normalization of the emission ratio at $t = 0$. *Error bars* represent SEM. (Adapted from Lindenburg 2013)

developed that can be used together with CFP-YFP-based sensors (Lindenburg et al. 2013). These red fluorescent FRET sensors (redCALWYs) were obtained by replacing cerulean and citrine by mOrange and mCherry, respectively. Functional sensors were only obtained after reengineering the surface of both fluorescent domains to promote association of mOrange and mCherry in the Zn^{2+}-free state. Red versions were created of eCALWY-1 and eCALWY4.

In vitro characterization of redCALWY-1 and redCALWY-4 yielded K_d values of 12.3 ± 2 pM and 234 ± 5 pM, respectively (Table 7.1), which are comparable to the affinities of their CFP-YFP counterparts, showing that the replacement of the fluorescent domains had no effect on the ligand-binding properties. In situ characterization showed that the redCALWY-1 showed a response to addition of TPEN and Zn^{2+} similar to the original eCALWY-1 (Fig. 7.2), with both high-affinity sensors being completely saturated with Zn^{2+} under normal physiological conditions. Similarly, the lower-affinity redCALWY-4 was found to be mostly empty when expressed in the cytosol of HeLa cells. To explore the feasibility of using these redCALWY variants together with CFP-YFP-based systems, the high-affinity eCALWY-1 (CFP/YFP) was coexpressed with the lower-affinity redCALWY-4 in the cytosol of HeLa cells (Fig. 7.4). As expected, addition of Zn^{2+}/pyrithione to the cells resulted in a decrease in the redCALWY-4 emission ratio without affecting the eCALWY-1 emission ratio. Subsequent addition of TPEN resulted in a quick response for redCALWY-4, followed by a slower increase in emission ratio for the high-affinity eCALWY-1 sensor. In this case, simultaneous expression of spectrally distinct Zn^{2+} sensors with different affinities in the same cellular compartment increases the range over which the Zn^{2+} concentration can be monitored. More importantly, the experiment showed the feasibility of monitoring Zn^{2+}

concentrations at different subcellular localizations that otherwise cannot be easily distinguished, such as the ER and the cytosol.

In conclusion, the accurate determination of the cytosolic free Zn^{2+} concentration using the eCALWY sensors relies on several factors:

1. *The availability of sensors with a range of Zn^{2+} affinities*. Although the free Zn^{2+} concentration can be determined based on the occupancy of a single Zn^{2+} sensor and its in vitro determined K_d, such determinations are inherently less reliable than measurements based on sensors with different affinities, particularly when the sensor's affinity is not close to the free Zn^{2+} concentration. In the case of the eCALWY sensors, determination of the free Zn^{2+} concentration using eCALWY-4 is therefore more reliable than using for example eCALWY-2.
2. *Accurate determination of R_{max} and R_{min}*. The absolute emission ratio observed for cells expressing the same sensor may vary substantially between cells for example because of varying contributions of background fluorescence. Accurate determination of Zn^{2+} concentration therefore requires the determination of the emission ratio in the absence of Zn^{2+} (R_{max} in this case) and in the presence of Zn^{2+} (R_{min}). Accurate determination of R_{max} and R_{min} is helped in this case by the relatively fast association and dissociation kinetics of the sensor, allowing the establishments of stable plateau values within minutes following TPEN addition or even seconds after addition of Zn^{2+}/pyrithione.
3. *In situ calibration*. The calculation of the free Zn^{2+} concentration is based on the experimentally observed occupancies and the K_d of the sensor, which is most accurately determined in vitro. However, to rule out the possibility that the Zn^{2+} affinity is strongly affected by the intracellular conditions such as macromolecular crowding, ideally the sensor's affinity is also determined in situ. In situ calibration of eCALWY-4 using the pore-forming protein α-toxin and a Zn^{2+} buffer solution revealed that the in situ K_d was only slightly lower than that obtained in vitro using purified sensor protein.

7.2.2.2 Sensors Based on Zinc Fingers: Zif- and Zap-Based FRET Sensors

Because zinc fingers (ZFs) display metal-dependent protein folding and they typically show a high affinity and specificity for Zn^{2+}, they provide an attractive class of Zn^{2+}-binding domains for FRET sensor development. Although Zn^{2+} in most ZF domains plays solely a structural role, several examples of ZnF domains have also been reported that act as Zn^{2+}-dependent transcriptional regulators. The Palmer group has developed FRET sensors based on several different zinc-finger domains, providing access to FRET sensors with a range of affinities. The first series of FRET sensors was constructed using a ZF domain derived from the mammalian transcription factor Zif268, which contains a Cys_2His_2 binding motif (Dittmer et al. 2009). This well-characterized ZF is known to be largely unstructured in the absence of the metal ion and only folds upon Zn^{2+} binding. Two sensors were constructed by flanking the ZF domain with CFP and YFP, one containing the

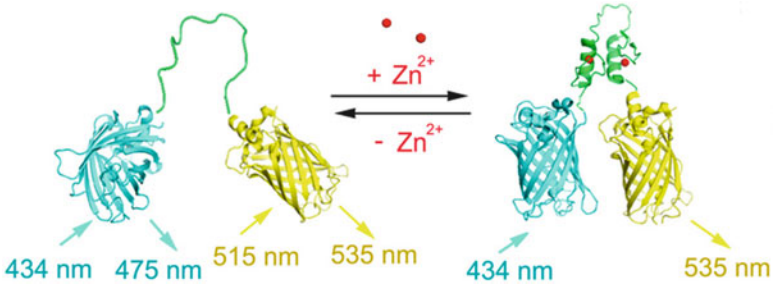

Fig. 7.5 Schematic representation of the high-affinity Zn^{2+} sensor ZapCY1, which consists of the first and second zinc finger of *Saccharomyces cerevisiae* Zap1 flanked by two fluorescent proteins, truncated CFP and citrine. (Adapted from Qin et al. 2013)

wild-type zinc-finger domain (ZifCY1) and a variant in which the cysteines in the original Cys2His2 motif were replace by two other histidines (ZifCY2). In vitro, a large increase in emission ratio was observed for both the ZifCY1 (2.2-fold) and the ZifCY2 (4-fold) sensors upon addition of Zn^{2+}. These in vitro Zn^{2+} titration experiments revealed Zn^{2+} affinities of 1.7 and ~160 μM for the ZifCY1 and ZifCY2 sensors, respectively. This affinity of the ZifCY1 sensor was surprisingly weak compared to the original Zif268 domain that contains a site with nanomolar affinity for Zn^{2+}. The origin of this large attenuation of Zn^{2+} affinity is not well understood but was attributed to the attachment of the fluorescent proteins. Both sensors were also tested by transiently expressing them in mammalian cell lines and monitoring their response to the addition of TPEN, followed by addition of digitonin (to permeabilize the cells) and subsequent Zn^{2+} addition. Unfortunately, the dynamic range of the ZifCY1 sensor was reduced to 25 %, possibly reflecting the effect of molecular crowding on decreasing the distance between the two fluorophores in the absence of zinc, resulting in an overall smaller ratiometric change. Despite the low Zn^{2+} affinity, the response of the ZifCY1 sensor was still used to estimate the cytosolic free Zn^{2+} concentration to be approximately 180 nM. This number merely reflected the lower limit of detection for this sensor, however, which also explained why higher sensor concentrations resulted in an apparent increase in the estimated intracellular Zn^{2+} level. This example illustrates the difficulty of measuring analyte concentrations that are outside the affinity range of a sensor, as the cytosolic Zn^{2+} concentration was subsequently shown to be 1,000 fold lower.

To overcome the low affinity and limited in situ dynamic range of the Zif268-based FRET sensors, Palmer and coworkers subsequently developed a series of FRET sensors based on zinc-finger domains from the yeast transcriptional regulator Zap1 (Qin et al. 2011). Instead of just a single ZF domain, these so-called Zap sensors consist of the first and second zinc fingers of *Saccharomyces cerevisiae* Zap1, which have low nanomolar affinity for Zn^{2+} (Fig. 7.5). FRET sensors based on these ZFs were actually first reported by Eide and coworkers (Qiao et al. 2006), who used them to learn more about the kinetics of Zn^{2+} binding and release to the

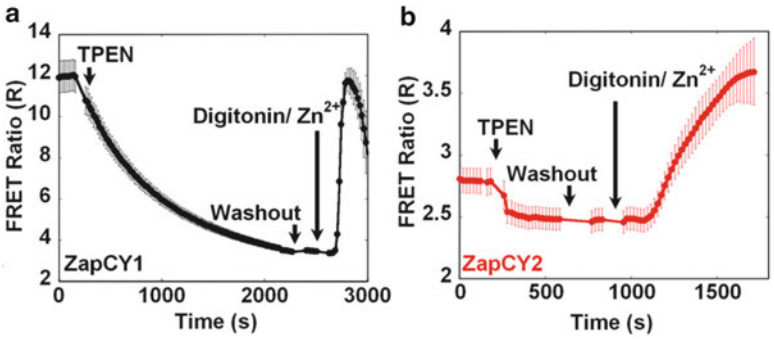

Fig. 7.6 FRET responses of ZapCY1 (**a**) and ZapCY2 (**b**) in the cytosol of HeLa cells ($n = 5$ cells). **a** ZapCY1 was fully saturated in the cytosol under resting conditions. **b** ZapCY2 was only partially saturated at the start of the experiment; free cytosolic Zn^{2+} concentration was estimated to be ~80 pM. (Adapted from Qin et al. 2011)

Zap1 zinc-finger domains. In the original study the sensors were not characterized in vitro and not used to measure the free Zn^{2+} concentration at the single-cell level. Palmer and coworkers improved these probes by introducing a truncated version of CFP and replacement of EYFP by the more pH stable citrine. To increase the dynamic range, the linker in the construct was also replaced by one previously used in genetically encoded Ca^{2+} sensors.

Determination of the Zn^{2+} affinity of the ZapCY1 sensor in vitro yielded a K_d of 2.5 pM at pH 7.4 (Fig. 7.6a). The Zn^{2+} affinity could be attenuated by replacing two of the cysteines in the zinc-finger domains by histidines again, yielding the ZapCY2 sensor, with a K_d of 811 pM. When ZapCY-1 was expressed in the cytosol of HeLa cells, a large fourfold decrease in emission ratio was observed upon treatment with a zinc-chelating reagent, which was completely reversed upon treatment of the cells with digitonin and excess Zn^{2+}. This response shows that the high-affinity ZapCY1 sensor was fully saturated under normal conditions. The lower-affinity ZapCY-2 sensor was only partially saturated in the cytosol under resting conditions (Fig. 7.6b), showed a 1.4-fold dynamic range, and could be used to estimate the free Zn^{2+} concentration in the cytosol to be ~80 pM, which is in the same range as determined using the eCALWY series.

A striking feature of the ZapCY-1 sensor is that prolonged incubations with TPEN are required to reach the Zn^{2+}-free state of the sensor. Part of this slow response may be an inherent feature of such high-affinity sensors, as the high-affinity eCALWY-1 sensor also showed a slower response than the intermediate-affinity eCALWY-4 sensor. Despite their similar affinities, the Zn^{2+}-free state of eCALWY-1 is reached within a few minutes, however, whereas this takes at least 30 min for ZapCY-1. The response of the lower-affinity ZapCY-2 to TPEN addition is much faster, making this the preferred Zap-based sensor for measuring cytosolic Zn^{2+} concentrations.

As for the eCALWY sensors, red-shifted variants have also been developed for the Zap-based FRET sensors. The CFP and YFP domains of the ZapCY1 and

ZapCY2 sensors were replaced by a variety of red-shifted donor and acceptor fluorescent domains, and sensor performance was tested for both cytosolic and nuclear-targeted sensors in HeLa cells, by doing an in situ calibration using TPEN and Zn^{2+}. Sensors with orange and red fluorescent domains, which are spectrally well separated from the CFP-YFP-based sensors, displayed relatively small changes in emission ratio of about 10 %. The highest in vivo dynamic range (40 %) was observed for sensors that used the green fluorescent protein Clover as donor and the red fluorescent protein mRuby2 as an acceptor. Using the latter sensors allowed the simultaneous measurement of Zn^{2+} concentration in different organelles, such as the nucleus and the cytosol, or the nucleus and the ER, Golgi, or mitochondria. The relatively modest dynamic range of the red-shifted sensor variants and the substantial spectral overlap between CFP/YFP- and Clover/mRuby2-based sensors precluded measurements in the same or overlapping intracellular compartments. The occupancies of the various sensors were sometimes found to be different, which suggests that replacing the fluorescent domains also affected Zn^{2+} affinity. Unfortunately, in vitro determination of the Zn^{2+} affinities of these new sensor variants was not reported.

7.2.2.3 eZinCh FRET Sensors and His-Tag-Based Sensors

The relatively high Zn^{2+} affinity of both the eCALWY and zinc-finger-based FRET sensors makes these the sensors of choice for imaging free cytosolic Zn^{2+} in mammalian cells. However, concentrations of free Zn^{2+} can differ substantially between cellular compartments, and the extracellular free Zn^{2+} concentration is known to be significantly higher. An example is provided by the insulin-secreting vesicles in pancreatic beta cells. The free Zn^{2+} concentrations in these vesicles was found to be high enough to completely saturate eCALWY-6, which at pH 6 binds Zn^{2+} with a K_d of 0.5 μM. Reliable measurements of these higher Zn^{2+} concentrations thus require the development of FRET sensors with sensitivities in the high nanomolar to micromolar region and sensors that cover a broader concentration range.

One example of these moderate-affinity sensors are the so-called ZinCh sensors (Evers et al. 2007). The ZinCh sensors do not contain separate Zn^{2+}-binding domains but consist of a fusion protein of ECFP and EYFP connected via a long flexible peptide linker of different lengths, in which two Zn^{2+}-coordinating amino acids (Y39H and S208C) were introduced at the dimer interface of both fluorescent domains (Fig. 7.7). A biphasic fourfold increase in emission ratio was observed upon addition of Zn^{2+}, corresponding to an increase in energy transfer efficiency from 50 % to 85 %. The first binding event ($K_d = 200$ nM at pH 8) involves the two Cys208 residues and results in formation of an intramolecular complex of ECFP and EYFP in a parallel orientation. After binding of Zn^{2+} to the high-affinity site, the two His39 are pre-organized to form a second, low-affinity Zn^{2+}-binding site ($K_{d2} \sim 88$ μM at pH 8.0), which results in a further increase in FRET. This sensor protein thus showed a large, fourfold increase in emission ratio over a broad range of Zn^{2+} concentrations between 100 nM and 1 mM. Importantly, ZinCh was shown

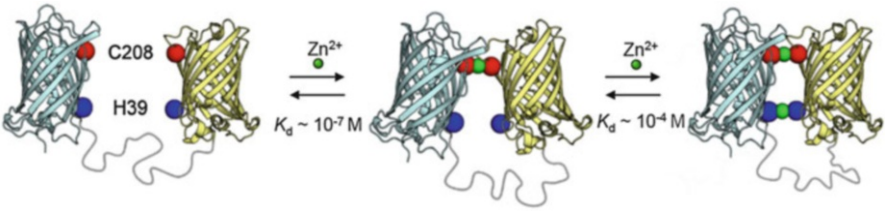

Fig. 7.7 Design of Zn^{2+}-chelating ECFP-EYFP chimera ZinCh-9. ECFP was fused to EYFP by a flexible $(GGSGGS)_9$ linker. A biphasic response was observed upon increasing Zn^{2+} concentrations, yielding a fourfold increase in emission ratio. (Adapted from Evers et al. 2007)

to be specific for Zn^{2+} over other divalent metal ions such as Cd^2, Ni^{2+}, Mg^2, and Ca^{2+}. More recently, the ECFP and EYFP domains were replaced by cerulean and citrine and the low-affinity site was deleted. This sensor, eZinCh-1, showed an even larger, eightfold increase in emission ratio and a similar Zn^{2+} affinity of $K_d = 258$ nM. The Zn^{2+} affinity of eZinCh-1 was also established under physiological relevant conditions, by determination of the Zn^{2+} affinity at pH 7.1 (cytosolic) and pH 6.0 (vesicular). Changing the pH from 8.0 to 7.1 resulted in a decrease in affinity to a K_d of 8.2 μM for Zn^{2+}, and K_d of ~250 μM at pH 6.0, most likely due to protonation of the cysteine residues ($pK_a = 8.3$) at lower pH. An initial effort to further increase the Zn^{2+} affinity of the eZinCh platform by introduction of additional flanking cysteine residues to create a tetrahedral Cys_4 site was not successful, as the Zn^{2+} affinities were in the same range as eZinch-1. This unimproved affinity suggests that only two of the four cysteines are involved in Zn^{2+} binding because the binding pocket is too large to tightly bind the Zn^{2+}. Indeed, Cd^{2+}, which has a larger ionic radius, was found to strongly bind to some of these Cys4 variants (Vinkenborg et al. 2011). Recently, our group found other eZinCh variants that display a substantially higher Zn^{2+} affinity, with a K_d of 1 nM at pH 7.1 and 200 nM at pH 6.0 (Hessels et al., unpublished results).

All the sensors discussed here rely at least partially on cysteines for Zn^{2+} binding, which renders these sensors redox sensitive and is the main reason for their pH sensitivity. Sensors based on histidine coordination are insensitive to oxidation and are predicted to be less sensitive to pH because of the lower pK_a of histidine. Based on the serendipitous discovery that Zn^{2+} forms a relatively stable 1:2 complex with His-tags, a FRET sensor was created by incorporating His-tags at the N- and C-termini of a fusion protein of ECFP and EYFP connected via a flexible peptide linker (Fig. 7.8). Addition of Zn^{2+} to this CLY9-2His sensor yielded a 1.6-fold increase in emission ratio, corresponding to a K_d of about 47 nM. The Zn^{2+} affinity of CLY9-2His is slightly higher than the afore-described ZinCh constructs. This sensor has not been used for intracellular imaging but has recently been applied to measure the free Zn^{2+} concentration in blood serum (Arts et al., unpublished results). This sensor variant may be improved further by replacing the ECFP and EYFP by cerulean and citrine and optimization of the linkers between the His-tags and the fluorescent domains. Such improved variants could be useful for intracellular imaging in oxidizing or acidic intracellular compartments.

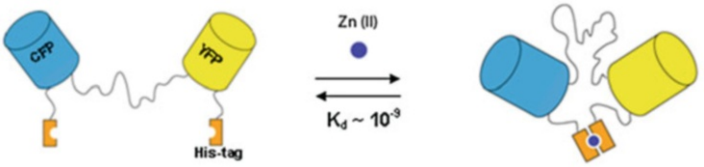

Fig. 7.8 Design of CLY9-2His containing both an N-terminal and a C-terminal His-tag. Zn^{2+} binding results in the formation of a compact intramolecular Zn^{2+} complex with a moderate Zn^{2+} affinity. (Adapted from Evers et al. 2008)

7.2.2.4 FRET Sensors Based on Binding of Fluorescently Labeled Sulfonamide to Carbonic Anhydrase

Thompson and coworkers have been developing fluorescent sensors based on carbonic anhydrase (CA), an enzyme with a single Zn^{2+}-binding site with picomolar affinity at pH 7.5 (Bozym et al. 2006). Carbonic anhydrase is one of the best studied enzymes, and a wealth of mutations are available that allow tuning of its Zn^{2+} affinity and Zn^{2+}-binding kinetics. In the original design, a FRET-based sensor was obtained by covalent attachment of Alexa Fluor 594 at a cysteine introduced at position 36, which acted as an acceptor for fluorescence from dapoxyl-sulfonamide, a fluorescent sulfonamide that binds strongly to the active site Zn^{2+}. The ratio of fluorescence observed at 617 nm obtained using dapoxyl excitation at 365 nm and direct AF594 excitation at 594 nm was used as a measure of the Zn^{2+} occupancy of the CA domain. To allow intracellular uptake of the fluorescently labeled CA, the probe was fused to a TAT peptide (Fig. 7.9).

Dissociation constants were obtained by determining calibration curves both on the microscope and a steady-state fluorometer, yielding $K_d \sim 70$ and ~137 pM, respectively. PC-12 cells were incubated with apoTAT-H36C-AF594-CA and dapoxyl-sulfonamide to measure free zinc levels inside the cell. A free Zn^{2+} concentration of 5 pM was obtained by direct comparison of the excitation ratio observed in the cell and the calibration curves. However, because no changes in emission ratio were observed upon addition of excess Zn^{2+} or strong Zn^{2+} chelators, in situ calibration of the sensor was not possible in this case, making the measurements more susceptible to variations in background fluorescence. Moreover, the performance of this system relies on the formation of a ternary complex between sensor, Zn^{2+}, and sulfonamide and thus assumes the presence of saturating concentrations of the latter of 1 μM. A fully genetically encoded version of the sensor protein was subsequently developed by fusing CA to dsRed2 as a FRET acceptor. This variant no longer requires protein transfection using the TAT peptide and could be targeted to the mitochondria of PC12 cells (McCranor et al. 2012). In situ calibration in isolated mitochondria revealed a K_d of 0.15 pM, which was substantially lower than the 17 pM determined for wtCA-dsRed2 in buffer and 70 pM determined for H36C-AF594-CA. Direct comparison of the excitation ratio yielded an apparent free Zn^{2+} concentration in the mitochondria of 0.15 pM.

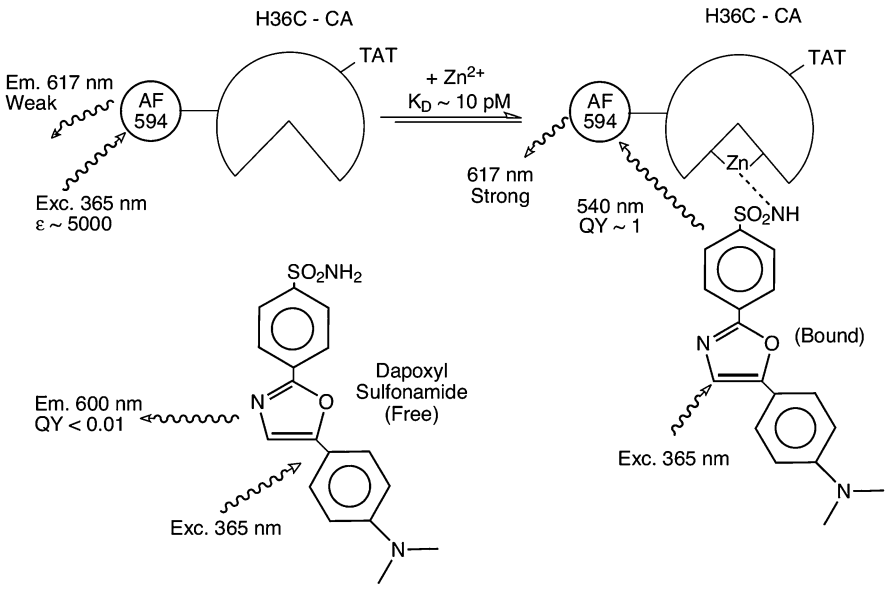

Fig. 7.9 Schematic representation of zinc ratiometric zinc determination with apoTAT-H36C-AlexaFluor 594 carbonic anhydrase and dapoxyl sulfonamide. In the absence of zinc, dapoxyl sulfonamide does not bind CA; therefore, no FRET occurs and very weak emission at 617 nm is observed. In the presence of zinc, dapoxyl sulfonamide binds to zinc, and FRET occurs from dapoxyl to the AlexaFluor 594. (Adapted from Bozym et al. 2006)

Both sensors just described are excitation ratiometric, which requires switching between two different lasers, and excitation of dapoxyl requires the use of 365-nm light, which may be damaging to the cells. Another sensor based on CA was therefore recently developed by the Thompson group (Zeng et al. 2013), in which the AF594 that is covalently attached to the CA acts as a donor. Chesapeake blue (CB) was coupled to the sulfonamide and can bind to the active protein in the presence of Zn^{2+}. In the absence of Zn^{2+}, the AF594 emits at 617 nm; when Zn^{2+} is bound to the protein, it promotes binding of the CB sulfonamide, resulting in emission at 650 nm. This red-shifted emission ratiometric sensor displayed a twofold change in emission ratio and bound Zn^{2+} with a $K_d \sim 5.8 \pm 3.1$ pM. The CB sulfonamide is highly charged, however, which prevents its application in intracellular imaging.

7.3 Applications of Genetically Encoded Sensors

In the previous section, the various types of genetically encoded fluorescent Zn^{2+} sensors were introduced and their in vitro and initial in situ characterization was described. In this section we discuss various subsequent applications such as their targeting to various subcellular compartments and their application in different cell types and organisms.

7.3.1 Subcellular Targeting of Genetically Encoded Sensors

One of the key distinguishing features of genetically encoded sensors is the relative ease by which they can be targeted to specific subcellular locations. Genetically encoded FRET sensors have been instrumental in establishing the concentration of free Zn^{2+} in a variety of cell types, revealing that cytosolic Zn^{2+} is well buffered between 0.1 and 1 nM. Successful determination of cytosolic Zn^{2+} concentrations critically depended on the availability of FRET sensors with the appropriate K_d and a robust calibration procedure to determine for each individual cell the emission ratio corresponding to Zn^{2+}-free and Zn^{2+}-bound state. In an effort to determine the free Zn^{2+} concentration in other organelles, several of the ZF-based FRET sensors developed in the Palmer laboratory were targeted to the ER, Golgi, and the mitochondrial matrix. Both the high-affinity ZapCY1 ($K_d \sim 2.5$ pM) and the low-affinity ZifCY1 ($K_d \sim 1.7$ μM) sensor were targeted to the lumen of the ER and the inner surface of the Golgi membrane (Qin et al. 2011). The targeting was confirmed by colocalization using commercially available markers to the desired organelles. HeLa cells expressing ER-ZapCY1 showed a slow and small decrease in emission ratio following addition of TPEN (Fig. 7.10a), followed by a much larger increase in emission ratio upon addition of excess Zn^{2+}. For cells expressing the low-affinity ER-ZifCY1 (Fig. 7.10b), no decrease in FRET was observed upon TPEN addition, suggesting that the free Zn^{2+} level in the ER was below the detection limit of this sensor. Equation 7.1 was used to calculate the free Zn^{2+} concentration in the ER under resting conditions by using R_{min} and R_{max} for calibration, yielding a free Zn^{2+} in the ER of 0.9 pM. The same experiments were performed in the Golgi (Fig. 7.10c, d), yielding a similarly low free Zn^{2+} of 0.6 pM.

These data would suggest that the free Zn^{2+} concentration in the ER and Golgi of mammalian cells is maintained at an even lower concentration than is present in the cytosol. One potential caveat of these measurements is the slow rate of Zn^{2+} release from the sensor upon TPEN addition, which makes it more difficult to accurately determine R_{min}. Very different results were recently obtained by the Rutter and Merkx groups, who used ER-targeted eCALWY-4 to probe the Zn^{2+} concentration in the ER. This probe, which has an affinity of 600 pM, was found to be mostly saturated with Zn^{2+} in a variety of cell lines and primary cardiomyocytes (Chabosseau et al. 2014). These results would suggest that the free Zn^{2+} concentration in the ER is at least 5 nM, which would be consistent with recent suggestions that the ER acts as a store for Zn^{2+} (Taylor et al. 2012). Taylor and coworkers recently proposed a model whereby phosphorylation of ZIP7 plays a role in release of Zn^{2+} from ER stores into the cytosol.

FRET sensors have also been targeted to the mitochondrial matrix. Palmer and coworkers appended an N-terminal mitochondrial targeting sequence from human cytochrome *c* oxidase subunit 8a to both Zif- and Zap-based FRET sensors, yielding

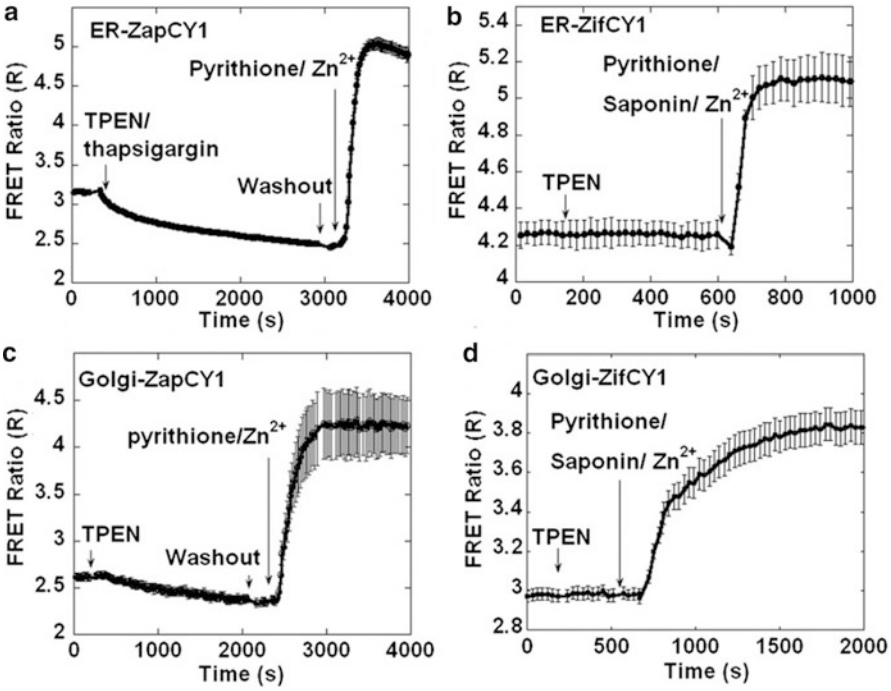

Fig. 7.10 Representative traces for ER-ZapCY1 (**a**) and ER-ZifCY1 (**b**). **a** The FRET ratio decreased upon addition of 150 μM TPEN/10 μM thapsigargin and increased with addition of 5 μM pyrithione and 10 nM Zn^{2+}. **b** No change in FRET ratio was observed upon TPEN addition, but addition of 25 μM pyrithione/saponin/500 μM Zn^{2+} resulted in an increase in emission ratio. Representative traces for Golgi-ZapCY1 (**c**) and Golgi-ZifCY1 (**d**). **c** The FRET ratio decreased upon addition of 150 μM TPEN and increased with addition of 5 μM pyrithione and 10 nM Zn^{2+}. **d** No change in FRET ratio was observed upon TPEN addition, but addition of 25 μM pyrithione/saponin/500 μM Zn^{2+} resulted in an increase in emission ratio. (Adapted from Qin et al. 2011)

mito-ZapCY1 and mito-ZifCY1. Colocalization studies using MitoTracker Red confirmed excellent targeting. Based on measurements with the low-affinity mito-ZifCY1, the mitochondrial free Zn^{2+} concentration was initially reported to be 680 nM. However, this number was found to be unreliable because of the poor dynamic range of mito-ZifCY1 as new variants with a larger dynamic range were found to be essentially unsaturated (Park et al. 2012; Dittmer et al. 2009). Targeting of the high-affinity ZapCY1 sensor to the mitochondria showed a response to TPEN and Zn^{2+}/pyrithione addition that is very similar to that of ER- and Golgi-targeted Zap-CY1; that is, addition of TPEN induces a small decrease in emission ratio over a period of an hour, whereas subsequent addition of excess Zn^{2+} results in a much larger and rapid increase in emission ratio. Based on this response and correcting for the even higher affinity of ZapCY1 at the mitochondrial pH of 8, the free mitochondrial Zn^{2+} concentration was estimated to be 0.22 pM in HeLa cells.

Somewhat higher sensor occupancies of 50 % were observed in MIN6 and primary neurons. These surprisingly low concentrations are 300 fold lower than what was recently determined using a small-molecule Zn^{2+} fluorescent sensor (Xue et al. 2012) that contained a triphenylphosphonium (TPP) group as an effective mitochondrial targeting ligand. The K_d of this probe was determined to be about 150 pM at mitochondrial pH and a free mitochondrial Zn^{2+} concentration of about 72 pM was measured in NIH 3T3 cells. Recent measurements of mitochondrial Zn^{2+} using the eCALW-4 sensor targeted to the mitochondrial matrix consistently yielded free Zn^{2+} concentrations of 200–300 pM in a number of cell lines and primary cells (Chabosseau et al. 2014). Thus, the ER and mitochondrial zinc concentrations determined using the eCALWY probes are three orders of magnitude higher than calculated using the targeted ZapCY1 probes (Park et al. 2012; Qin et al. 2011). This discrepancy is likely not the result of an error in determining the Zn^{2+} affinities of one of the sensors, as the eCALWY sensors and ZapCY1/CY2 sensors give much more consistent results when applied in the cytosol. A striking feature of the ZapCY1 sensor is its slow dissociation kinetics, which required more than 1 h incubation in TPEN to achieve the fully Zn^{2+}-depleted state,(Qin et al. 2011) compared to 2–3 min for the equivalent eCALWY probe (Fig. 7.2). In general, rapid equilibration makes the determination of $R_{0\%}$ and $R_{100\%}$ less sensitive to baseline drift, and prolonged incubations with TPEN have also been reported to be cytotoxic (Hashemi et al. 2007; Donadelli et al. 2008) Nonetheless, why the eCALWY- and ZapCY-based sensors behave so differently when targeted to the ER and mitochondria remains to be explained. One way to resolve these discrepancies might be to use FRET sensors that have alternative binding mechanisms. The current sensors depend on cysteines for Zn^{2+} coordination, which may result in formation of disulfide bonds or misfolding in the oxidizing environment of the ER lumen.

Secretory vesicles in many cell types contain highly elevated Zn^{2+} concentrations including those involved in neurotransmission, the prostate, and the insulin-containing vesicles of pancreatic β cells. The only example of a genetically encoded sensor targeting a secretory vesicle has been reported by Vinkenborg et al. Both the high-affinity sensors eCALWY-1 and eCALWY-6 and the low-affinity eZinCh-1 sensor were targeted to the secretory granules of INS-1(832/13) cells by fusion to vesicle-associated membrane protein 2 (VAMP2). Zn^{2+} is known to be important for insulin storage and secretion, and stable insulin Zn^{2+} complexes are formed in the acidic (pH 6.0) interior of these vesicles. Mutations in Znt8, a Zn^{2+}-specific importer protein that is exclusively localized in the secretory granules, have also been linked to the development of diabetes (Murgia et al. 2009). Colocalization studies of these vesicular-targeted constructs showed exclusive localization in insulin-containing granules (Fig. 7.11a). Emission ratios of the cells expressing either one of the sensors were monitored; for the eCALWY variants, low emission ratios were observed, indicating that the sensors were already fully saturated with Zn^{2+} at the start of the experiment. On the other hand, the low affinity eZinCh-1 appeared to be empty at this stage. None of the sensors showed changes in emission ratio upon addition of either TPEN or Zn^{2+}/pyrithione, suggesting that at high free

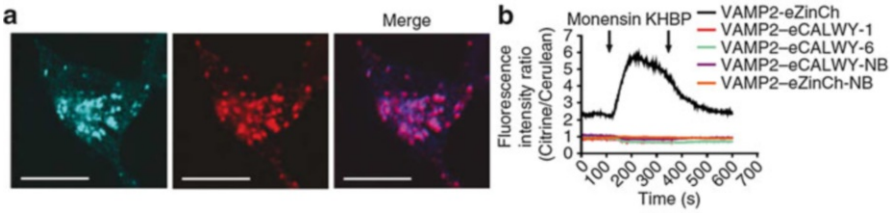

Fig. 7.11 Subcellular targeting of Zn^{2+} probes to insulin-storing vesicles. **a** Confocal laser microscopy images of INS-1(832/13) cells transfected with plasmids encoding VAMP2-eCALWY-1 (*left*) and neuropeptide Y-mCherry (*middle*). **b** Ratiometric response of INS-1 (832/13) cells expressing different VAMP2 constructs to 10 μM monensin (*1*), followed by buffer without compounds (*2*). (Adapted from Vinkenborg et al. 2009)

Zn^{2+} concentrations TPEN is unable to remove the Zn^{2+} from the high-affinity eCALWY variants ($K_d = 0.5$ μM at pH 6.0 for eCALWY-6). At the same time, it is probably also difficult to raise the free Zn^{2+} concentration inside the vesicles to the millimolar (mM) concentrations that are required to saturate the low-affinity eZinCh-1 sensor (K_d 250 μM at pH 6.0; Table 7.1). To test the hypothesis that eZinCh-1 was indeed nearly empty because of the low vesicular pH, cells were treated with the Na^+/H^+ exchanger monensin, which transiently increases the pH from 6 to 7. As expected, a reversible increase in emission ratio was observed for cells expressing VAMP2-eZinCh-1 as a result of simultaneous Zn^{2+} release from the insulin-Zn^{2+} and increase in affinity of eZinCh-1 (Fig. 7.11b). Although this experiment established the functionality of vesicular-targeted eZinCh-1, it also showed that accurate determination of (fluctuations in) vesicular free Zn^{2+} concentrations requires the development of less pH sensitive FRET sensors with a Zn^{2+} affinity of about 10 μM at pH 6.0.

7.3.2 Applications in Primary Cells and Plants

Genetically encoded fluorescent Zn^{2+} sensors have been mostly used in cell lines using transient transfection. To study Zn^{2+} homeostasis in primary pancreatic β cells, Rutter and coworkers cloned several eCALWY-variants into a pShuttle vector to allow transfection of primary cells using adenovirus (Bellomo et al. 2011). pShuttle-eCALWY constructs were digested with Pme1 and electroporated into competent BJ5183-AD-1 cells. Recombined pADeasy1 clones were screened, and positive clones were then digested with Pac1 and transfected into HEK293 cells for the generation of adenoviral particles. Primary islet cells were infected with eCALWY-4-expressing adenovirus and used to measure free cytosolic Zn^{2+}. High glucose concentrations were found to induce an increase in the cytosolic free Zn^{2+} concentration. Cytosolic free Zn^{2+} concentrations were found to be twofold higher in cells 24 h after being treated with high glucose concentrations (16.7 mM) compared to cells treated with low (3 mM) glucose concentrations,

853 pM Zn^{2+} versus 452 pM Zn^{2+}, respectively. These findings are in contrast with the work done by Zalewski et al., who observed a decrease in cytosolic Zn^{2+} in islet cells in response to glucose (Zalewski et al. 1994). They used a synthetic probe (Zinquin) to perform their measurements, however, which partly localizes in granules and other membrane-bound organelles where the free Zn^{2+} concentration is higher than in the cytosol. Therefore, the observed free Zn^{2+} concentrations and changes may be caused by degranulation of cells in response to glucose. Other primary cells in which FRET sensors have been used include primary neurons (Park et al. 2012) and cardiomyocytes (Chabosseau et al. 2014).

Several techniques have been used to investigate cellular distribution of total Zn^{2+} in plants, but until recently genetically encoded sensors have not been used in plants (Lanquar et al. 2005). The chemical probe Zinpyr-1 was used to study the role of Zn^{2+} in plants (Sinclair et al. 2007), but several issues were apparent, including problems with cellular penetration, control over intracellular localization, and long-term imaging possibilities. The Frommer group therefore recently constructed transgenic *Arabidopsis thaliana* lines that each constitutively express different cytosolic eCALWY variants (Lanquar et al. 2014). Low or no fluorescence was observed in the Col-O ecotype, indicating transgene-induced silencing. Therefore, the eCALWY variants were also expressed in the rgr6 line, which is deficient in transgene-induced silencing. Transgenic plant lines showing high and homogeneous fluorescence in this genetic background were selected. These eCALWY-expressing *Arabidopsis* lines looked normal and healthy and did not show a change in bulk Zn concentration. To avoid background fluorescence and easy imaging, Zn^{2+} homeostasis was imaged in root cells using the so-called RootChip setup. To determine the cytosolic Zn^{2+} concentration, the sensor occupancy was measured for each sensor as a function of the K_d of the sensor. For the plants grown in the presence of normal concentrations of Zn^{2+} (5 μM), the free cytosolic Zn^{2+} concentration was determined to be about 420 pM, the same value was found in mammalian cells using the same FRET sensors (Fig. 7.12). A concentration of about 2 nM was found for plants grown at excess Zn^{2+}, and the estimated free cytosolic Zn^{2+} concentration in starved root cells was about 1.5 nM, although a high standard error was observed under Zn^{2+}-depleted conditions. Monitoring the dynamic response of cytosolic Zn^{2+} to external supply suggested the involvement of high- and low-affinity uptake systems as well as release from internal stores.

7.4 Conclusion

Genetically encoded Zn^{2+} sensors have proven themselves as a valuable alternative to the use of synthetic fluorescent dyes for monitoring intracellular Zn^{2+} homeostasis and signaling. FRET-based fluorescent sensor proteins are attractive because they do not require cell-invasive procedures, they allow ratiometric detection, their concentration can be tightly controlled, and they can be targeted to different locations in the cell. FRET-based sensors developed by our group and others

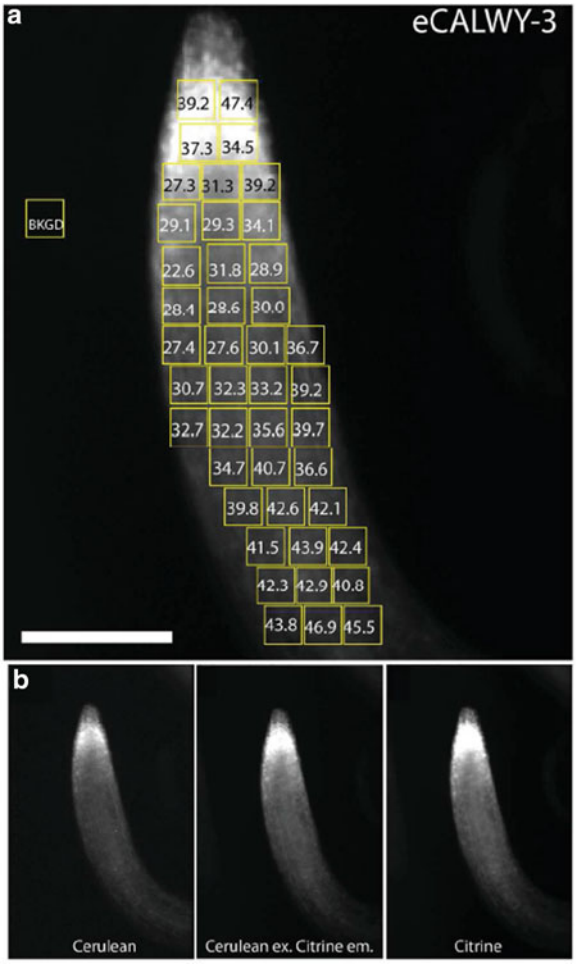

Fig. 7.12 Images displaying the expression of eCALWY-3 in *Arabidopsis thaliana* roots. **a** The numbers reflect sensor occupancy values calculated for each region of interest. **b** Expression pattern of the sensor in each channel for the same root: cerulean emission (*left*), citrine emission upon cerulean excitation (*middle*), and citrine emission (*right*). (Adapted from Lanquar et al. 2014)

have been instrumental to establish that the cytosolic levels of free zinc in mammalian cells are tightly regulated at around 0.5 nM. An important recent addition to the toolbox of sensors is the development of red-shifted variants, which permits the simultaneous imaging of Zn^{2+} in different cellular compartments such as the ER and the cytosol, but also provides an opportunity to study the spatiotemporal relationship between Zn^{2+} signaling and other intracellular signaling pathways. Targeting of FRET sensors to organelles has revealed conflicting results, with one sensor type (ZapCY-1) reporting extremely low levels of free Zn^{2+} in the ER, Golgi, and mitochondria, whereas much higher levels (>1,000 fold) have been observed for the eCALWY system when targeted to the ER and mitochondria in different cell lines and primary cells. These conflicting results may be resolved by the development of new FRET sensors based on different binding mechanisms. Other items on the to-do list include the development of probes that are redox

insensitive, less pH sensitive, and have affinities tuned to specific applications, such as measuring Zn^{2+} in secretory vesicles, extracellular Zn^{2+} in the brain, and Zn^{2+} in blood plasma. Their genetic encoding should allow easy distribution of these probes throughout the research community and the generation of transgenic organisms to study Zn^{2+} homeostasis and signaling in a variety of model organisms.

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References

Barondeau DP, Kassmann CJ, Tainer JA, Getzoff ED (2002) Structural chemistry of a green fluorescent protein Zn biosensor. J Am Chem Soc 124:3522–3524

Bellomo EA, Meur G, Rutter GA (2011) Glucose regulates free cytosolic Zn^{2+} concentration, Slc39 (Zip), and metallothionein gene expression in primary pancreatic islet beta-cells. J Biol Chem 286:25778–25789

Bozym RA, Thompson RB, Stoddard AK, Fierke CA (2006) Measuring picomolar intracellular exchangeable zinc in Pc-12 cells using a ratiometric fluorescence biosensor. ACS Chem Biol 1:103–111

Chabosseau P, Tuncay E, Meur G, Bellomo EA, Hessels, AM, Hughes S, Johnson PRV, Bugliani M, Marchetti P, Turan B, Lyon AR, Merkx M, Rutter GA (2014) ACS Chem Biol in press (doi:10.1021/cb5004064)

Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. J Biol Chem 281:24085–24089

Dittmer PJ, Miranda JG, Gorski JA, Palmer AE (2009) Genetically encoded sensors to elucidate spatial distribution of cellular zinc. J Biol Chem 284:16289–16297

Domaille DW, Que EL, Chang CJ (2008) Synthetic fluorescent sensors for studying the cell biology of metals. Nat Chem Biol 4:168–175

Donadelli M, Dalla Pozza E, Costanzo C, Scupoli MT, Scarpa A, Palmieri M (2008) Zinc depletion efficiently inhibits pancreatic cancer cell growth by increasing the ratio of antiproliferative/proliferative genes. J Cell Biochem 104:202–212

Evers TH, Appelhof MA, De Graaf-Heuvelmans PT, Meijer EW, Merkx M (2007) Ratiometric detection of Zn(II) using chelating fluorescent protein chimeras. J Mol Biol 374:411–425

Evers TH, Appelhof MA, Meijer EW, Merkx M (2008) His-tags as Zn(II) binding motifs in a protein-based fluorescent sensor. Protein Eng Des Sel 21:529–536

Hashemi M, Ghavami S, Eshraghi M, Booy EP, Los M (2007) Cytotoxic effects of intra and extracellular zinc chelation on human breast cancer cells. Eur J Pharmacol 557:9–19

Ho LH, Riuffin RE, Murgia C, Li XL, Krilis SA, Zalewski PD (2004) Labile zinc and zinc transporter Znt4 in mast cell granules: role in regulation Nf-Kb translocation. J Immunol 172:7750–7760

Hutton JC, Penn EJ, Peshavaria M (1983) Low-molecular weight constituents of isolated insuline-secretory granules: bivalent-cations, adenine-nucleotides and inorganic phosphates. Biochem J 210:297–305

Kikuchi K (2010) Design, synthesis and biological application of chemical probes for bio-imaging. Chem Soc Rev 39:2048–2053

Krezel A, Maret W (2006) Zinc-buffering capacity of a eukaryotic cell at physiological P_{zn}. J Biol Inorg Chem 11:1049–1062

Lanquar V, Lelievre F, Bolte S, Hames C, Alcon C, Neumann D, Vansuyt G, Curie C, Schroder A, Kramer U, Barbier-Brygoo H, Thomine S (2005) Mobilization of vacuolar iron by Atnramp3 and Atnramp4 is essential for seed germination on low iron. EMBO J 24:4041–4051

Lanquar V, Grossmann G, Vinkenborg JL, Merkx M, Thomine S, Frommer WB (2014) Dynamic imaging of cytosolic zinc in *Arabidopsis* roots combining fret sensors and rootchip technology. New Phytol 202:198–208

Lindenburg LH, Hessels AM, Ebberink EH, Arts R, Merkx M (2013) Robust red FRET sensors using self-associating fluorescent domains. ACS Chem Biol 8:2133–2139

Linkous DH, Flinn JM, Koh JY, Lanzirotti A, Bertsch PM, Jones BF, Giblin LJ, Frederickson CJ (2008) Evidence that the Znt3 protein controls the total amount of elemental zinc in synaptic vesicles. J Histochem Cytochem 56:3–6

Liu J, Karpus J, Wegner SV, Chen PR, He C (2013a) Genetically encoded copper(I) reporters with improved response for use in imaging. J Am Chem Soc 135:3144–3149

Liu X, Li J, Hu C, Zhou Q, Zhang W, Hu M, Zhou J, Wang J (2013b) Significant expansion of the fluorescent protein chromophore through the genetic incorporation of a metal-chelating unnatural amino acid. Angew Chem Int Ed Engl 52:4805–4809

McCranor BJ, Bozym RA, Vitolo MI, Fierke CA, Bambrick L, Polster BM, Fiskum G, Thompson RB (2012) Quantitative imaging of mitochondrial and cytosolic free zinc levels in an in vitro model of ischemia/reperfusion. J Bioenerg Biomembr 44:253–263

Miranda JG, Weaver AL, Qin Y, Park JG, Stoddard CI, Lin MZ, Palmer AE (2012) New alternately colored fret sensors for simultaneous monitoring of Zn^{2+} in multiple cellular locations. PLoS One 7:E49371

Mizuno T, Murao K, Tanabe Y, Oda M, Tanaka T (2007) Metal-ion-dependent GFP emission in vivo by combining a circularly permutated green fluorescent protein with an engineered metal-ion-binding coiled-coil. J Am Chem Soc 129:11378–11383

Murgia C, Devirgiliis C, Mancini E, Donadel G, Zalewski P, Perozzi G (2009) Diabetes-linked zinc transporter Znt8 is a homodimeric protein expressed by distinct rodent endocrine cell types in the pancreas and other glands. Nutr Metab Cardiovasc Dis 19:431–439

Nolan EM, Lippard SJ (2009) Small-molecule fluorescent sensors for investigating zinc metalloneurochemistry. Acc Chem Res 42:193–203

Park JG, Qin Y, Galati DF, Palmer AE (2012) New sensors for quantitative measurement of mitochondrial Zn^{2+}. ACS Chem Biol 7:1636–1640

Qiao W, Mooney M, Bird AJ, Winge DR, Eide DJ (2006) Zinc binding to a regulatory zinc-sensing domain monitored in vivo by using fret. Proc Natl Acad Sci USA 103:8674–8679

Qin Y, Dittmer PJ, Park JG, Jansen KB, Palmer AE (2011) Measuring steady-state and dynamic endoplasmic reticulum and Golgi Zn^{2+} with genetically encoded sensors. Proc Natl Acad Sci USA 108:7351–7356

Qin Y, Miranda JG, Stoddard CI, Dean KM, Galati DF, Palmer AE (2013) Direct comparison of a genetically encoded sensor and small molecule indicator: implications for quantification of cytosolic Zn^{2+}. ACS Chem Biol 8:2366–2371

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

Sinclair SA, Sherson SM, Jarvis R, Camakaris J, Cobbett CS (2007) The use of the zinc-fluorophore, zinpyr-1, in the study of zinc homeostasis in *Arabidopsis* roots. New Phytol 174:39–45

Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P (2012) Protein kinase Ck2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel Zip7. Sci Signal 5:ra11

Valko M, Morris H, Cronin MT (2005) Metals, toxicity and oxidative stress. Curr Med Chem 12:1161–1208

Van Dongen EM, Dekkers LM, Spijker K, Meijer EW, Klomp LW, Merkx M (2006) Ratiometric fluorescent sensor proteins with subnanomolar affinity for Zn(II) based on copper chaperone domains. J Am Chem Soc 128:10754–10762

Van Dongen EMWM, Evers TH, Dekkers LM, Meijer EW, Klomp LWJ, Merkx M (2007) Variation of linker length in ratiometric fluorescent sensor proteins allows rational tuning of Zn(II) affinity in the picomolar to femtomolar range. J Am Chem Soc 129:3494–3495

Vinkenborg JL, Nicolson TJ, Bellomo EA, Koay MS, Rutter GA, Merkx M (2009) Genetically encoded fret sensors to monitor intracellular Zn^{2+} homeostasis. Nat Methods 6:737–740

Vinkenborg JL, Van Duijnhoven SM, Merkx M (2011) Reengineering of a fluorescent zinc sensor protein yields the first genetically encoded cadmium probe. Chem Commun (Camb) 47:11879–11881

Wegner SV, Arslan H, Sunbul M, Yin J, He C (2010) Dynamic copper(I) imaging in mammalian cells with a genetically encoded fluorescent copper(I) sensor. J Am Chem Soc 132:2567–2569

Xue L, Li G, Yu C, Jiang H (2012) A ratiometric and targetable fluorescent sensor for quantification of mitochondrial zinc ions. Chemistry 18:1050–1054

Zalewski PD, Millard SH, Forbes IJ, Kapaniris O, Slavotinek A, Betts WH, Ward AD, Lincoln SF, Mahadevan I (1994) Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc. J Histochem Cytochem 42:877–884

Zeng HH, Matveeva EG, Stoddard AK, Fierke CA, Thompson RB (2013) Long wavelength fluorescence ratiometric zinc biosensor. J Fluoresc 23:375–379

Chapter 8
Zinc Signal in Brain Functions

Atsushi Takeda

Abstract Brain zinc homeostasis is strictly controlled under healthy conditions, indicating the importance of zinc for physiological functions in the brain. Zinc in the synaptic vesicles is released from glutamatergic (zincergic) neuron terminals and serves as a signal factor (Zn^{2+} signal) in the intracellular (cytosol) compartment, in addition to the extracellular compartment. The synaptic Zn^{2+} signal is dynamically linked to neurotransmission and participates in synaptic plasticity such as long-term potentiation (LTP) and cognitive function. The hypothalamic-pituitary-adrenal (HPA) axis activity, that is, glucocorticoid secretion, is linked to cognitive function and can potentiate glutamatergic neuron activity. The modification of synaptic Zn^{2+} signal by the HPA axis activity, which is enhanced by stress and aging, is likely to be linked to cognitive function. On the other hand, abnormal Zn^{2+} influx into postsynaptic neurons, which is induced by the abnormal glutamatergic (zincergic) neuron activity, induces neuronal death and is involved in neurological disorders such as stroke/ischemia, temporal lobe epilepsy, Alzheimer's disease, and amyotrophic lateral sclerosis. Therefore, the homeostasis of synaptic Zn^{2+} signal is critical in both functional and pathological aspects. This chapter summarizes the physiological significance of intracellular Zn^{2+} signaling in brain functions, especially in cognition.

Keywords Excitotoxicity • Glucocorticoid • Glutamate • Hippocampus • HPA axis • Memory • Synaptic Zn^{2+} • Zinc deficiency

8.1 Introduction

The importance of zinc in human health has been widely reported (Frederickson et al. 2005; Cole and Lifshitz 2008; Maret and Sandstead 2008). Zinc deficiency in children is a nutritional and health problem in both developing and developed countries (Black 1998; Bryan et al. 2004). Approximately 50 % of the world population is at risk of zinc deficiency (Brown et al. 2001), and 10 % of the

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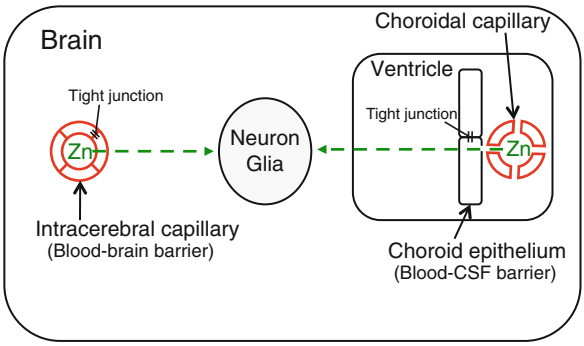

Fig. 8.1 Zinc transport into the brain parenchyma cells through the brain barrier system. Blood–brain and the blood–cerebrospinal fluid (CSF) barriers exist in the tight junction between brain capillary endothelial cells and the tight junction between the choroidal epithelial cells, respectively

North American population consumes less than half of the recommended daily allowance for zinc (King et al. 2000).

More than 300 proteins require zinc for their functions in microorganisms, plants, and animals. Zinc powerfully influences cell division and differentiation (Vallee and Falchuk 1993; Prasad 2008). Zinc is essential for brain growth and its function. Zinc concentration in the adult brain reaches around 200 μM (Markesbery et al. 1984). Extracellular zinc concentration in the adult brain is estimated to be much less than 1 μM (Weiss et al. 2000). Zinc concentration in the cerebrospinal fluid (CSF) is approximately 0.15 μM (Hershey et al. 1983), and that in the plasma is approximately 15 μM. Zinc transport from the plasma to the brain extracellular fluid and the cerebrospinal fluid is strictly regulated by the brain barrier system, that is, the blood–brain and blood–CSF barrier (Fig. 8.1). The brain barrier system serves for zinc homeostasis in the brain (Takeda 2000, 2001). Zinc homeostasis in the brain is closely associated with neurological diseases (Capasso et al. 2005; Mocchegiani et al. 2005) and is spatiotemporally altered in the process of neurological disease (Barnham and Bush 2008).

Approximately 80 % of the total brain zinc exists as zinc metalloproteins and the rest is histochemically reactive, as revealed by Timm's sulfide-silver staining method (Frederickson 1989; Frederickson and Danscher 1990), based on the data that the removal of zinc transporter (ZnT)3 protein, which is responsible for the movement of zinc from the cytoplasm into synaptic vesicles (Palmiter et al. 1996), results in a 20 % reduction of the total amount of zinc in the brain (Cole et al. 1999). Zinc is relatively concentrated in the hippocampus and amygdala (Fig. 8.2b, c) (Takeda et al. 1995). Both regions are enriched with histochemically reactive zinc, and histochemically reactive zinc predominantly exists in the presynaptic vesicles and serves as a signal factor (Zn^{2+} signal) in the cytosolic compartment as well as the extracellular compartment. Zn^{2+} is released with glutamate in a calcium- and impulse-dependent manner from glutamatergic (zincergic) neuron terminals in the hippocampus (Fig. 8.3); all the mossy fibers, and approximately 50 % of the Schaffer collateral where strong staining by Timm's method shows zinc is contained in the presynaptic vesicles (Fig. 8.2d). In contrast, the perforant pathway is minimally stained by Timm's method. Synaptic Zn^{2+} signal serves as an

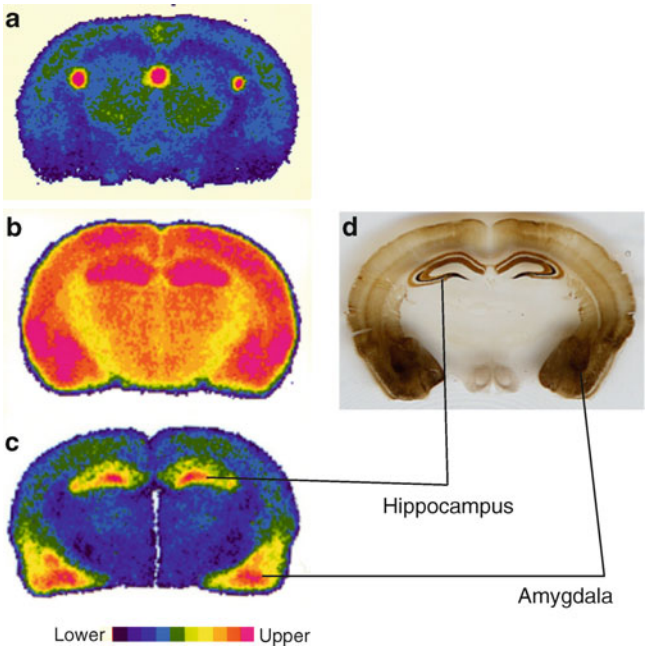

Fig. 8.2 ^{65}Zn distribution and Timm's stain in the brain. ^{65}Zn distribution in coronal sections 1 h (**a**), 12 days (**b**), and 30 days (**c**) after i.v. injection of $^{65}ZnCl_2$ into rats. (**d**) Timm's stain in a mouse coronal section. Note that ^{65}Zn concentrations are considerably correlated with Timm's stain intensity

endogenous neuromodulator of several important receptors including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor, *N*-methyl-D-aspartate (NMDA) receptors, and γ-amino butyric acid (GABA) receptors (Smart et al. 1994; Nakashima and Dyck 2009).

The hippocampus and amygdala participate in cognitive and emotional behavior. Synaptic plasticity such as long-term potentiation (LTP) is believed to be a cellular mechanism in learning and memory and has been widely studied at the glutamatergic synapses in the brain, especially in the hippocampus (Fig. 8.3). For processing of information in memory, glutamatergic neurons compose the neural circuit in the hippocampus and amygdala. The hypothalamic-pituitary-adrenal (HPA) axis activity, that is, glucocorticoid secretion, is linked to cognitive and emotional functions and can potentiate glutamatergic neuron activity (Sandi 2011). The modification of synaptic Zn^{2+} signal by HPA axis activity, which is enhanced by stress and aging, is likely to be linked to cognitive and emotional behavior (Takeda and Tamano 2009, 2012). On the other hand, abnormal Zn^{2+} influx into postsynaptic neurons, which is induced by the abnormal glutamatergic (zincergic) neuron activity, induces neuronal death and is involved in neurological disorders such as stroke/ischemia, temporal lobe epilepsy, Alzheimer's disease, and amyotrophic lateral sclerosis (Takeda 2011a). Therefore, the homeostasis of

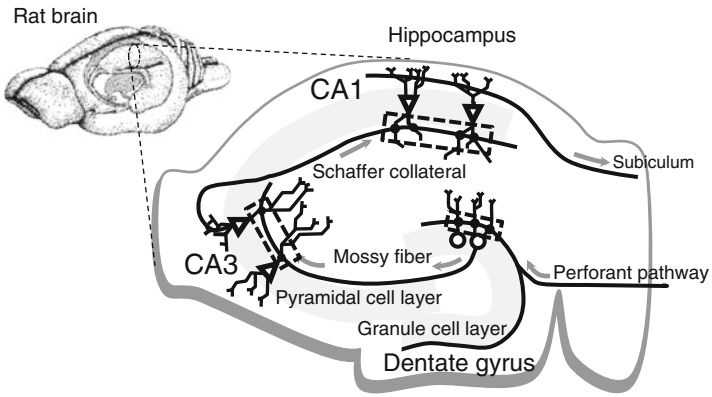

Fig. 8.3 Glutamatergic neural circuit in the hippocampus. Three synapses, i.e., the parforant pathway, mossy fiber, and Schaffer collateral synapses, where surrounded by *dotted lines* are glutamatergic. A subclass of the three synapses contains zinc in synaptic vesicles as described in the text

synaptic Zn^{2+} signal is critical for both functional and pathological aspects (Takeda 2011b; Takeda et al. 2013a). This chapter summarizes the physiological significance of intracellular Zn^{2+} signaling in brain functions, especially in cognitive and emotional behavior.

8.2 Zinc Homeostasis and the Brain Barrier System

Plasma zinc is largely bound to proteins such as albumin and is thought to be a major pool of zinc in the living body to transfer zinc to the tissues and organs including the brain. The regulation of zinc homeostasis in the living body is tightly controlled by both intestinal absorption and intestinal and renal excretions (King et al. 2000). Zrt-Irt-like proteins (ZIP), which are responsible for the transport of zinc into the cytoplasm, and the ZnT family, which are responsible for the transport of zinc out of the cytoplasm, are involved in the absorption and excretions of zinc to maintain zinc homeostasis in the living body (Cousins et al. 2006; Fukada and Kambe 2011). However, the absorption and excretions of zinc in the living body are synergistic, and the overall mechanism to control zinc homeostasis through the actions of ZIP and ZnT proteins is unknown.

A very stable environment via the brain barrier system is necessary for brain functions. The blood–brain barrier, which consists of the tight junction between the brain capillary endothelial cells (Fig. 8.1) (Abbott 2005), is completed around 2 weeks and 4 months after birth in rats and humans, respectively. The blood–CSF barrier, which consists of the tight junction between the choroidal epithelial cells (Fig. 8.1), is functional in early development (Johansson et al. 2008). Zinc is transported into the brain through the blood–brain and the blood–CSF barriers

(Takeda et al. 1994a). The blood–CSF barrier seems to be important for zinc transport into the hippocampus (Takeda et al. 1994b). Zinc transport into the brain is very slow (Fig. 8.2), compared to that into the peripheral tissues.

The cells forming the brain barrier system express ZIP and ZnT and maintain zinc homeostasis in the brain (Belloni-Olivi et al. 2009). These transporters have specialized functions for cellular zinc transport. However, their precise role in zinc transport across the brain barrier system is unknown (Colvin et al. 2003; Nakashima and Dyck 2009). Zinc concentration in the brain increases with development after birth and is maintained constant in the adult brain (about 200 μM) (Markesbery et al. 1984). It has been reported that ZIP such as ZIP1, ZIP4, ZIP6, ZIP7, ZIP10, and ZIP14, and ZnT proteins such as ZnT1, ZnT3, and ZnT10, are involved in not only brain zinc homeostasis but also brain growth (Takeda et al. 2013a). Furthermore, Timm's stain is hardly observed in the rat brain just after birth and the intensity of the stain increases with brain maturation (Valente and Auladell 2002; Valente et al. 2002), indicating that Zn^{2+} signal is involved in not only the development and maturation of neural circuits but also its function.

Zinc concentration in the brain is hardly affected by exposure to zinc. In a blood–brain barrier model, the expression of zinc transporters such as ZnT-1 and ZnT-2 and metallothioneins, zinc-binding proteins, are changed to respond to a moderately excessive zinc environment (Bobilya et al. 2008). On the other hand, dietary zinc deficiency, which readily reduces plasma zinc, facilitates zinc uptake into the brain via changes in zinc transporter expression in the brain such as ZnT-1 and LIV-1 (SLC39A6 or ZIP6) (Chowanadisai et al. 2005), resulting in the maintenance of zinc concentration in the brain. However, zinc concentration is decreased in the hippocampus when 8-week-old rats are fed a zinc-deficient diet for 12 weeks (Takeda et al. 2001). In spite of the tight regulation of the brain barrier system, hippocampal zinc is susceptible to chronic zinc deficiency. In young rats after 1-week zinc deprivation, plasma zinc concentration is less than 50 % of the control (Takeda et al 2007a). It is possible that the decrease in plasma zinc leads to the decrease in CSF zinc, followed by decrease in hippocampal zinc.

The astrocytes directly contact the brain capillary endothelial cells and are a possible sensor to maintain zinc homeostasis in the brain (Fig. 8.4). It is possible that the astrocytes sense the decrease in plasma zinc, which serves to prevent the loss of brain zinc in cooperation with the brain capillary endothelial cells. In addition to the communication via zinc between astrocytes and capillary endothelial cells, the communication via zinc between neurons and astrocytes might be important for intracellular zinc homeostasis, which is critical for cellular function (Bertoni-Freddari et al. 2008). Zn^{2+} is a possible tool for communications; synaptic Zn^{2+} dynamically moves along with neuronal activity and modulates synaptic neurotransmission (as described next) (Ueno et al. 2002) and is potentially involved in communications. Cooperative metabolism between neurons and astrocytes is reported concerning energy metabolism (Magistretti 2006) and utilization of neurotransmitters such as glutamate (Voutsinos-Porche et al. 2003).

On the other hand, brain capillary endothelial cells also play an active part in the process of many neurological diseases. The blood–brain barrier is modified or

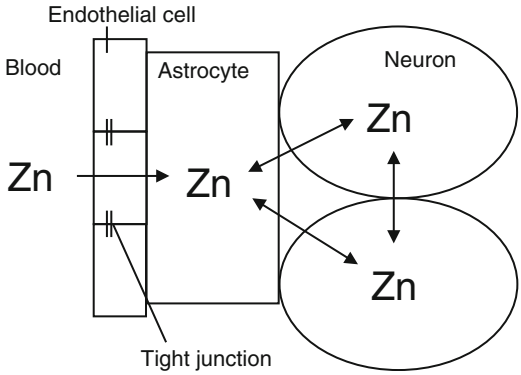

Fig. 8.4 Communication via zinc between endothelial cells, astrocytes, and neurons. Endothelial cells are primarily surrounded by astrocytes. Astrocytes might be involved in zinc supply to neurons and sense the decrease in plasma zinc via communication with the brain capillary endothelial cells

disrupted in the process of neurological diseases such as neoplasia, stroke/ischemia, epilepsy, and dementia (Ballabh et al. 2004). Increase in blood–brain barrier permeability elicits the dyshomeostasis of metals such as zinc and iron, which has been implicated in many neurodegenerative diseases (Yokel 2006).

8.3 Synaptic Zn^{2+} Homeostasis

If zinc concentration in the brain extracellular fluid is equal to that in the cerebrospinal fluid, it is about 150 nM and approximately 1,000-fold of the total brain zinc concentration. Zinc concentration, especially Zn^{2+} concentration, in the synaptic cleft, especially in zincergic synapses, is estimated to be higher than that in the brain extracellular fluid, because the regions where zincergic synapses exist is intensely stained with ZnAF-2, a membrane-impermeable zinc indicator (Fig. 8.5e–h) (Takeda et al. 2012a). The synaptic cleft is surrounded with the processes of astrocytes and might be convenient to maintain a steady concentration of zinc and neurotransmitters.

Zinc concentration in brain extracellular fluid, as measured by the in vivo microdialysis, is increased with brain development (Takeda et al. 2013a). However, the increase is suppressed under chronic zinc deficiency (Takeda et al. 2006b); in the hippocampus, extracellular zinc concentration is significantly lower than that of the control rats after 4-week zinc deprivation because of the increase in extracellular zinc concentration in the control (Takeda et al. 2003a, b). The intensity of Timm's stain of zinc-deficient rats is also lower than that of the control rats after 4-week zinc deprivation (Fig. 8.5a–d) (Takeda et al. 2006a). The chronic decrease in plasma zinc concentration may suppress the increase in zinc concentration in the brain extracellular fluid, followed by suppression of the increase in the intensity of Timm's stain.

Some fractions of the total zinc in the plasma seem to be responsive to zinc deficiency. One of these is potentially free Zn^{2+} that is estimated to be as low as

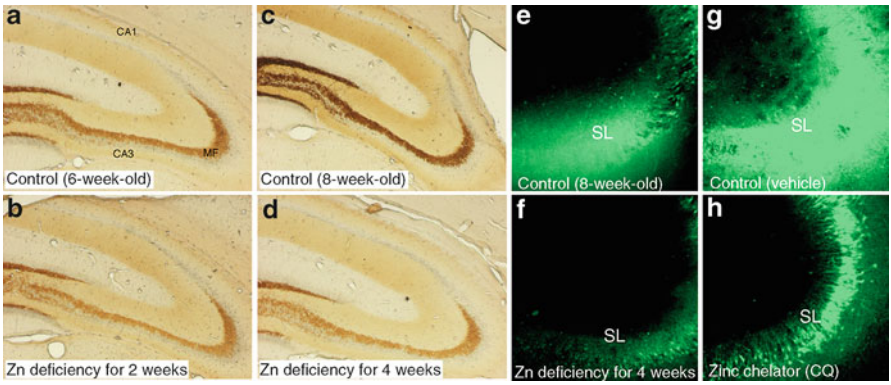

Fig. 8.5 Decrease in synaptic Zn^{2+} in the hippocampus. Rats (4-week-old) were fed a control (**a**, **c**) and zinc-deficient (**b**, **d**) diet for 2–4 weeks. The coronal sections of the brain show Timm's stain in the hippocampus. Mossy fibers (MF) from dentate granule cells are strongly stained. The increase in Timm's stain intensity is obviously suppressed in mossy fibers and other hippocampal regions after 4-week zinc deficiency (**b**, **d**). Extracellular (synaptic cleft) Zn^{2+}, which was measured with ZnAF-2, a membrane-impermeable zinc indicator, is decreased in the hippocampal CA3 region, especially in mossy fibers (stratum lucidum, *SL*), of rats after 4-week zinc deficiency (**e**, **f**). Extracellular (synaptic cleft) Zn^{2+} is also decreased in the hippocampal CA3 region, especially in mossy fibers of rats 6 h after i.p. injection of clioquinol (CQ, 30 mg/kg), a lipophilic zinc chelator (**g**, **h**)

10^{-9}–10^{-10} M (Magneson et al. 1987). The idea is supported by the data that extracellular Zn^{2+} levels detected with ZnAF-2 are lower in the hippocampus of zinc-deficient rats than that of the control rats (Fig. 8.5e, f) (Takeda et al. 2012a). Interestingly, Zn^{2+} level in the brain extracellular fluid, which is estimated to be 10^{-8} M (Frederickson et al. 2006), is higher than that in the plasma. In brain extracellular fluid, the high rate of Zn^{2+} level compared to total zinc concentration seems to be associated with synaptic Zn^{2+} signal in the brain. It is possible that extracellular Zn^{2+} serves as a pool for zinc in the synaptic vesicle and is involved in synaptic Zn^{2+} homeostasis (Fig. 8.6), although the chemical form of vesicular zinc is unknown.

The basal Zn^{2+} concentrations are extremely low in the intracellular (cytosol) compartment ($<10^{-9}$ M) (Sensi et al. 1997; Colvin et al. 2008). ZnT proteins such as ZnT1, ZnT3, and ZnT10, and ZIP such as ZIP4 and ZIP6, are involved in the control of Zn^{2+} levels in the cytosolic compartment, especially in the static (basal) circumstance (Emmetsberger et al. 2010). Some of these transporters transport cytosolic Zn^{2+} into different subcellular organelles, such as mitochondria, lysosomes, endosomes, and Golgi apparatus, probably to maintain the static Zn^{2+} levels in the cytosolic compartment (Sensi et al. 2003; Danscher and Stoltenberg 2005; Colvin et al. 2006). In contrast, it is possible that Zn^{2+} release from the subcellular organelles through synaptic neurotransmission serves as a Zn^{2+} signal that participates in synaptic function (Stork and Li 2010). Zn^{2+} levels except for vesicular Zn^{2+} are estimated to be less than 5 % of the total Zn^{2+} amount in the hippocampus

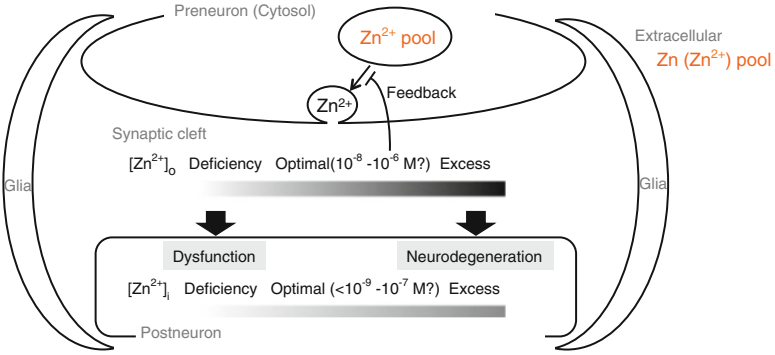

Fig. 8.6 Synaptic Zn^{2+} homeostasis. Zn^{2+} released from neuron terminals serves as a signal factor in both extracellular and cytosolic compartments. It is estimated that the functional signaling occurs in the optimal ranges of extracellular Zn^{2+} concentration $[Zn^{2+}]_o$ and intracellular Zn^{2+} concentration $[Zn^{2+}]_i$. Both the lack and excess of Zn^{2+} signal affect synaptic neurotransmission. The synaptic vesicle and the extracellular compartment seem to be the pool of Zn^{2+}

and cerebral cortex (Lee et al. 2011). ZnT1 is a major Zn^{2+} transporter on the plasma membrane and may participate in cytosolic Zn^{2+} homeostasis in neurons by transporting Zn^{2+} from the somata to the extracellular space (Sekler et al. 2002) It has been reported that ZnT1 restricts excessive accumulation of Zn^{2+} in the cytosolic compartment (Nolte et al. 2004), resulting in protecting neurons from Zn^{2+} toxicity in neurological diseases such as transient forebrain ischemia (Aguilar-Alonso et al. 2008). Tissue plasminogen activator, a secreted serine protease, is excitotoxic and increases lysosomal sequestration of the increased Zn^{2+} in the cytosolic compartment through interaction with ZIP4, which might also lead to protecting neurons from Zn^{2+} toxicity (Emmetsberger et al. 2010). Spatiotemporal control of the Zn^{2+} signal via ZIP and ZnT maintains the steady environment in both the extracellular and cytosolic compartments.

8.4 Functional and Neurotoxic Zn^{2+} Signaling

Zn^{2+} is released from zincergic neuron terminals and Zn^{2+} concentration is increased in the synaptic cleft (extracellular compartment), followed by increase in the cytosol [intracellular compartment (Fig. 8.7)]. Zn^{2+} released is quickly taken up into presynaptic and postsynaptic neurons and astrocytes. Calcium channels such as calcium-permeable AMPA/kainate receptors are involved in Zn^{2+} influx during neuronal excitation (Weiss et al. 2000; Jia et al. 2002; Takeda et al. 2007b). On the basis of the finding that the low-affinity site ($IC_{50} \approx 20$ μM at −40 mV) of NMDA receptors is bound by Zn^{2+} as a NMDA receptor blocker, extracellular Zn^{2+} concentration after tetanic stimulation is estimated to range between 10 and 100 μM (Vogt et al. 2000). In contrast, Kay (2003) reports that extracellular Zn^{2+}

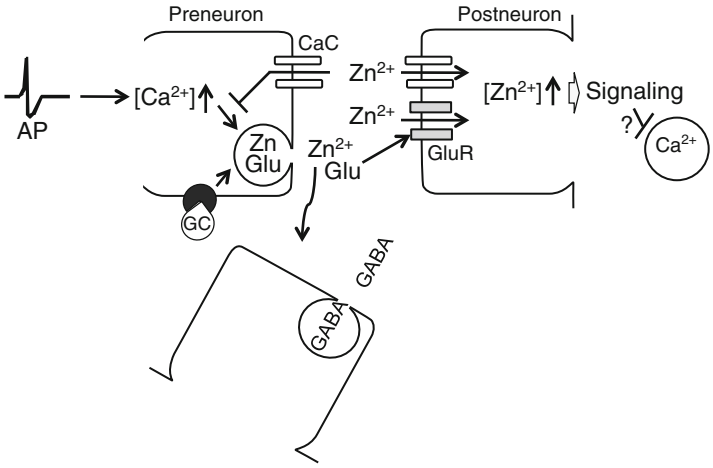

Fig. 8.7 Glutamatergic neurotransmission and Zn^{2+} signaling in the hippocampus. Zn^{2+} released from zincergic neuron terminals is immediately taken up into presynaptic and postsynaptic neurons through calcium-permeable channels (CaC and GluR). Zn^{2+} release can be enhanced by presynaptic glucocorticoid (GC) signal via mineralocorticoid or glucocorticoid receptors. Intracellular Zn^{2+} signal is critical for cognitive function, although the optimal range for intracellular Zn^{2+} signal is controversial (Fig. 8.6)

concentration after mossy fiber excitation may be nanomolar in an experiment using the fluorimetric FluoZin-3 (K_D, 15 nM) in conjunction with a zinc chelator. Hippocampal LTP modulation by exogenous Zn^{2+} suggests that endogenous Zn^{2+} reaches approximately low micromolar concentrations in the extracellular compartment after tetanic stimulation (Fig. 8.6). Therefore, the extracellular concentration of Zn^{2+} reached after depolarization, which is dependent on frequency of depolarizing stimulation (Ueno et al. 2002), is a matter of debate. The optimal range of Zn^{2+} signal in the cytosolic compartment also remains to be determined (Fig. 8.6).

In the extracellular compartment, Zn^{2+} modulates glutamatergic and GABAergic neuron activity (Fig. 8.7). Zn^{2+} potentiates AMPA/kainate receptors, while blocking Ca^{2+} current through calcium channels such as NNDA receptors and voltage-dependent calcium channels (VDCC). Zn^{2+} released from zincergic neuron terminals may serve as a negative feedback factor against glutamate release (Minami et al. 2006; Takeda et al. 2007c) (Fig. 8.7). In the cytosolic compartment, Zn^{2+} can decrease not only the basal Ca^{2+} level (Takeda et al. 2007b) but also Ca^{2+} levels increased by metabotropic glutamate receptors. Crosstalk of Zn^{2+} signaling to Ca^{2+} signaling via calcium channels may modulate presynaptic and postsynaptic neuronal activity (Takeda and Tamano 2009, 2010).

Excessive activation of glutamate receptors by excess of extracellular glutamate leads to a number of deleterious consequences, including impairment of calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition, and secondary excitotoxicity (Danbolt 2001; Dong et al. 2009).

Glutamate excitotoxicity, a final common pathway for neuronal death, is observed in numerous pathological processes such as stroke/ischemia, temporal lobe epilepsy, Alzheimer's disease, and amyotrophic lateral sclerosis. Zn^{2+} plays a neuroprotective role in glutamate-induced excitotoxicity (Bancila et al. 2004; Cohen-Kfir et al. 2005; Takeda et al. 2007d). However, excess of extracellular Zn^{2+}, which is induced under glutamate excototoxicity, is harmful; excessive Zn^{2+} influx into postsynaptic neurons is involved in neurodegeneration under pathological conditions. Calcium-permeable AMPA receptors may play a key role for Zn^{2+} influx in postsynaptic neurodegeneration (Liu et al. 2004; Noh et al. 2005; Weiss 2011). Therefore, synaptic Zn^{2+} signaling is involved in both functional and pathological aspects.

8.5 Zn^{2+} Signal and Cognition

Synaptic Zn^{2+} signal is involved in synaptic plasticity such as LTP in the hippocampus and amygdala. Enhanced plasticity in zincergic synapses is associated with cortical modification after exposure to enriched environment. These data suggest the synaptic Zn^{2+} signal is involved in cognitive and emotional behavior through the modulation of synaptic plasticity (Takeda and Tamano 2009).

Targeted deletion of the ZnT3 prevents vesicular Zn^{2+} uptake (Cole et al. 1999) and ablates Zn^{2+} release into the extracellular space by action potentials. There are close correlations between vesicular Zn^{2+} levels and ZnT3 protein expression (Palmiter et al. 1996). Zn^{2+} transport into the synaptic vesicle is ZnT3 dependent and is important in constructing a major pool of Zn^{2+} signal (Fig. 8.6). In the initial experiment, ZnT3KO mice were able to recognize novel or displaced objects normally (Cole et al. 2001). However, Adlard et al. (2010) report that ZnT3KO mice exhibit significant cognitive deficits at 6 months of age but not at 3 months of age. Three- to 4-month-old ZnT3KO mice also exhibit significant deficits in contextual discrimination and spatial working memory (Martel et al. 2010; Sindreu et al. 2011). Furthermore, ZnT3 is also involved in associative fear memory and extinction, but not in innate fear, consistent with the role of synaptic Zn^{2+} in amygdala synaptic plasticity (Martel et al. 2011). Therefore, it is likely that Zn^{2+} signaling is involved in cognitive and emotional behavior, even in ZnT3KO. The pool of Zn^{2+} might be other subcellular organelles or zinc-binding proteins such as metallothionein in ZnT3KO mice. On the other hand, recognition memory deficit is observed in wild-type animals when the acute loss of synaptic Zn^{2+} is induced by treatment with lipophilic zinc chelators (Takeda et al. 2010a). Chelation of extracellular Zn^{2+} by CaEDTA in the amygdala increases the time of freezing behavior in the contextual recall of fear (Takeda et al. 2010b). The amount of Zn^{2+} functioning as a signal factor seems to be less available in ZnT3KO mice than in wild-type mice.

Saito et al. (2000) reported that age-dependent reduction of Zn^{2+} levels in synaptic vesicles of the mossy fibers induced by low expression of ZnT3 causes glutamatergic excitotoxicity in the hippocampal neurons and the deterioration of

learning and memory in senescence-accelerated mouse prone 10 (SAMP10). There are also age-dependent reductions in ZnT3 expression and synaptic Zn^{2+} levels in the hippocampal mossy fibers of human amyloid precursor protein-transgenic (Tg2576) mice, suggesting that extensive modifications of the brain Zn^{2+} pool, particularly synaptic (vesicular) Zn^{2+}, underlie the neuronal dysfunction characteristic of Alzheimer disease (Lee et al. 2012). Furthermore, there is a significant age-related decline in cortical ZnT3 levels from age 48 to 91 in healthy people (Adlard et al. 2010), and ZnT3 levels are more markedly decreased in Alzheimer's disease cortex. On the other hand, Zn^{2+} released from zincergic neurons is known to mediate parenchymal and cerebrovascular amyloid formation in Tg2576 mice (Lee et al. 2002; Friedlich et al. 2004; Stoltenberg et al. 2007). The transsynaptic movement of Zn^{2+} may be severely compromised in Alzheimer's disease both by the lack of ZnT3 expression and by being sequestered in amyloid. Adlard et al. (2010) reported that the genetic ablation of ZnT3 may represent a phenocopy for the synaptic and memory deficits of Alzheimer's disease. Deshpande et al. (2009) postulated that the sequestration of Zn^{2+} in oligomeric amyloid-β (Aβ)–Zn complexes may lead to a reduction in Zn^{2+} availability at the synapse, resulting in a loss of the modulatory activity by Zn^{2+} and leading to cognitive decline in Alzheimer's disease. Synaptic Zn^{2+} signal is dynamically functional for learning and memory in the healthy brain (Takeda and Tamano 2012), and also change in Zn^{2+} availability may participate in cognitive decline in dementia such as Alzheimer's disease (Bush 2013).

8.6 Glucocorticoid Signal and Zn^{2+} Signal

The hippocampus is enriched with corticosteroid receptors and is the major target region of corticosteroids (Joëls 2008). Mineralocorticoid receptors and glucocorticoid receptors are colocalized in CA1 and CA2 pyramidal cells and in dentate gyrus granule cells. In the CA3 pyramidal cells, on the other hand, mineralocorticoid receptors are abundantly expressed and glucocorticoid receptors are much less expressed (Ozawa 2005). Mineralocorticoid receptors are extensively occupied with low levels of corticosterone, and glucocorticoid receptors are particularly activated after exposure to stress (Joëls et al. 2008; Sandi 2011). Dentate granule cells require corticosterone levels at the physiological range; a selective degeneration of dentate gyrus granule cells is observed in the hippocampus after adrenalectomy (Joëls 2007).

An increase in serum corticosterone level induces a rapid increase in corticosterone level in the hippocampus in parallel with an increase in extracellular glutamate level (Venero and Borrell 1999). Corticosterone-induced increase in extracellular glutamate levels in the hippocampus seems to be exerted through the action of membrane-associated mineralocorticoid receptors or glucocorticoid receptors, which increase glutamate release probability in synaptic activation (Fig. 8.7) (Karst et al. 2005; Musazzi et al. 2010). The rapid effects of corticosterone on

glutamatergic transmission seem to be linked to the diverse effects on synaptic plasticity and memory processes in the hippocampus. The increase in glutamate release probability through the action of corticosterone leads to increasing the amount of glutamate released during learning and increasing the activation of postsynaptic glutamate receptors. Corticosterone can contribute to increase the efficacy of glutamatergic transmission by AMPA receptor insertion at synaptic sites through both rapid and delayed (genomic) effects. These effects are the advantage in the process of synaptic plasticity such as LTP and also learning and memory (Sandi 2011). Therefore, it is estimated that corticosterone increases Zn^{2+} release probability from zincergic neuron terminals through the rapid nongenomic effect in the hippocampus (Takeda et al. 2012b) (Fig. 8.7), while requiring intracellular Zn^{2+} signal for the genomic effect (Takeda and Tamano 2012). Although the evidence is limited, it is likely that synaptic Zn^{2+} signal cooperates with corticosteroid signal in learning and memory.

In contrast, glutamate accumulates in the extracellular compartment at high levels through corticosterone-mediated blockade of glutamate transporter activity when corticosterone is abnormally secreted under severe stress circumstances. The abnormal corticosterone secretion also contributes to abnormal glutamate release from neuron terminals. These effects facilitate long-term depression (LTD), and impair LTP and memory processing (Wong et al. 2007; Howland and Wang 2008). Excess of synaptic Zn^{2+} signal induced by corticosterone or stress is also involved in impairment of LTP (Takeda et al. 2012b), possibly followed by the impairment of learning and memory (Takeda et al. 2011). Furthermore, prolonged exposure of animals to a high level of corticosterone presumably makes dentate granule cells more vulnerable to delayed cell death (Joëls 2007). Stress and corticosterone strongly inhibit adult hippocampal neurogenesis (Mirescu and Gould 2006), which participates in learning and memory, and decreased neurogenesis has been implicated in the pathogenesis of anxiety and depression. Neurogenesis-deficient mice show increased food avoidance in a novel environment after acute stress, increase behavioral despair in the forced swim test, and decrease sucrose preference, a measure of anhedonia; adult hippocampal neurogenesis buffers stress responses and depressive behavior (Snyder et al. 2011). The hippocampus provides negative control of the HPA axis (Carroll et al. 1981; Holsboer 1983; Arana et al. 1985). The HPA axis activity is disrupted in approximately 50 % of human depressives. This disruption is generally presented as a dexamethasone-mediated negative feedback resistance to cortisol secretion. It is reported that a small subset of neurons within the dentate gyrus are critical for hippocampal negative control of the HPA axis. Interestingly, zinc-deficient diet is a stressor and feeding a zinc-deficient diet induces abnormal HPA axis activity, which underlies brain dysfunction and abnormal behavior under dietary zinc deficiency, as described next.

8.7 Neuropsychological Symptoms in Zinc Deficiency

When young mice and rats are fed a zinc-deficient diet, reductions in total food intake are observed within approximately 3 days. The decrease in plasma zinc is also observed at this time (Ohinata et al. 2009). Zinc deficiency results in food intakes that are less than 50 % of normal. Zinc homeostasis in the brain is not easily disrupted by dietary zinc deficiency (Takeda et al. 2005a). However, zinc deficiency decreases synaptic Zn^{2+} levels in the hippocampus (Fig. 8.5e–h), followed by decrease in hippocampal zinc concentration, suggesting that Zn^{2+} is the most susceptible to zinc deficiency in zinc components. On the other hand, zinc deficiency activates the HPA axis before decreasing synaptic Zn^{2+} levels in the hippocampus. Serum corticosterone concentration is significantly increased in young mice and rats fed a zinc-deficient diet for 2 weeks (Takeda et al. 2007a, 2008). It is possible that the decrease in plasma zinc, possibly plasma Zn^{2+}, leads to abnormal corticosterone secretion through HPA axis activation, followed by reductions in total food intake.

Abnormal glucocorticoid secretion has been reported in many neuropsychiatric disorders including depression (Whiteford et al. 1987; Starkman et al. 1992; Seed et al. 2000). It is thought that exposure to chronic stress precipitates or exacerbates neuropsychiatric disorders. In human depressives, smaller volumes of the hippocampus are associated with abnormal cortisol secretion (Bremner et al. 2000). Interestingly, it is reported that human depressives are zinc deficient (Maes et al. 1994; Nowak et al. 2005). Depression-like behavior is increased in zinc-deficient animals (Takeda et al. 2011). Repeated corticosterone injections induce anxiety and depression-like behavior in mice and rats. Antiglucocorticoids, similar to conventional antidepressants, may recover depressive symptoms by boosting hippocampal neurogenesis. Zinc deficiency impairs neuronal precursor cell proliferation in the hippocampus (Corniola et al. 2008; Suh et al. 2009). It is likely that abnormal corticosterone secretion is associated with depressive symptoms in zinc deficiency through the changes in hippocampal function (Takeda and Tamano 2009).

8.8 Potentiation of Glutamate Excitotoxicity by Glucocorticoids

Glutamate concentration in brain extracellular fluid is estimated to be about 2 μM and that in the synaptic vesicles of glutamatergic neuron terminals is markedly high (~100 mM) (Meldrum 2000). The hippocampus is relatively susceptible to glutamate excitotoxicity in the brain. Neuronal death in the hippocampus is observed in temporal lobe epilepsy, in which seizures frequently originate in the hippocampus. The increase in extracellular glutamate in the hippocampus may trigger spontaneous seizures in patients with complex partial epilepsy (During and Spencer 1993).

It has been reported that zinc homeostasis in the brain is associated with the etiology and manifestation of epileptic seizures (Takeda and Tamano 2009). Zinc concentration in the brain, especially in the hippocampus, is decreased by epileptic seizures (Assaf and Chung 1984; Takeda et al. 2003c). Seizure susceptibility of El (epilepsy) mice is increased by zinc deficiency and decreased by zinc loading (Fukahori and Itoh 1990). Kainate and NMDA-induced seizures are enhanced in mice and rats after 4-week zinc deprivation, in which synaptic Zn^{2+} levels are decreased (Takeda et al. 2003b, 2005b, 2009), and hippocampal cell death is observed more after treatment with kainate in zinc deficiency (Takeda et al. 2005c). An in vivo microdialysis experiment indicates that an enhanced release of glutamate associated with a decrease in GABA concentration is a possible mechanism for the increased seizure susceptibility and cell death in zinc deficiency (Takeda et al. 2003b). Abnormal corticosterone secretion may also be involved in the increased seizure susceptibility in zinc-deficient epilepsy animals. Furthermore, the decrease in Zn^{2+} level, which is induced with zinc chelators, enhances susceptibility to spontaneous seizures in Noda epileptic rats (Takeda et al. 2013b), suggesting that the Zn^{2+} signal has a protective action against the development of seizures (Takeda et al. 2003d).

In global ischemia, the concentration of extracellular potassium transiently reaches 75 mM, followed by the increase in extracellular glutamate (Hansen and Zeuthen 1980). The increase in extracellular glutamate induced with 100 mM KCl is enhanced in zinc-deficient young rats (Takeda et al. 2003a, 2008). The neurological symptoms associated with glutamate excitotoxicity may be precipitated or aggravated by zinc deficiency (Stoltenberg et al. 2007). A factor of the aggravation is the increase in corticosterone secretion, which potentiates glutamate excitotoxicity (Takeda and Tamano 2012). The decrease in brain extracellular Zn^{2+} may also aggravate glutamate excitotoxicity in zinc deficiency.

Selective increase in the nocturnal levels of cortisol is observed in aged humans (Landfield and Eldridge 1994). Plasma zinc level decreases with aging in humans. The increase in the basal levels of intracellular Ca^{2+} and modification of Ca^{2+} signaling are observed in both aged (Billard 2006; Foster 2007) and zinc-deficient animals (Takeda et al. 2009; Tamano et al. 2009). Aged animals and humans seem to be more susceptible to glutamate excitotoxicity as well as zinc-deficient animals. It is likely that glucocorticoids modify the dynamics of both Zn^{2+} signal and Ca^{2+} signal and that the increased glucocorticoid secretion underlies dysfunctions in both zinc deficiency and aging, which may increase the risk of neurological diseases such as depression and Alzheimer's disease (Takeda and Tamano 2010). As a matter of fact, high levels of cortisol are found in Alzheimer's disease as well as depression. In Alzheimer's disease patients, cognitive deficits (such as memory) and psychological symptoms (such as anxiety) are associated with an early deregulation of the HPA axis (Brureau et al. 2013). Therefore, it is possible that excess of intracellular Zn^{2+} signal through abnormal cortisol secretion is involved in cognitive deficits in both normal aging and neurological diseases such as dementia.

8.9 Perspective

There are two major pools of Zn^{2+}, that is, the synaptic vesicle and the extracellular compartment, in the brain (Fig. 8.6). Synaptic Zn^{2+} homeostasis seems to be controlled by the two pools and is critical for synaptic function (Takeda et al. 2013a). The synaptic Zn^{2+} signal is involved in both cognitive function and dysfunction. On the other hand, Zn^{2+} exists in the plasma at an extremely low concentration, and plasma Zn^{2+} levels are potentially linked to HPA axis activity. HPA axis activity is increased with aging, and the increases are superimposed in neurological diseases such as depression and Alzheimer's disease. It is likely that that the modification of synaptic Zn^{2+} signaling through the HPA axis activation is involved in not only cognitive function but also cognitive decline. The molecular mechanism of intracellular Zn^{2+} signaling in cognition seems to be important to understand cognitive decline in normal aging and also in neurological symptoms such as dementia. An important issue that remains to be addressed is how homeostasis of Zn^{2+} is controlled in the brain and what determines the threshold between adaptive and maladaptive Zn^{2+} signaling under diverse circumstances.

References

Abbott NJ (2005) Dynamics of CNS barriers: evolution, differentiation, and modulation. Cell Mol Neurobiol 25:5–23

Adlard PA, Parncutt JM, Finkelstein DI, Bush AI (2010) Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease? J Neurosci 30:1631–1636

Aguilar-Alonso P, Martinez-Fong D, Pazos-Salazar NG, Brambila E, Gonzalez-Barrios JA, Mejorada A, Flores G, Millan-Perezpeña L, Rubio H, Leon-Chavez BA (2008) The increase in zinc levels and upregulation of zinc transporters are mediated by nitric oxide in the cerebral cortex after transient ischemia in the rat. Brain Res 1200:89–98

Arana GW, Baldessarini RJ, Ornsteen M (1985) The dexamethasone suppression test for diagnosis and prognosis in psychiatry. Arch Gen Psychiatry 42:1193–1204

Assaf SY, Chung S-H (1984) Release of endogeneous Zn^{2+} from brain tissue during activity. Nature (Lond) 308:734–735

Ballabh P, Braun A, Nedergaard M (2004) The blood–brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis 16:1–13

Bancila V, Nikonenko I, Dunant Y, Bloc A (2004) Zinc inhibits glutamate release via activation of pre-synaptic K_{ATP} channels and reduces ischaemic damage in rat hippocampus. J Neurochem 90:1243–1250

Barnham KJ, Bush AI (2008) Metals in Alzheimer's and Parkinson's diseases. Curr Opin Chem Biol 12:222–228

Belloni-Olivi L, Marshall C, Laal B, Andrews GK, Bressler J (2009) Localization of zip1 and zip4 mRNA in the adult rat brain. J Neurosci Res 87:3221–3230

Bertoni-Freddari C, Fattoretti P, Casoli T, Di Stefano G, Giorgetti B, Balietti M (2008) Brain aging: the zinc connection. Exp Gerontol 43:389–393

Billard JM (2006) Ageing, hippocampal synaptic activity and magnesium. Magnes Res 19:199–215

Black MM (1998) Zinc deficiency and child development. Am J Clin Nutr 68:464S–469S

Bobilya DJ, Gauthier NA, Karki S, Olley BJ, Thomas WK (2008) Longitudinal changes in zinc transport kinetics, metallothionein and zinc transporter expression in a blood-brain barrier model in response to a moderately excessive zinc environment. J Nutr Biochem 19:129–137
Bremner JD, Narayan M, Anderson ER, Staib LH, Miller HL, Charney DS (2000) Hippocampal volume reduction in major depression. Am J Psychiatry 157:115–118
Brown KH, Wuehler SE, Peerson JM (2001) The importance of zinc in human nutrition and estimation of the global prevalence of zinc deficiency. Food Nutr Bull 22:113–125
Brureau A, Zussy C, Delair B, Ogier C, Ixart G, Maurice T, Givalois L (2013) Deregulation of hypothalamic-pituitary-adrenal axis functions in an Alzheimer's disease rat model. Neurobiol Aging 34:1426–1439
Bryan J, Osendarp S, Hughes D, Calvaresi E, Baghurst K, van Klinken JW (2004) Nutrients for cognitive development in school-aged children. Nutr Rev 62:295–306
Bush AI (2013) The metal theory of Alzheimer's disease. J Alzheimers Dis 33:S277–S281
Capasso M, Jeng JM, Malavolta M, Mocchegiani E, Sensi SL (2005) Zinc dyshomeostasis: a key modulator of neuronal injury. J Alzheimers Dis 8:93–108
Carroll BJ, Feinberg M, Greden JF, Tarika J, Albala AA, Haskett RF, James NM, Kronfol Z, Lohr N, Steiner M, de Vigne JP, Young E (1981) A specific laboratory test for the diagnosis of melancholia: standardization, validation and clinical utility. Arch Gen Psychiatry 38:15–22
Chowanadisai W, Kelleher SL, Lönnerdal B (2005) Zinc deficiency is associated with increased brain zinc import and LIV-1 expression and decreased ZnT-1 expression in neonatal rats. J Nutr 135:1002–1007
Cohen-Kfir E, Lee W, Eskandari S, Nelson N (2005) Zinc inhibition of gamma-aminobutyric acid transporter 4 (GAT4) reveals a link between excitatory and inhibitory neurotransmission. Proc Natl Acad Sci USA 102:6154–6159
Cole CR, Lifshitz F (2008) Zinc nutrition and growth retardation. Pediatr Endocrinol Rev 5:889–896
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
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
Colvin RA, Fontaine CP, Laskowski M, Thomas D (2003) Zn^{2+} transporters and Zn^{2+} homeostasis in neurons. Eur J Pharmacol 479:171–185
Colvin RA, Laskowski M, Fontaine CP (2006) Zinquin identifies subcellular compartmentalization of zinc in cortical neurons. Relation to the trafficking of zinc and the mitochondrial compartment. Brain Res 1085:1–10
Colvin RA, Bush AI, Volitakis I, Fontaine CP, Thomas D, Kikuchi K, Holmes WR (2008) Insights into Zn^{2+} homeostasis in neurons from experimental and modeling studies. Am J Physiol Cell Physiol 294:C726–C742
Corniola RS, Tassabehji NM, Hare J, Sharma G, Levenson CW (2008) Zinc deficiency impairs neuronal precursor cell proliferation and induces apoptosis via p53-mediated mechanisms. Brain Res 1237:52–61
Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. J Biol Chem 281:24085–24089
Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105
Danscher G, Stoltenberg M (2005) Zinc-specific autometallographic in vivo selenium methods: tracing of zinc-enriched (ZEN) terminals, ZEN pathways, and pools of zinc ions in a multitude of other ZEN cells. J Histochem Cytochem 53:141–153
Deshpande A, Kawai H, Metherate R, Glabe CG, Busciglio J (2009) A role for synaptic zinc in activity-dependent A-beta oligomer formation and accumulation at excitatory synapses. J Neurosci 29:4004–4015

Dong XX, Wang Y, Qin ZH (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin 30:379–387
During MJ, Spencer DD (1993) Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. Lancet 341:1607–1610
Emmetsberger J, Mirrione MM, Zhou C, Fernandez-Monreal M, Siddiq MM, Ji K, Tsirka SE (2010) Tissue plasminogen activator alters intracellular sequestration of zinc through interaction with the transporter ZIP4. J Neurosci 30:6538–6547
Foster TC (2007) Calcium homeostasis and modulation of synaptic plasticity in the aged brain. Aging Cell 6:319–325
Frederickson CJ (1989) Neurobiology of zinc and zinc-containing neurons. Int Rev Neurobiol 31:145–238
Frederickson CJ, Danscher G (1990) Zinc-containing neurons in hippocampus and related CNS structures. Prog Brain Res 83:71–84
Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6:449–462
Frederickson CJ, Giblin LJ, Krezel A, McAdoo DJ, Muelle RN, Zeng Y, Balaji RV, Masalha R, Thompson RB, Fierke CA, Sarvey JM, Valdenebro M, Prough DS, Zornow MH (2006) Concentrations of extracellular free zinc (pZn)e in the central nervous system during simple anesthetization, ischemia and reperfusion. Exp Neurol 198:285–293
Friedlich AL, Lee JY, van Groen T, Cherny RA, Volitakis I, Cole TB, Palmiter RD, Koh JY, Bush AI (2004) Neuronal zinc exchange with the blood vessel wall promotes cerebral amyloid angiopathy in an animal model of Alzheimer's disease. J Neurosci 24:3453–3459
Fukada T, Kambe T (2011) Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics 3:662–674
Fukahori M, Itoh M (1990) Effects of dietary zinc status on seizure susceptibility and hippocampal zinc content in the El (epilepsy) mouse. Brain Res 529:16–22
Hansen AJ, Zeuthen T (1980) Extracellular ion concentrations during spreading depression and ischemia in the rat brain cortex. Acta Physiol Scand 113:437–445
Hershey CO, Hershey LA, Varnes A, Vibhakar SD, Lavin P, Strain WH (1983) Cerebrospinal fluid trace element content in dementia: clinical, radiologic, and pathologic correlations. Neurology 33:1350–1353
Holsboer F (1983) The dexamethasone suppression test in depressed patients: clinical and biochemical aspects. J Steroid Biochem 19:251–257
Howland JG, Wang YT (2008) Synaptic plasticity in learning and memory: stress effects in the hippocampus. Prog Brain Res 169:145–158
Jia Y, Jeng JM, Sensi S, Weiss JH (2002) Zn^{2+} currents are mediated by calcium-permeable AMPA/kainite channels in cultured murine hippocampal neurons. J Physiol (Lond) 543:35–48
Joëls M (2007) Role of corticosteroid hormones in the dentate gyrus. Prog Brain Res 163:355–370
Joëls M (2008) Functional actions of corticosteroids in the hippocampus. Eur J Pharmacol 583:312–321
Joëls M, Karst H, DeRijk R, de Kloet ER (2008) The coming out of the brain mineralocorticoid receptor. Trends Neurosci 31:1–7
Johansson PA, Dziegielewska KM, Liddelow SA, Saunders NR (2008) The blood–CSF barrier explained: when development is not immaturity. Bioessays 30:237–248
Karst H, Berger S, Turiault M, Tronche F, Schütz G, Joëls M (2005) Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. Proc Natl Acad Sci USA 102:19204–19207
Kay AR (2003) Evidence for chelatable zinc in the extracellular space of the hippocampus, but little evidence for synaptic release of Zn. J Neurosci 23:6847–6855
King JC, Shames DM, Woodhouse LR (2000) Zinc homeostasis in humans. J Nutr 130:1360S–1366S
Landfield PW, Eldridge JC (1994) Evolving aspects of the glucocorticoid hypothesis of brain aging: hormonal modulation of neuronal calcium homeostasis. Neurobiol Aging 15:579–588

Lee JY, Cole TB, Palmiter RD, Suh SW, Koh JY (2002) 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 JS, Byun HR, Palmiter RD, Koh JY (2011) Dependence of the histofluorescently reactive zinc pool on zinc transporter-3 in the normal brain. Brain Res 1418:12–22
Lee JY, Cho E, Seo JW, Hwang JJ, Koh JY (2012) Alteration of the cerebral zinc pool in a mouse model of Alzheimer disease. J Neuropathol Exp Neurol 71:211–222
Liu S, Lau L, Wei J, Zhu D, Zou S, Sun HS, Fu Y, Liu F, Lu Y (2004) Expression of Ca(2+)-permeable AMPA receptor channels primes cell death in transient forebrain ischemia. Neuron 43:43–55
Maes M, D'Haese PC, Scharpé S, D'Hondt P, Cosyns P, De Broe ME (1994) Hypozincemia in depression. J Affect Disord 31:135–140
Magistretti PJ (2006) Neuron-glia metabolic coupling and plasticity. J Exp Biol 209:2304–2311
Magneson GR, Puvathingal JM, Ray WJ Jr (1987) The concentrations of free Mg^{2+} and free Zn^{2+} in equine blood plasma. J Biol Chem 262:11140–11148
Maret W, Sandstead HH (2008) Possible roles of zinc nutriture in the fetal origins of disease. Exp Gerontol 43:378–381
Markesbery WR, Ehmann WD, Alauddin M, Hossain TIM (1984) Brain trace element concentrations in aging. Neurobiol Aging 5:19–28
Martel G, Hevi C, Friebely O, Baybutt T, Shumyatsky GP (2010) Zinc transporter 3 is involved in learned fear and extinction, but not in innate fear. Learn Mem 17:582–590
Martel G, Hevi C, Kane-Goldsmith N, Shumyatsky GP (2011) Zinc transporter 3 is involved in learned fear and extinction, but not in innate fear. Behav Brain Res 223:233–238
Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130:1007S–1015S
Minami A, Sakurada N, Fuke S, Kikuchi K, Nagano T, Oku N, Takeda A (2006) Inhibition of presynaptic activity by zinc released from mossy fiber terminals during tetanic stimulation. J Neurosci Res 83:167–176
Mirescu C, Gould E (2006) Stress and adult neurogenesis. Hippocampus 16:233–238
Mocchegiani E, Bertoni-Freddari C, Marcellini F, Malavolta M (2005) Brain, aging and neurodegeneration: role of zinc ion availability. Prog Neurobiol 75:367–390
Musazzi L, Milanese M, Farisello P, Zappettini S, Tardito D, Barbiero VS, Bonifacino T, Mallei A, Baldelli P, Racagni G, Raiteri M, Benfenati F, Bonanno G, Popoli M (2010) Acute stress increases depolarization-evoked glutamate release in the rat prefrontal/frontal cortex: the dampening action of antidepressants. PLoS One 5:e8566
Nakashima AS, Dyck RH (2009) Zinc and cortical plasticity. Brain Res Rev 59:347–373
Noh KM, Yokota H, Mashiko T, Castillo PE, Zukin RS, Bennett MV (2005) Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. Proc Natl Acad Sci USA 102:12230–12235
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
Nowak G, Szewczyk B, Pilc A (2005) Zinc and depression. An update. Pharmacol Rep 57:713–718
Ohinata K, Takemoto M, Kawanago M, Fushimi S, Shirakawa H, Goto T, Asakawa A, Komai M (2009) Orally administered zinc increases food intake via vagal stimulation in rats. J Nutr 139:611–616
Ozawa H (2005) Steroid hormones, their receptors and neuroendocrine system. J Nippon Med School 72:316–325
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
Prasad AS (2008) Zinc in human health: effect of zinc on immune cells. Mol Med 14:353–357

Saito T, Takahashi K, Nakagawa N, Hosokawa T, Kurasaki M, Yamanoshita O, Yamamoto Y, Sasaki H, Nagashima K, Fujita H (2000) Deficiencies of hippocampal Zn and ZnT3 accelerate brain aging of rat. Biochem Biophys Res Commun 279:505–511

Sandi C (2011) Glucocorticoids act on glutamatergic pathways to affect memory processes. Trends Neurosci 34:165–176

Seed JA, Dixon RA, McCluskey SE, Young AH (2000) Basal activity of the hypothalamic-pituitary-adrenal axis and cognitive function in anorexia nervosa. Eur Arch Psychiatry Clin Neurosci 250:11–15

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 LMT, 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 15:9554–9564

Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH (2003) Modulation of mitochondrial function by endogenous Zn^{2+} pools. Proc Natl Acad Sci USA 100:6157–6162

Sindreu C, Palmiter RD, Storm DR (2011) Zinc transporter ZnT-3 regulates presynaptic Erk1/2 signaling and hippocampus-dependent memory. Proc Natl Acad Sci USA 108:3366–3370

Smart TG, Xie X, Krishek BJ (1994) Modulation of inhibitory and excitatory amino acid receptor ion channels by zinc. Prog Neurobiol 42:393–441

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature (Lond) 476:458–461

Starkman MN, Gebarski SS, Berent S, Schteingart DE (1992) Hippocampal formation volume, memory dysfunction, and cortisol levels in patients with Cushing's syndrome. Biol Psychiatry 32:756–765

Stoltenberg M, Bush AI, Bach G, Smidt K, Larsen A, Rungby J, Lund S, Doering P, Danscher G (2007) Amyloid plaques arise from zinc-enriched cortical layers in APP/PS1 transgenic mice and are paradoxically enlarged with dietary zinc deficiency. Neuroscience 150:357–369

Stork CJ, Li YV (2010) Zinc release from thapsigargin/IP3-sensitive stores in cultured cortical neurons. J Mol Signal 5:5

Suh SW, Won SJ, Hamby AM, Fan Y, Sheline CT, Tamano H, Takeda A, Liu J (2009) Decreased brain zinc availability reduces hippocampal neurogenesis in mice and rats. J Cereb Blood Flow Metab 29:1579–1588

Takeda A (2000) Movement of zinc and its functional significance in the brain. Brain Res Rev 34:137–148

Takeda A (2001) Zinc homeostasis and functions of zinc in the brain. Biometals 14:343–352

Takeda A (2011a) Insight into glutamate excitotoxicity from synaptic zinc homeostasis. Int J Alzheimers Dis 2011:491597

Takeda A (2011b) Zinc signaling in the hippocampus and its relation to pathogenesis of depression. Mol Neurobiol 44:167–174

Takeda A, Tamano H (2009) Insight into zinc signaling from dietary zinc deficiency. Brain Res Rev 62:33–34

Takeda A, Tamano H (2010) Zinc signaling through glucocorticoid and glutamate signaling in stressful circumstances. J Neurosci Res 88:3002–3010

Takeda A, Tamano H (2012) Proposed glucocorticoid-mediated zinc signaling in the hippocampus. Metallomics 4:614–618

Takeda A, Akiyama T, Sawashita J, Okada S (1994a) Brain uptake of trace metals, zinc and manganese, in rats. Brain Res 640:341–344

Takeda A, Sawashita J, Okada S (1994b) Localization in rat brain of the trace metals, zinc and manganese, after intracerebroventricular injection. Brain Res 658:252–254

Takeda A, Sawashita J, Okada S (1995) Biological half-lives of zinc and manganese in rat brain. Brain Res 695:53–58

Takeda A, Minami A, Takefuta S, Tochigi M, Oku N (2001) Zinc homeostasis in the brain of adult rats fed zinc-deficient diet. J Neurosci Res 63:447–452
Takeda A, Hirate M, Tamano H, Oku N (2003a) Release of glutamate and GABA in the hippocampus under zinc deficiency. J Neurosci Res 72:537–542
Takeda A, Hirate M, Tamano H, Nishibaba D, Oku N (2003b) Susceptibility to kainate-induced seizures under dietary zinc deficiency. J Neurochem 85:1575–1580
Takeda A, Hirate M, Tamano H, Oku N (2003c) Zinc movement in the brain under kainite-induced seizures. Epilepsy Res 54:123–129
Takeda A, Minami A, Seki Y, Oku N (2003d) Inhibitory function of zinc against excitation of hippocampal glutamatergic neurons. Epilepsy Res 57:169–174
Takeda A, Tamano H, Tochigi M, Oku N (2005a) Zinc homeostasis in the hippocampus of zinc-deficient young adult rats. Neurochem Int 46:221–225
Takeda A, Tamano H, Oku N (2005b) Involvement of unusual glutamate release in kainate-induced seizures in zinc-deficient adult rats. Epilepsy Res 66:137–143
Takeda A, Tamano H, Nagayoshi A, Yamada K, Oku N (2005c) Increase in hippocampal cell death after treatment with kainate in zinc deficiency. Neurochem Int 47:539–544
Takeda A, Itoh H, Tamano H, Oku N (2006a) Responsiveness to kainate in young rats after 2-week zinc deprivation. Biometals 19:565–572
Takeda A, Itoh H, Imano S, Oku N (2006b) Impairment of GABAergic neurotransmitter system in the amygdala of young rats after 4-week zinc deprivation. Neurochem Int 49:746–750
Takeda A, Tamano H, Kan F, Itoh H, Oku N (2007a) Anxiety-like behavior of young rats after 2-week zinc deprivation. Behav Brain Res 177:1–6
Takeda A, Fuke S, Minami A, Oku N (2007b) Role of zinc influx via AMPA/kainate receptor activation in metabotropic glutamate receptor-mediated calcium release. J Neurosci Res 85:1310–1317
Takeda A, Fuke S, Tsutsumi W, Oku N (2007c) Negative modulation of presynaptic activity by zinc released from Schaffer collaterals. J Neurosci Res 85:3666–3672
Takeda A, Minami A, Sakurada N, Nakajima S, Oku N (2007d) Response of hippocampal mossy fiber zinc to excessive glutamate release. Neurochem Int 50:322–327
Takeda A, Yamada K, Tamano H, Fuke S, Kawamura M, Oku N (2008) Hippocampal calcium dyshomeostasis and long-term potentiation in 2-week zinc deficiency. Neurochem Int 52:241–246
Takeda A, Itoh H, Nagayoshi A, Oku N (2009) Abnormal calcium mobilization in hippocampal slices of epileptic animals fed a zinc-deficient diet. Epilepsy Res 83:73–80
Takeda A, Takada S, Ando M, Itagaki K, Tamano H, Suzuki M, Iwaki H, Oku N (2010a) Neuroscience 171:443–450
Takeda A, Tamano H, Imano S, Oku N (2010b) Increases in extracellular zinc in the amygdala in acquisition and recall of fear experience and their roles in response to fear. Neuroscience 168:715–722
Takeda A, Takada S, Nakamura M, Suzuki M, Tamano H, Ando M, Oku N (2011) Transient increase in Zn^{2+} in hippocampal CA1 pyramidal neurons causes reversible memory deficit. PLoS One 6:e28615
Takeda A, Tamano H, Ogawa T, Takada S, Ando M, Oku N, Watanabe M (2012a) Significance of serum glucocorticoid and chelatable zinc in depression and cognition in zinc deficiency. Behav Brain Res 226:259–264
Takeda A, Suzuki M, Tamano H, Takada S, Ide K, Oku N (2012b) Involvement of glucocorticoid-mediated Zn^{2+} signaling in attenuation of hippocampal CA1 LTP by acute stress. Neurochem Int 60:394–399
Takeda A, Nakamura M, Fujii H, Tamano H (2013a) Synaptic Zn^{2+} homeostasis and its significance. Metallomics 5:417–423
Takeda A, Iida M, Ando M, Nakamura M, Tamano H, Oku N (2013b) Enhanced susceptibility to spontaneous seizures of Noda epileptic rats by loss of synaptic Zn^{2+}. PLoS One 8:e71374

Tamano H, Kan F, Kawamura M, Oku N, Takeda A (2009) Behavior in the forced swim test and neurochemical changes in the hippocampus in young rats after 2-week zinc deprivation. Neurochem Int 55:536–541

Ueno S, Tsukamoto M, Hirano T, Kikuchi K, Yamada MK, Nishiyama N, Nagano T, Matsuki N, Ikegaya Y (2002) Mossy fiber Zn^{2+} spillover modulates heterosynaptic *N*-methyl-D-aspartate receptor activity in hippocampal CA3 circuits. J Cell Biol 158:215–220

Valente T, Auladell C (2002) Developmental expression of ZnT3 in mouse brain: correlation between the vesicular zinc transporter protein and chelatable vesicular zinc (CVZ) cells. Glial and neuronal CVZ cells interact. Mol Cell Neurosci 21:189–204

Valente T, Auladell C, Pérez-Clausell J (2002) Postnatal development of zinc-rich terminal fields in the brain of the rat. Exp Neurol 174:215–229

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

Venero C, Borrell J (1999) Rapid glucocorticoid effects on excitatory amino acid levels in the hippocampus: a microdialysis study in freely moving rats. Eur J Neurosci 11:2465–2473

Vogt K, Mellor J, Tong G, Nicoll R (2000) The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron 26:187–196

Voutsinos-Porche B, Bonvento G, Tanaka K, Steiner P, Welker E, Chatton JY, Magistretti PJ, Pellerin L (2003) Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. Neuron 37:275–286

Weiss JH (2011) Ca permeable AMPA channels in diseases of the nervous system. Front Mol Neurosci 4:42

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

Whiteford HA, Peabody CA, Thiemann S, Kraemer HC, Csernansky JG, Berger PA (1987) The effect of age on baseline and postdexamethasone cortisol levels in major depressive disorder. Biol Psychiatry 22:1029–1032

Wong TP, Howland JG, Robillard JM, Ge Y, Yu W, Titterness AK, Brebner K, Liu L, Weinberg J, Christie BR, Phillips AG, Wang YT (2007) Hippocampal long-term depression mediates acute stress-induced spatial memory retrieval impairment. Proc Natl Acad Sci USA 104:11471–11476

Yokel RA (2006) Blood–brain barrier flux of aluminum, manganese, iron and other metals suspected to contribute to metal-induced neurodegeneration. J Alzheimers Dis 10:223–253

Chapter 9
The Clinical Implications of Impaired Zinc Signaling in the Brain

Sara M. Hancock, Ashley I. Bush, and Paul A. Adlard

Abstract Zinc is an essential requirement for normal cellular processes and is involved in the function of more than 300 enzymes. Currently, it is estimated that approximately 10 % of the proteins encoded by the human genome contain zinc. Zinc is necessary for neuromodulation, synaptic transmission, intracellular signal transduction, and myriad other processes. The clinical implications of impaired zinc signaling are, therefore, far reaching, with profound central and peripheral disorders arising as a result of either an acute or a long-term dyshomeostasis in normal zinc levels. Targeting zinc as a therapy is now emerging as a tantalizing, although especially difficult, approach to a variety of diseases, including central nervous system disorders. This chapter reviews the clinical implications of impaired zinc signaling in the brain.

Keywords Brain • Central nervous system disorders • Zinc • Zinc homeostasis • Zinc signals • Zinc transporters

9.1 Introduction

Zinc, which was first described as an essential trace ion by Raulin in 1869, is now recognized as essential for life. It is a stable divalent cation that is involved in the normal function of more than 300 enzymes (being required for catalytic, co-catalytic, and structural functions of enzymes) and which is also found in approximately 10 % of all human proteins (Andreini et al. 2006). Zinc is indispensable for a multitude of normal cellular functions and organ systems, having roles in processes as diverse as DNA synthesis, cognitive function, growth, development, and immunity. As a result, zinc levels are tightly regulated throughout the body. However, when the normal homeostatic systems become impaired or fail,

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altered zinc levels can arise within different cellular compartments to then precipitate a diverse range of biological sequelae. As the body is well equipped to excrete excess zinc when required, humans are generally more likely to suffer from issues relating to zinc deficiency. Indeed, zinc deficiency, typically resulting from malnutrition, aging, or disease (e.g., cancer or diabetes), is a leading life-threatening factor in developing countries, and is becoming increasingly prevalent in developed countries, particularly in the aged population.

The human body contains 2 to 3 mg zinc, which is the second most abundant metal in mammalian tissues, second only to iron (Wang et al. 2005; Paoletti et al. 2009), with almost 90 % of that found in muscle and bone (Wastney et al. 1986). A high level of zinc has also been found in other organs including the brain, heart, kidney, liver, prostate, pancreas, lung, skin, and gastrointestinal (GI) tract. Although dietary sources of zinc come from a range of foods including vegetables and dairy products, animal flesh remains the primary source of bioavailable zinc for humans. Red meats such as beef and veal, in particular, have significantly higher zinc levels than white meats such as chicken, pork, and fish. Furthermore, phytates and dietary fibers in legumes and some vegetables actually inhibit zinc absorption in the intestine. Thus, a balanced diet is required to maintain optimal zinc nutrition.

9.2 Zinc Homeostatic Pathways

Before discussing the clinical implications of impaired zinc signaling, we briefly review the basic homeostatic mechanisms that control zinc levels throughout the body, as this is relevant to understanding the biological sequelae of a failure in the maintenance of normal zinc levels.

9.2.1 General Zinc Handling

Maintaining a constant state of cellular zinc nutrition is essential for normal function (King et al. 2000). In the periphery, zinc homeostasis is a highly regulated and coordinated process that involves uptake through intestinal epithelial cells and reabsorption via the kidneys; changes in the absorption/excretion of zinc in the GI tract are the primary mechanisms for maintaining zinc homeostasis in the body (King et al. 2000). Excess zinc is eliminated through excretion into the feces (Krebs 2000; Zhang et al. 2008). Research on zinc homeostasis has focused on intestinal and liver zinc transport processes (Levenson and Tassabehji 2007); however, data on zinc transport within the central nervous system (CNS) are increasingly emerging in the literature.

9.2.2 Zinc in the Brain

The brain has a large zinc content (Vasto et al. 2008), where it is essential for the correct functioning of more than 2,000 transcription factors and is a cofactor for more than 300 enzymes (Takeda 2000; Levenson and Tassabehji 2007; Jeong and Eide 2013). Here, zinc is involved in the structural conformation of about 70 % of proteins, and is present in almost half of the glutamatergic synapses in the cortex and limbic nuclei (Karol et al. 2010). Zinc is released into the synapse alongside glutamate during excitation, whereupon it interacts with ion channels, synaptic receptors, and transporters. Therefore, zinc is considered to be essential for the activity of enzymes and for modulating both synaptic transmission and plasticity.

It is well known that the majority of zinc in the brain is bound to macromolecules such as enzymes and transcription factors, although a small number of zinc ions (approximately 10–15 % of total brain Zn) exists as "free" or chelatable zinc (Levenson and Tassabehji 2007). Within the brain, zinc homeostasis is controlled by three main families of proteins that each have distinct tissue- and cell-level patterns of localization and expression (Hennigar and Kelleher 2012): these are the metallothioneins (MTs), zinc- and iron-like regulatory proteins (ZIPs), and zinc transporters (ZnTs). The presenilins have also recently been demonstrated to influence zinc concentrations (Greenough et al. 2013).

9.2.3 Metallothioneins

Currently, there are four MT isoforms that control cytosolic zinc concentrations through binding and distributing Zn^{2+} (Mocchegiani et al. 2001) (they also coordinate a variety of other metal ions, including copper). Metallothioneins are composed of a single polypeptide chain of 61–68 amino acids, and there are several isoforms, named MT-1, MT-2, MT-3, and MT-4, the first three of which are synthesized in the central nervous system (CNS) (Manso et al. 2011). MT-1 and MT-2 are widely expressed in all tissues, are present throughout the brain and spinal cord, and are primarily expressed by astrocytes, although neurons and reactive microglia are capable of synthesis. MT-3 is predominantly found in the CNS, although its precise localization is still debated (Manso et al. 2011). Similar to MT-3, MT-4 is a minor MT species and is found in stratified epithelial cells (Thirumoorthy et al. 2011).

9.2.4 Zinc- and Iron-Like Regulatory Proteins

The ZIP proteins have been divided into two subfamilies. Subfamily I refers to ZIPs that are mostly present in plant species, and subfamily II contains the ZIPs that are present in mammals, of which 14 are currently known in humans (Liuzzi and

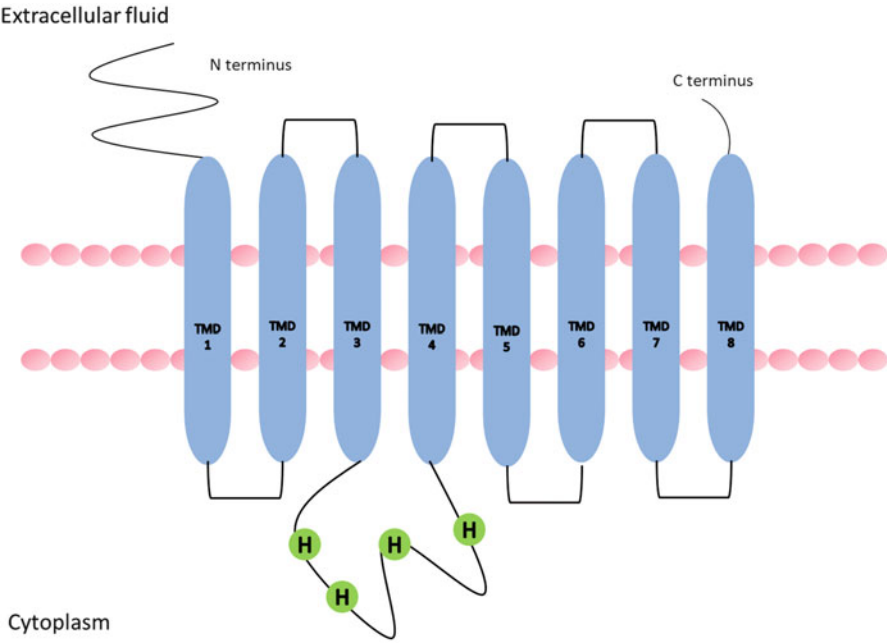

Fig. 9.1 Proposed molecular zinc- and iron-like regulatory protein (ZIP) structure showing eight trans-membrane domains (*TMD*s) and a long histidine (*H*)-rich domain, TMDs *3* and *4*

Cousins 2004). The primary role of the ZIPs is considered to be cellular zinc uptake into cells or export from vesicles or organelles (Levenson and Tassabehji 2007). Most ZIP proteins have a specific tissue-level pattern of expression and contain eight transmembrane domains and extracellular N- and C-termini, as illustrated in Fig. 9.1. Zinc transport activity has been confirmed for ZIPs 1–8 and -14 via transfection of DNA into mammalian cells (Cousins et al. 2006). Within the CNS, ZIP1 and ZIP3 appear to be the main facilitators of zinc uptake, although ZIP1 is more abundant in the brain, suggesting it is the primary neuronal zinc uptake mechanism (Levenson and Tassabehji 2007). Still, little is known about the structure, function, and localization of ZIPs 9–13, although ZIP12 is likely localized to the brain and ZIP13 is thought to be intracellular. For a summary table of tissue distributions of the ZIPs, see Eide (2004).

9.2.5 Zinc Transporters

There are currently ten known ZnT proteins in humans (Kambe 2012; Liuzzi and Cousins 2004), which function to regulate both the influx and efflux of zinc ions in cells and cellular compartments such as vesicles, mitochondria, and the Golgi apparatus. These transporters are predicted to have six transmembrane domains with cytoplasmic amino- and carboxy-termini (Fig. 9.2).

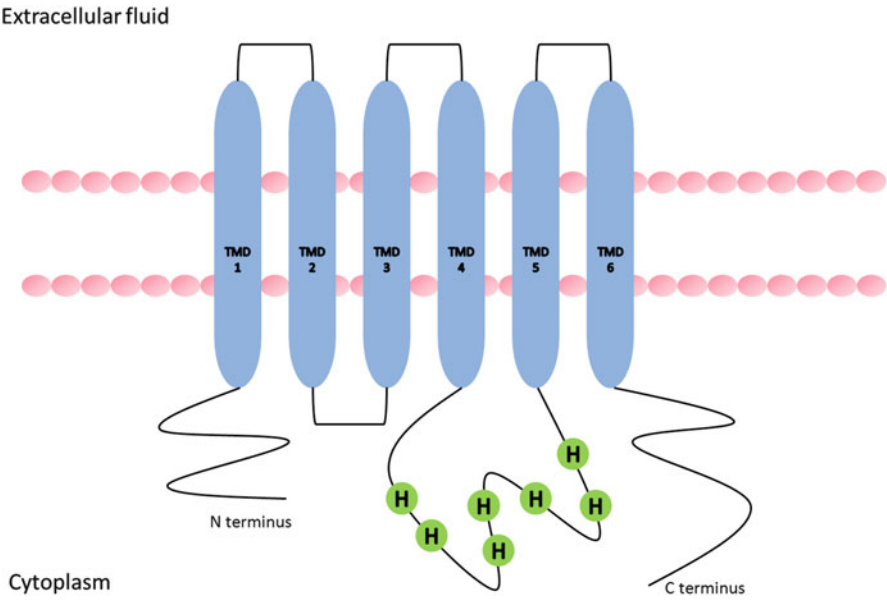

Fig. 9.2 Proposed molecular zinc transporter (ZnT) structure illustrating six *TMD*s and a long histidine (*H*)-rich loop between TMDs *4* and *5*

ZnT1, which was the first mammalian zinc transporter to be identified and characterized, is ubiquitously expressed and localized to the plasma membrane. ZnT2 is specific to secretory vesicles and is upregulated by high dietary zinc intake (Lichten and Cousins 2009). ZnT3 is similar to ZnT2 in function, in its role of loading Zn^{2+} into pre-synaptic vesicles. However, ZnT3 transcripts are limited to the brain with a high abundance in the hippocampus and cortex. ZnT4 has a high expression in the mammary gland, brain, and intestinal epithelial cells, where it has been detected at the trans-Golgi network (TGN) and is involved in vesicular secretory functions. Of the ZnTs, the exception to the rule is ZnT5, which has 15 transmembrane domains; it is ubiquitously expressed and interacts with ZnT6 (found only in brain and lung) to form a complex that transports zinc into a secretory pathway. Both ZnT5 and ZnT6 are located at the TGN. ZnT7 is expressed throughout the large and small intestine (regions of zinc absorption) and is involved in transcellular zinc movement through polarized epithelial cells. ZnT8 is localized to the membrane of insulin storage granules, and a direct relationship between glucose-induced insulin secretion and ZnT8 has been suggested (Cousins and Lichten 2011). There has been little research into the localization and role of ZnT9 and ZnT10, although ZnT9 has been associated with cytosol and nuclear fractions whereas it has been suggested that ZnT10 may be restricted to fetal development.

There is low expression of ZnT 2, 5, 7, and 8 in the brain; ZnT 1, 3, 4, and 6 are more highly expressed. These latter proteins are essential for modulating intracellular cytosolic zinc (ZnT 4 and ZnT 6) as well as zinc export (ZnT1 and ZnT3).

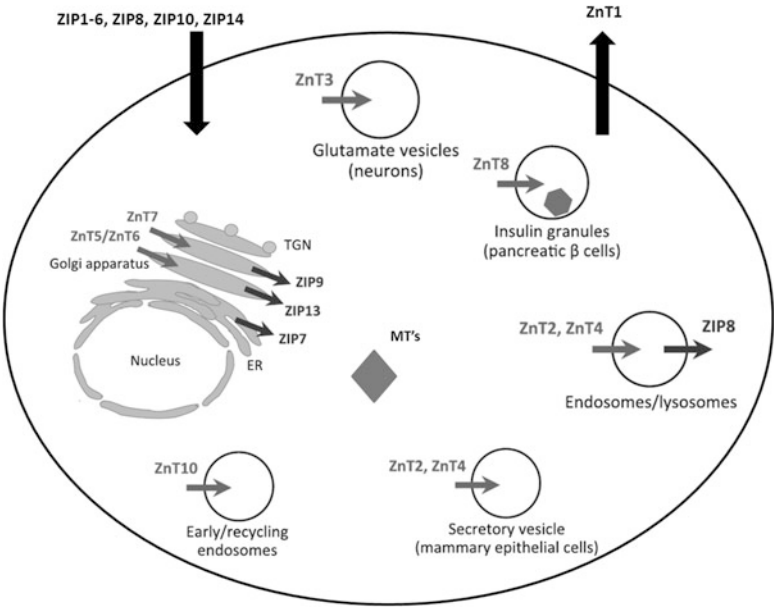

Fig. 9.3 Illustration of the proteins responsible for the cellular homeostasis of zinc. *MT*, metallothionein

ZnT1 and ZnT3 differ from the other ZnTs in that ZnT1 is present on the plasma membrane while ZnT3 is located on presynaptic vesicular membranes. A diagram more clearly demonstrating the homeostatic proteins is shown in Fig. 9.3. For more detailed descriptions of the ZIPs and ZnTs, see Lichten and Cousins (2009).

In summary, the regulation of zinc is tightly controlled via a range of specialized homeostatic mechanisms. When these processes are working correctly, the zinc-dependent proteins and signaling cascades present within the cell can function to maintain normal healthy tissue. However, such a large number of homeostatic proteins also provides increased opportunities for disruptions to occur. In this chapter we focus on the implications of these disruptions in the CNS. We now review the mechanisms and also the cellular and clinical outcomes of zinc dysregulation.

9.3 Zinc Signaling

As previously mentioned, zinc is a potent neuromodulator and has recently been suggested to have neurotransmitter qualities resulting from its role in synaptic signaling. Zinc is capable of directly influencing postsynaptic receptors and has been shown to exert effects in both neuronal and glial cells. It is able to do this via

its release into the synaptic cleft upon cellular activation, whereupon it can aid in regulating neuronal processes such as long-term potentiation (LTP) and long-term depression (LTD) by acting on *N*-methyl-D-aspartate (NMDA) receptors (Takeda and Tamano 2012). These postsynaptic actions have led to the suggestion that zinc is a signaling molecule capable of acting both intracellularly and extracellularly. Following its release alongside glutamate, zinc is taken up into adjacent cells through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) channels that trigger the generation of reactive oxygen species (ROS), which are known to directly contribute to oxidative stress (Fukada et al. 2011). By behaving in this manner, synaptically released zinc is able to regulate synaptic function and plasticity and has been labeled an "atypical neurotransmitter" (Haase and Rink 2011). Moreover, zinc has been identified as an endogenous G protein-coupled receptor agonist through ZIP14 regulation (Fukada et al. 2011), further supporting its identification as a neurotransmitter.

Different types of zinc signals have been observed that are thought to aid in regulating a variety of cellular functions. These zinc signals have a huge number of targets including receptors, second messengers, kinases, phosphatases, caspases, and transcription factors. The number of proteins influenced by zinc signals and the effects these signals can induce suggest there may be even more as yet unidentified proteins that zinc can affect. Previous research has defined zinc signals as a change in the concentration of intracellular free (chelatable) zinc, which then effects the transduction or processing of information at the cellular level (Haase and Rink 2011). Hirano and colleagues (2008) suggested the definitions of "transcription-independent early zinc signaling (EZS)" and "transcription-dependent late zinc signaling (LZS)" to describe signaling mechanisms also referred to as fast, intermediate (zinc wave), or slow (lasting for days). Currently, three major sources for zinc signals have been identified: zinc uptake from the extracellular environment, reversible storage of zinc in vesicles (synaptic or intracellular), and the oxidative release of zinc from storage proteins (Haase and Rink 2011). Fast signals, such as those mentioned here, occur after electrical extracellular stimulation whereas slow signals depend upon changes in the expression of proteins involved in zinc homeostasis (transporters, storage proteins, etc.). These slow signals have been predominantly observed in cell proliferation and differentiation where zinc uptake is the required signal (Li and Maret 2009). It should be noted here that uptake of zinc for signaling purposes should involve an active transport mechanism (Haase and Rink 2011).

Currently, it remains unclear how a relatively nonspecific change in intracellular free zinc can somehow regulate multiple signaling pathways. Despite advances in research techniques, the measurements used remain crude and only give average zinc concentration values for entire cells or whole populations of cells. To better understand how zinc signaling goes wrong and therefore how to specifically treat it, further improvements in research techniques are needed.

9.3.1 How Does Impaired Signaling Arise and What Are the Clinical Implications?

It is now known that there are a wide range of effectors of impaired zinc signaling, including phenomena such as mutations in key zinc transporters or homeostatic pathways. The most common cause, however, is undoubtedly diet. Although perhaps not so prevalent in wealthier countries and populations, malnutrition remains rampant throughout the developing world. First recognized as essential for humans in 1963, zinc deficiency is thought to affect approximately 40 % of the global population (Maret and Sandstead 2006). The results of nutritional zinc deficiency include growth retardation, hypogonadism in males, rough skin, and neurosensory disorders (Prasad et al 1963), as well as anorexia (Shay and Mangan 2000). This broad suite of symptoms arises from decreased zinc bioavailability throughout different tissues in the body. Although the brain has some resistance to dietary zinc deficiency through the blood–brain barrier (BBB), meaning zinc homeostasis in the brain is not easily disrupted (Takeda and Tamano 2009), it will ultimately succumb, which will be reflected by neuronal dysfunction that leads to cognitive deficits and other neuropsychiatric disturbances. Such cognitive impairments, which frequently accompany many chronic diseases (Fukada et al. 2011), are often responsive to zinc supplementation (Nyaradi et al. 2013).

One area of debate that remains in the literature is whether aging itself results in a global or tissue-specific zinc deficit. According to the current literature, brain zinc concentrations rise from birth to adulthood, then remain stable throughout life within a specific concentration range (Roberts et al. 2012). In comparison, plasma zinc levels, although constant through adulthood, decline with advancing age. Irrespective of whether there is an age-dependent change in total brain zinc, a number of reports now support an age-related decline in key zinc transporters, suggesting that there may be discrete cellular compartments that become zinc deficient with age. Although further research is essential, a study by (Wong et al 2013) showed an age-related decrease in ZIP6 that enhanced proinflammatory responses. Decreased ZnT4 and ZnT6 have been shown in individuals with mild cognitive impairment (MCI) (Smith et al. 2006), whereas in both normal aging and Alzheimer's disease (AD) there is a decline in ZnT3 protein levels (Adlard et al. 2010). In humans, it is particularly difficult to determine if changes in zinc are caused by zinc dyshomeostasis or if zinc dyshomeostasis is caused by decreased dietary zinc intake, a known problem in the elderly (Mocchegiani and Malavolta 2011). Furthermore, the neuronal uptake of zinc is dependent on a variety of factors including voltage-gated calcium (Ca^{2+}) channels and intracellular sodium (Na) (Tapiero and Tew 2003) that could fatigue with age, although uptake via ZnT2 has been suggested to be energy independent (Gaither and Eide 2001). Thus, a decline in transporter/importer levels, in combination with decreased efficiency of remaining transporters, will foster a zinc-deficient cellular milieu that may result in a host of deficits, including impaired learning and memory and cognitive decline.

Table 9.1 Tissue-specific expression of zinc- and iron-like regulatory proteins (ZIPs) and the related pathological conditions

Protein	Pathology/disease
ZIP	
ZIP4	Acrodermatitis enteropathica
ZIP6	Cancer progression markers for estrogen receptor-positive cancers (breast cancer)
ZIP7	Breast cancer
ZIP8	T-cell immune responses
ZIP10	Breast cancer
ZIP12	Schizophrenia
ZIP13	Ehlers–Danlos syndrome
ZIP14	T-cell immune responses
ZnT	
ZnT3	Alzheimer's disease; cognitive decline
ZnT4	Prostate disease/cancer
ZnT5	Allergic responses
ZnT8	Diabetes

As mentioned earlier, mutations in zinc transporters/importers are also capable of damaging zinc signaling processes, resulting in a variety of disease states: these are summarized in Table 9.1. We briefly describe the most robustly described CNS-related effects of these ZnT and ZIP mutations.

Acrodermatitis enteropathica (AE) is a disease resulting from a genetic mutation in ZIP4 causing intestinal zinc malabsorption. The disease is recognizable within a few months postpartum by the distinctive skin lesions around the mouth and anus and a failure to thrive (Perafan-Riveros et al. 2002). Similar symptoms are apparent in acquired zinc deficiency. Despite the presence of other mechanisms of intestinal zinc absorption, the zinc deficiency caused by AE results in both physical and neurological deficits such as behavioral changes, depression, and irritability (Sehgal and Jain 2000). When left untreated, AE is fatal.

Various cancers have been linked to zinc transporters, with ZIP6, ZIP7, and ZIP10 suggested to have a role in breast cancer. Additionally, ZIP6 is expressed in numerous cancer cell lines, leading to the hypothesis that it is involved in cancer progression. Currently it remains unclear if and how alterations in these transporters affect cancer pathology or the neurological consequences. Downregulation of ZnT4 is associated with prostate disease and prostate cancer; however, functions related to ZnT4 have received little attention in the literature and therefore require further exploration. There is a considerable amount of epidemiological evidence supporting a relationship between diabetes, zinc, and ZnT8 in humans (Cousins and Lichten 2011), because ZnT8 is localized to the membrane of insulin storage granules in the pancreas and single-nucleotide polymorphisms (SNPs) in ZnT8 have been associated with reduced insulin secretion following glucose stimulation (Staiger et al. 2007). It is well known that diabetes can have neurological consequences, and diabetes is a risk factor for dementia and AD (Liu et al. 2009).

Reductions in motor speed, psychomotor efficiency, learning, and reasoning have been shown in diabetics from mid-life to older ages, but this has yet to be assessed in younger diabetics (Seaquist 2010). It is also unclear how changes in zinc are relevant to these deficits. Allergy and immune responses have also been linked to zinc and zinc transporters; research has focused on ZIP8 and ZIP14 (close paralogues of each other) in T-cell immune responses. The expression of ZIP8 is influenced by immune mediators whereas ZIP14 is expressed in T cells and is capable of transporting zinc. Both have been demonstrated in mouse brain to be responsive to inflammation (Galvez-Peralta et al. 2014). The spondylocheiro dysplastic form of Ehlers–Danlos syndrome is a heritable connective tissue disorder characterized by joint hypermobility, hyperelasticity of skin, as well as hypotonia of skeletal muscles, and is caused by mutations in the ZIP13 gene (Giunta et al. 2008; Jeong et al. 2012). Neurological deficits are yet to be determined as the biological characterization of ZIP13 is an ongoing process (Bin et al. 2011).

Other diseases that can influence zinc signaling include stroke, trauma, and heart attack (Nolte et al. 2004; Grabrucker et al. 2011), which can also result in zinc-dependent CNS abnormalities. For example, depression and schizophrenia (Levenson and Tassabehji 2007), AD, amyotrophic lateral sclerosis (ALS), Down's syndrome, multiple sclerosis (Grabrucker et al. 2011), normal age-related cognitive decline (Adlard et al. 2013), and anorexia have all been linked to impaired zinc homeostasis. In AD, increased expression of ZnT1, ZnT3, ZnT4, ZnT5, ZnT6, and ZnT7 has been demonstrated in Aβ plaques and nearby amyloid angiopathic vessels (Zhang et al. 2008), although levels in surrounding tissues may be different. The CNS effects of zinc abnormalities are myriad: impaired signaling pathways, activation of the hypothalamic-pituitary axis (HPA), learning and memory deficits, and downregulated immune responses, to name a few. In addition, pneumonia causes abnormally low plasma zinc concentrations (Netsky et al. 1969). Alcoholism results in decreased liver zinc concentrations (McBean et al. 1972). Infectious diseases and acute myocardial infarction have also been reported to cause changes in zinc metabolism (McBean et al. 1972). For most of these disorders, the mechanisms through which zinc abnormality translates to a CNS deficit are not entirely clear and require more research.

We have discussed the human conditions that arise from impaired zinc signaling, but to gain further insights into these and the potential role of both zinc and zinc transporter mutations in the CNS, disease models are necessary. Mouse models remain the most used and perhaps most ideal tool for researching the effects of zinc deficiency. Mice with targeted disruptions in ZIP1, ZIP2, or ZIP3 are sensitive to zinc deficiency during pregnancy, and ZIP13 knockout mice experience disorganization in hard and connective tissues such as bones and teeth. A ZIP14 knockout results in retarded growth and impaired gluconeogenesis (Fukada et al. 2011). The ablation of the ZnT3 gene in mice has resulted in the most relevant CNS phenotype, as rodents develop a robust cognitive phenotype with very specific pre- and postsynaptic signaling alterations that drive deficits in learning and memory (Adlard et al. 2010; Sindreu et al. 2011; Martel et al. 2011). Other genetic modifications include loss-of-function mutations in both ZnT4 and ZnT2. This change

results in zinc deficiencies in the milk produced by the females, which in turn may result in a variety of clinical implications in both the peripheral and central nervous systems. Essentially, alterations in zinc transporters at either a genetic or molecular level, as a result of mutation or dietary deficiency, will impact the body and the brain in a multitude of ways, with many of these still to be discovered.

9.4 Zinc Signaling as a Therapeutic Strategy

For reasons of its prolific role throughout the nervous system, zinc signaling is an obvious therapeutic target. However, it is this extensive involvement with a multiplicity of proteins, enzymes, and signaling cascades that makes it a particularly difficult target to modulate to achieve a defined clinical outcome. Haase and Rink (2011) suggested that to regulate zinc and zinc signaling a prerequisite to treatment must be the sequestration of the zinc signal of interest. As zinc deficiency is prevalent across many diseases and physiological events, zinc supplementation should be an appropriate treatment option. However, zinc absorption is dependent on interactions with other nutrients (phytates, copper, iron, calcium) (Mocchegiani and Malavolta 2011). Hence, scientists have attempted to treat diseases with metal-modulating drugs. Two classes of interest are metal chelators and metal ionophores/chaperones, which have primarily been tested in models of age-related disorders such as AD. Chelators are compounds that bind to metal ions, decreasing their bioavailability (Ding and Lind 2009). However, currently there is little evidence for a therapeutic benefit in pathological aging. Building on the concept of metal chelators, other compounds have recently come to light that can modulate cellular metal concentrations. These compounds have been termed "metal ionophores" or "chaperones," and the two most extensively studied, particularly in relationship to their anti-AD potential, are clioquinol and PBT2 (both belonging to the 8-OH quinolone class of compounds). Both compounds have been shown to have positive effects on both pathology and cognition in a variety of transgenic mouse models of AD (Adlard et al. 2008, 2010). One of the benefits of this latter approach is that a much more targeted modulation of zinc can be achieved, as compared to simple zinc chelation or supplementation. It should also be noted that in AE, zinc supplementation can reduce physical symptoms, but it cannot repair neurological damage. Moreover, long-term zinc supplementation may also lead to a copper deficiency, which appears to be the result of competitive absorption of zinc and copper within enterocytes (Tapiero and Tew 2003; Mocchegiani and Malavolta 2011). This absorption is regulated by MTs that have a higher binding affinity for copper. Increased dietary zinc intake results in upregulation of MT protein expression, causing increased copper binding and excretion of the MT–copper complex. This finding further highlights the need for specifically targeted zinc treatments that will allow better control of potential side effects. Hence, zinc supplementation and zinc-modulating therapies must be used within a combination of treatments that are specifically targeted to a particular deficit.

9.5 Conclusion

Here we have discussed the critical role of zinc in a range of physiological processes wherein it is essential for life through its ability to act as a neurotransmitter and impact molecular and cellular changes essential for cognition, growth, immunity, DNA synthesis, and cell signaling. We have also discussed the diversity of diseases and neurological disorders that have been demonstrated to involve zinc and zinc dyshomeostasis. Research has also demonstrated the differing methods of zinc signaling that result in specific cellular and protein changes. Although this signaling represents a potential therapeutic target, further research and technical advances are required to ensure specificity of treatments that can reduce potential side effects while repairing only the dysfunctional zinc signal.

References

Adlard PA, Cherny RA, Finkelstein DI, Gautier E, Robb E, Cortes A et al (2008) Rapid restoration of cognition in Alzheimer's transgenic mice with 8-hydroxy quinoline analogs is associated with decreased interstitial Aβ. Neuron 59:43–55

Adlard PA, Parncutt JM, Finkelstein DI, Bush AI (2010) Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits in Alzheimer's disease? J Neurosci 30(5):1631–1636

Adlard PA, Sedjahtera A, Gunawan L, Bray L, Hare D, Lear J et al (2013) A novel approach to rapidly prevent age-related cognitive decline. Aging Cell. doi:10.1111/acel.12178

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196–201

Bin B-H, Fukada T, Hosaka T, Yamasaki S, Ohashi W, Hojyo S et al (2011) Biochemical characterisation of human ZIP13 protein: a homo-dimerized zinc transporter involved in the spondylocheiro dysplatic Ehlers–Danlos syndrome. J Biol Chem 286(46):40255–40265

Cousins RJ, Lichten LA (2011) Zinc transporters. In: Rink L (ed) Zinc in human health. IOS Press, Amsterdam, pp 136–162

Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking and signals. J Biol Chem 281(34):24085–24089

Ding W-Q, Lind SE (2009) Metal ionophores: an emerging class of anticancer drugs. Life 61 (11):1013–1018

Eide DJ (2004) The SLC39 family of metal ion transporters. Eur J Physiol 447:796–800

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signalling in health and diseases. J Biol Inorg Chem 16:1123–1134

Gaither LA, Eide DJ (2001) Eukaryotic zinc transporters and their regulation. In: Maret W (ed) Zinc biochemistry, physiology and homeostasis. Springer, Dordrecht, pp 65–84

Galvez-Peralta M, Wang Z, Bao S, Knoell DL, Nebert DW (2014) Tissue-specific induction of mouse ZIP8 and ZIP14 divalent cation/bicarbonate symporters by, and cytokine response to, inflammatory signals. Int J Toxicol 33:246–258

Giunta C, Elcioglu NH, Albrecht B, Eich G, Chambaz C, Janecke AR et al (2008) Spondylocheiro dysplastic form of the Ehlers–Danlos syndrome: an autosomal-recessive entity caused by mutations in the zinc transporter gene SLC39A13. Am J Hum Genet 82(6):1290–1305

Grabrucker AM, Rowan A, Garner CC (2011) Brain-delivery of zinc-ions as potential treatment for neurological diseases: mini review. Drug Deliv Lett 1(1):13–23

Greenough MA, Camakaris J, Bush AI (2013) Metal dyshomeostasis and oxidative stress in Alzheimer's disease. Neurochem Int 62(5):540–555

Haase H, Rink L (2011) Zinc signalling. In: Rink L (ed) Zinc in human health. IOS Press, Amsterdam, pp 94–117

Hennigar SR, Kelleher SL (2012) Zinc networks: the cell-specific compartmentalization of zinc for specialised functions. Biol Chem 393:565–578

Hirano T, Murakami M, Fukada T, Nishida K, Yamasaki S, Suzuki T (2008) Roles of zinc and zinc signalling in immunity: zinc as an intracellular signalling molecule. Adv Immunol 97:149–176

Jeong J, Eide DJ (2013) The SLC39 family of zinc transporters. Mol Asp Med 34:612–619

Jeong J, Walker JM, Wang F, Park JG, Palmer AE, Giunta C et al (2012) Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers–Danlos syndrome. Proc Natl Acad Sci USA 109(51):E3530–E3538

Kambe T (2012) Molecular architecture and function of ZnT transporters. Curr Top Membr 69:199–220

Karol N, Brodski C, Bibi Y, Kaisman T, Forberg M, Hershfinkel M et al (2010) Zinc homeostatic proteins in the CNS are regulated by crosstalk between extracellular and intracellular zinc. J Cell Physiol 224:567–574

King JC, Shames DM, Woodhouse LR (2000) Zinc homeostasis in humans. Am Soc Nutr Sci 1360S–1366S

Krebs NF (2000) Overview of zinc absorption and excretion in the human gastrointestinal tract. J Nutr 130(5):1374S–1377S

Levenson CW, Tassabehji NM (2007) Role and regulation of copper and zinc transport proteins in the central nervous system. In: Lajtha A (ed) Handbook of neurochemistry and molecular neurobiology. Plenum, New York

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr 29:153–176

Liu Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong C-X (2009) Brain glucose transporters, O-GlcNAcylation and phosphorylation of tau in diabetes and Alzheimer's disease. J Neurochem 111(1):242–249

Li Y, Maret W (2009) Transient fluctuations of intracellular zinc ions in cell proliferation. Exp Cell Res 315:2463–2470

Liuzzi JP, Cousins RJ (2004) Mammalian zinc transporters. Annu Rev Nutr 24:151–172

Manso Y, Adlard PA, Carrasco J, Vasak M, Hidalgo J (2011) Metallothionein and brain inflammation. J Biol Inorg Chem 16:1103–1113

Maret W, Sandstead HH (2006) Zinc requirements and the risks and benefits of zinc supplementation. J Trace Elem Med Biol 20(1):3–18

Martel G, Hevi C, Kane-Goldsmith N, Shumyatsky GP (2011) Zinc transporter ZnT3 is involved in memory dependent on the hippocampus and perirhinal cortex. Behav Brain Res 223:233–238

McBean LD, Dove JT, Halsted JA, Smith JC Jr (1972) Zinc concentration in human tissues. Am J Clin Nutr 25:672–676

Mocchegiani E, Malavolta M (2011) Zinc and ageing. In: Rink L (ed) Zinc in human health. IOS Press, Amsterdam, pp 325–346

Mocchegiani E, Giaconni R, Cipriano C, Muzzioli M, Fattoretti P, Bertoni-Freddari C et al (2001) Zinc-bound metallothioneins as potential biological markers of ageing. Brain Res Bull 55 (2):147–153

Netsky MG, Harrison WH, Brown M, Benson C (1969) Tissue zinc and human disease. Relationship of zinc content of kidney, liver and lung to atherosclerosis and hypertension. Am J Clin Pathol 51:358

Nolte C, Gore A, Sekler I, Kresse W, Hershfinkel M, Hoffman A et al (2004) ZnT1 expression in astroglial cells protects against zinc toxicity and slows the accumulation of intracellular zinc. Glia 43:145–155

Nyaradi A, Li J, Hickling S, Foster J, Oddy WH (2013) The role of nutrition in children's neurocognitive development, from pregnancy through childhood. Front Hum Neurosci 7:97
Paoletti P, Vergnano AM, Barbour B, Casado M (2009) Zinc at glutamatergic synapses. Neuroscience 158:126–136
Perafan-Riveros C, Franca FS, Alves CF, Sanches JA Jr (2002) Acrodermatitis enteropathica: case report and review of the literature. Pediatr Dermatol 19(5):426–431
Prasad AS, Miale A, Farid Z, Schulert A, Sandstead HH (1963) Zinc metabolism in patients with syndrome of iron deficiency anemia, hypogonadism and dwarfism. J Lab Clin Med 61:537–549
Raulin J (1869) Chemical studies on vegetation. Am Sci Nat 11:93–99
Roberts BR, Ryan TM, Bush AI, Masters CL, Duce JA (2012) The role of metallobiology and amyloid-β peptides in Alzheimer's disease. J Neurochem 120(suppl 1):149–166
Sehgal VN, Jain S (2000) Acrodermatitis enteropathica. Clin Dermatol 18(6):745–748
Seaquist ER (2010) The final frontier: How does diabetes affect the brain? Diabetes 59:4–5
Shay NF, Mangan HF (2000) Neurobiology of zinc-influenced eating behaviour. J Nutr 130 (5S suppl):1493S–1499S
Sindreu C, Palmiter RD, Storm DR (2011) Zinc transporter ZnT-3 regulates presynaptic Erk1/2 signaling and hippocampus-dependent memory. Proc Natl Acad Sci USA 108:3366–3370
Smith JL, Xiong S, Markesbery WR, Lovell MA (2006) Altered expression of zinc transporters 4- and 6- in mild cognitive impairment, early and late Alzheimer's disease brain. Neuroscience 140:879–888
Staiger H, Machicao F, Stefan N, Tschritter O, Thamer C, Kantartzis K et al (2007) Polymorphisms within novel risk loci for type 2 diabetes determine beta-cell function. Plos One 9:e832
Takeda A (2000) Zinc homeostasis and functions of zinc in the brain. Biometals 14:343–351
Takeda A, Tamano H (2009) Insight into zinc signalling from dietary zinc deficiency. Brain Res Rev 62:33–44
Takeda A, Tamano H (2012) Proposed glucocorticoid-mediated zinc signalling in the hippocampus. Metallomics 4:614–618
Tapiero H, Tew KD (2003) Trace elements in human physiology and pathology: zinc and metallothioneins. Biomed Pharmacother 57(9):399–411
Thirumoorthy N, Shyam SA, Manisethil Kuma KT, Ganesh GNK, Chatterjee M (2011) A review of metallothioneins isoforms and their role in pathophysiology. World J Surg Oncol 9(54)
Vasto S, Candore G, Listi F, Balistreri CR, Colonna-Romano G, Malavolta M et al (2008) Inflammation, genes and zinc in Alzheimer's disease. Brain Res Revs 58:96–105
Wang Z-Y, Stoltenberg M, Huang L, Danscher G, Dahlstrom A, Shi Y et al (2005) Abundant expression of zinc transporters in Bergman glia of mouse cerebellum. Brain Res Bull 64:441–448
Wong CP, Magnusson KR, Ho E (2013) Increased inflammatory response in aged mice is associated with age-related zinc deficiency and zinc transporter dysregulation. J Nutri Biochem 24:353–359
Wastney ME, Aamodt RL, Rumble WF, Henkin RI (1986) Kinetic analysis of zinc metabolism and its regulation in normal humans. Am J Physiol 251:R398–R408
Zhang L-H, Wang X, Zheng Z-H, Ren H, Stoltenberg M, Danscher G et al (2008) Altered expression and distribution of zinc transporters in APP/PS1 transgenic mouse brain. Neurobiol Aging 31:74–87

Chapter 10
Zinc Signals in Immunology

Lothar Rink and Martina Maywald

Abstract Zinc is known to be an essential trace element that is highly important for all proliferating cells in the human body, especially for the immune system. Free zinc influences several signaling pathways, such as Toll-like receptor 4 or T-cell receptor signaling, by binding reversibly to regulatory sites in signaling proteins, resulting in a change of free zinc concentrations that can affect signal transduction; thus, cellular responses can be altered. Zinc signals have been observed in cells of the innate as well as of the adaptive immune system, that is, neutrophil granulocytes, mast cells, monocytes, dendritic cells, and T cells, mostly in changes of the cytoplasmic zinc concentration. To characterize zinc signals one can distinguish them by the timescale in which they take place. First, zinc signals can occur within a few seconds to minutes, and are therefore called fast zinc signals. Second, a slightly slower type of zinc signal is known and described as "zinc wave." Third, some zinc signals occur on a timescale significantly longer than the others. In these cases, the signals are typically involved in altered expression of proteins involved in zinc homeostasis.

Zinc signals occurring in different cell types and signaling pathways that are mentioned in this chapter are classified regarding the specific discrimination of fast zinc signal, zinc wave, and late zinc signal.

Keywords Adaptive and innate immunity • Signal transduction • TLR-4 • Zinc • Zinc deficiency • Zinc homeostasis

The essentiality of zinc for animals was recognized in 1934, but it was not until the early 1960s that zinc was known to be essential for humans. Compared to other metal ions with similar chemical properties, zinc is relatively harmless (Fosmire 1990). Moreover, it has been shown that only exposure to high doses has toxic effects, making acute zinc intoxication a rare event. The uptake of cytotoxic doses of exogenous zinc is prevented by systemic homeostasis and efficient regulatory mechanisms on the cellular level. Intracellular zinc has an important role in cytotoxic events in single cells and is involved in various functions in numerous

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cell and organ systems (Prasad 2009). Virtually all immune cells are modulated by zinc in vitro and in vivo.

In this chapter, we discuss the importance of zinc signals in the immune system and the interactions of zinc with major signaling pathways that are responsible for the regulation of cellular functions, as well as the influence of zinc deficiency and zinc supplementation on zinc signaling in various immune cells.

10.1 Zinc Homeostasis and the Immune System

Zinc is considered to be relatively nontoxic to humans, and the human body contains a total amount of 2 to 4 g zinc (Haase and Rink 2010). In general there is no specialized zinc storage system in the human body, and thus one needs a daily intake of zinc to achieve a steady state for proper immune function. The distribution of zinc in the human body differs significantly among various tissues and organs, ranging from 10 μg/g dry weight in the brain (1.5 % of total body zinc) up to 100 μg/g in the bones (29.0 % of total body zinc). Other organs containing estimable concentrations of zinc include liver, prostate, kidney, skin, lung, heart, pancreas, and the gastrointestinal tract. Oral uptake of zinc results in absorption throughout the small intestine, and distribution subsequently occurs via the serum, where zinc concentrations reach 10–18 μM, comparable to around 1 μg/g (0.1 % of total body zinc) (Mills 1989). It is abundantly bound with low affinity to albumin (60 %), with high affinity to α_2-macroglobulin (30 %), and to transferrin (10 %) (Rink and Gabriel 2000). Thus, it is a predominantly intracellular ion (Haase and Maret 2009), distributed between the cell nucleus (30–40 %), cytoplasm, organelles, and vesicles (50 %) (Vallee and Falchuk 1993), including zinc-specific vesicles called "zincosomes" (Beyersmann and Haase 2001). These vesicles segregate high amounts of zinc upon stimulation, for example, with growth factors (Haase and Maret 2003). Metallothioneins (MT) play an important role in zinc homeostasis by complexing about 20 % of intracellular zinc. MTs are characterized by a low molecular weight of 6–7 kDa, high cysteine content, and their ability to complex metal ions. They are ubiquitously expressed proteins, and each MT can bind up to seven zinc ions. Although all zinc ions are bound in similar tetrathiolate coordination environments, the affinities of the sites for zinc range with a logK from 7.7 to 11.8. Thus, MT can act as a cellular zinc buffer over several orders of magnitude that range from picomolar to nanomolar concentrations as a consequence of the different affinities of the metal ion-binding sites (Krezel and Maret 2007). The dynamic regulation of cellular zinc homeostasis results from the synthesis of the apo-form thionein (T) in response to raised intracellular zinc levels by triggering the metal-response element-binding transcription factor (MTF)-1. MTF-1 is a cellular zinc ion sensor and regulates transcription of genes, whose promoters contain metal-response elements, because at least two of its six zinc fingers are not constitutively bound to zinc, and thus these sense available free zinc by reversible binding, resulting in stabilization of the zinc-finger domains

(Radtke et al. 1993). Furthermore, oxidation of cysteine residues alters the number of metal-binding sites, thus connecting redox and zinc metabolism (Laity and Andrews 2007).

Despite the fact that the plasma zinc pool is very small, it is highly mobile and immunologically important. Similar to serum zinc, most of cellular zinc is bound to proteins, leaving only a minor part of intracellular zinc, which is also referred to as labile, mobile or "free zinc," loosely bound or unbound. Free zinc is an operative term, used to distinguish zinc involved in signal transduction from the tightly protein-bound zinc, which is thermodynamically unavailable. The term is used in this chapter with the understanding that it is chemically incorrect, because zinc ions will never be completely without ligands, but form complexes with amino acids, glutathione, phosphate, or other low molecular weight ligands, when not bound to proteins.

So far, more than 300 enzymes and even more proteins are identified to contain zinc. Based on a screening for well-known zinc-binding sequences, it is estimated that up to 10 % of the encoded proteins in the human body may contain zinc (Andreini et al. 2006). The zinc proteome consists of two major groups of comparable size, enzymes and transcription factors. Together, these form more than 90 % of the zinc proteome, indicating that the vast majority of zinc is required for catalysis and transcriptional regulation (Andreini et al. 2009).

Tightly protein-bound zinc is required for catalytic, co-catalytic, and structural functions of enzymes (Vallee and Falchuk 1993). In some enzymes it is important for structural integrity, and in others it is the central ion for enzymatic activity, but sometimes both functions are involved. Moreover, zinc is involved in the stabilization of structural domains, such as in zinc fingers and related structures and enables interaction of proteins or nucleic acids, as for many transcription factors (Maret 2006).

First, understanding of the mechanism by which zinc uptake into the cell takes place is important. It has been shown that exogenous zinc enters the cell within minutes (Wellinghausen et al. 1996). Cellular zinc underlies an efficient homeostatic control to avoid excessive accumulation. Therefore, two families of eukaryotic zinc transporters are known to date: the Zip and ZnT proteins. The Zrt-like, Irt-like protein (Zip) family comprises 14 genes designated solute carrier family 39 (SLC39) A1 to A14 that transport zinc into the cytosol, whereas the zinc transporter (ZnT) family of 10 genes (SLC30A1 to SLC30A10) transports zinc in the opposite direction (Cousins et al. 2006; Eide 2006). The same transporter families also regulate the intracellular distribution of zinc into the endoplasmatic reticulum, mitochondria, and Golgi network (see Chap. 3). In addition, different possible mechanisms for zinc uptake such as facilitated diffusion via amino acids and anionic exchange or calcium-conducting channels have been reported (Bentley 1992; Hogstrand et al. 1996). To date, additionally members of four distinct superfamilies, that is, voltage-gated calcium channels (VGCC) (Gyulkhandanyan et al. 2006), glutamatergic receptors (Jia et al. 2002), nicotinic acetylcholine receptors (Ragozzino et al. 2000), and TRP channels (Dong et al. 2008) have been shown to transport zinc through the cell membrane.

However, free zinc contributes to the regulation of numerous cellular processes and influences several signaling pathways by binding reversibly to regulatory sites in signaling proteins; this results in a change of the free zinc concentration that can affect signaling pathways, and thus cellular responses can be altered (Ibs and Rink 2003). Furthermore, zinc shows intracellular fluctuation after stimulation (Haase et al. 2006a), assuming a complex interaction between zinc homeostasis and signal transduction, where zinc may have similar functions as the second messenger calcium (Williams 1984).

Free zinc is crucial for the appropriate development and function of innate as well as adaptive immunity, but the importance of zinc for proper immune function is most obvious in zinc-deficient individuals.

Although intoxication by excessive exposure is rare, zinc deficiency is widespread and has been known to have a detrimental impact on growth, neuronal development, and immunity, and in severe cases its consequences are lethal. Human zinc deficiency has been reported the first time in 1961, when Iranian males were diagnosed with symptoms including growth retardation, skin abnormalities, hypogonadism, and mental lethargy that led back to nutritional zinc deficiency (Prasad et al. 1961). Later, additional studies showed remarkably similar features and manifested zinc deficiency as a potentially widespread problem in developing countries as well as in industrial nations (Prasad et al. 1963; Sandstead 1991).

Zinc deficiency manifests itself on different levels and can be subdivided into either severe or marginal zinc deficiency. Moreover, zinc deficiency can be either inherited or acquired. On the one hand, severe zinc deficiency is caused by metabolic disorders regarding zinc uptake as well as by other sources such as parenteral nutrition without zinc.

Zinc deficiency results in complex immune defects, leading to various immune disorders (see Chap. 11). This condition is observed in its most severe form in the zinc malabsorption syndrome acrodermatitis enteropathica, an autosomal recessive disorder characterized by periorificial and acral dermatitis, mucocutaneous lesions, failure to thrive, alopecia, diarrhea, neuropsychological disturbances, weight loss, and frequent severe infections with fungi, viruses, and bacteria that is based in most cases on a mutation of the intestinal zinc uptake protein Zip 4 (Kury et al. 2002; Wang et al. 2002).

Acquired severe zinc deficiency has been observed in patients receiving total parental nutrition without zinc supplementation, following excessive alcohol consumption, severe malabsorption, and iatrogenic causes such as treatment with histidine or penicillamine (Prasad 1985). The arising symptoms are mostly similar to those in acrodermatitis enteropathica. Patients who become zinc deficient while receiving total parenteral nutrition without zinc supplementation develop abnormalities that include lymphopenia, decreased ratios of $CD4^+$ to $CD8^+$ T cells, decreased NK cell activity, and increased monocyte cytotoxicity, but these are readily corrected by proper zinc supplementation (Allen et al. 1983).

In another group of inherited disorders of zinc metabolism the zinc plasma level is above 3 μg/ml, more than three times the physiological level, while iron and

copper levels stay normal (Failla et al. 1982; Fessatou et al. 2005; Smith et al. 1976). Even though this exceeds the amount that can be found in serum after zinc intoxication, symptoms range from none to severe anemia, growth failure, and systemic inflammation (Fessatou et al. 2005; Smith et al. 1976; Saito et al. 2002; Sampson et al. 1997). The increased zinc levels have been attributed to excessive binding to serum proteins such as albumin (Failla et al. 1982; Smith et al. 1976) or to an overexpression of calprotectin, a zinc-binding S100 protein (Fessatou et al. 2005; Saito et al. 2002). Therefore, the large amount of zinc in the serum of these patients that is attached to proteins and thus biologically available may be depleted (Sampson et al. 1997). Furthermore, moderate zinc deficiency can also accrue as a consequence of sickle cell disease (Prasad 1981). Hyperzincuria and a high protein turnover caused by increased hemolysis lead to moderate zinc deficiency in these patients, causing clinical manifestations typical for zinc deficiency, such as growth retardation, hypogonadism in males, hyperammonemia, and cell-mediated immune disorder (Prasad 2002) connected with thymic atrophy (Dardenne et al. 1984).

Affected subjects show reduced lymphocyte proliferation response to mitogens, anergy, thymic atrophy, a selective decrease in $CD4^+$ T cells, and deficient thymic hormone activity (Prasad 2000). In general, zinc deficiency leads to an increased susceptibility to infection and parasitic disease, and is furthermore the fifth leading cause of mortality and morbidity in developing countries (WHO 2002).

Marginal zinc deficiency can be characterized by slight weight loss, oligospermia, and hyperammonemia (Prasad 1985). It may be caused by nutritional zinc deficiency consequent to high consumption levels of zinc-chelating phosphates, lignins, and phytates that counteract zinc absorption, by malabsorption syndrome, or by sickle cell anemia.

Moreover, parallels exist between the decline in immune function in the elderly and zinc deficiency even in industrialized countries. A significant percentage of elderly show reduced serum zinc levels, and zinc supplementation studies indicate that this deficiency contributes to increased susceptibility to infectious diseases. It also occurs frequently during pregnancy and lactation, because of rapid growth, in vegetarians, or in persons with renal insufficiency (Brieger and Rink 2010; Haase and Rink 2010).

The overall frequency of zinc deficiency worldwide is expected to be higher than 20 % (Wuehler et al. 2005). In developing countries, it may affect more than 2 billion people (Prasad et al. 1961, 1963; Cavdar et al. 1983). Furthermore, it has been estimated that only 42 % of the elderly ($\geq$71 years) in the United States have adequate zinc intake (Briefel et al. 2000). This widespread occurrence combined with the variety of clinical manifestations makes zinc deficiency a serious nutritional problem, which has a far greater impact on human health than the relatively infrequent zinc intoxication.

Detractions in zinc homeostasis affect multiple aspects of the immune system including hematopoiesis, cell-cycle progression, and immune-regulating molecules. Intracellular killing, cytokine production, and ROS (reactive oxygen species) synthesis are all affected by zinc deficiency. It also affects adversely the growth and

function of T and B cells by dysregulation of basic biological functions at the cellular level. Moreover, zinc is needed for DNA synthesis, RNA transcription, cell division, and cell activation. Programmed cell death (apoptosis) is also elevated in the absence of adequate zinc levels. Also, secretion and function of cytokines, the basic messengers of the immune system, are adversely affected by zinc deficiency. The ability of zinc to function as a pro-antioxidant and to stabilize membranes suggests that it has a role in prevention of free radical-induced injury during inflammatory processes.

All in all, zinc signals contribute to the regulation of virtually all immune cells (see Chap. 11), to numerous cellular processes, and influence several signaling pathways by binding reversibly to regulatory sites in signaling proteins. Thus, zinc is crucial for the appropriate development and function of innate as well as adaptive immunity, as is further described next.

10.2 Types of Zinc Signals

Free zinc influences several signaling pathways by binding reversibly to regulatory sites in signaling proteins, resulting in a change of free zinc concentrations that can affect signaling pathways. Thus, cellular responses can be altered (Haase and Rink 2009a).

It has been shown that activation of T cells causes an intracellular redistribution of zinc and an activation of protein kinase C (PKC) (Csermely et al. 1987, 1988). Since that time, zinc signals have been observed in different immune cells, that is, T cells (Kaltenberg et al. 2010; Yu et al. 2011; Lee et al. 2008; Aydemir et al. 2009), dendritic cells (Kitamura et al. 2006), monocytes (Haase et al. 2008), neutrophil granulocytes (Hasan et al. 2013), and mast cells (Yamasaki et al. 2007), mostly in changes of cytoplasmic zinc concentration (Haase and Rink 2009a).

One way to characterize zinc signals is by the timescale in which they take place. First, zinc signals can occur within a few seconds to minutes by triggering receptors such as Toll-like receptor (TLR)-4. Zinc acts as second messenger, comparable to calcium, and influences several signaling pathways directly. Therefore, the zinc signal is independent from the synthesis of proteins such as Zips and ZnTs and is thus called the fast zinc signal.

Second, a slightly slower type of zinc signal is known that is described as “zinc wave” and investigated in mast cells. Here, crosslinking of FcεRI induces a release of free zinc from the perinuclear area, including the endoplasmic reticulum. Comparable to the fast zinc signal, zinc acts also as a second messenger but is induced indirectly depending on calcium influx (see Chap. 5). However, zinc signals and the zinc wave also have a function as second messenger; the altered cytoplasmic zinc concentration influences other signaling pathways also, comparable to the role of late zinc signals.

Third, some zinc signals occur on a significantly longer timescale. In these cases, the late signals are typically involved in altered expression of proteins involved in

Table 10.1 Characterization of zinc signals concerning the timescale during which they take place

Zinc signal	Duration	Effect
Fast zinc signal	Seconds to minutes	Influence of cGMP by inhibition of PDE in monocytes/macrophages
		MKP inhibition in monocytes/macrophages
		PMA-induced NET-formation in PMN
		Expression of IL-8 in epithelial cells
		Lck recruitment to TCR-activating complex
		Zinc release from lysosomes in T cells
		T-cell activation by APC
		Homodimerization and activation of Lck in T cells
		Redistribution of zinc from nucleus/mitochondria to cytosol/microsomes in T cells
		IL-2-mediated zinc signal
Zinc wave	Minutes	Zinc release from perinuclear area in mast cells
Altered zinc homeostasis (late zinc signal)	Hours	Adherence of monocytes to endothelial cells
		Influence of mRNA transcription, e.g., IL-2, A20 in T cells, monocytes/macrophages
		MAPK activation in airway epithelial cells and monocytes/macrophages
		Negative regulation of TRIF pathway in macrophages Change of Zip/ZnT expression in DCs and T cells
		Inhibition of adenylate cyclase transcription in T cells
		Influence of cytokine production, e.g. IL-2 in T cells
		Upregulation of Akt phosphorylation in T cells
		PTEN inhibition in T cells
		Stat 3/6 phosphorylation in B and T cells
		Induction/stabilization of regulatory T cells
		Reduced cytokine production, e.g., IFN-γ in T cells

Zinc signals occurring within a few seconds to minutes are called fast zinc signals and are independent of the synthesis of proteins such as Zips and ZnTs. A slightly slower type of zinc signal, described as a "zinc wave," occurs after a few minutes. Zinc signals that take place on a timescale significantly longer (hours) are typically involved in altered expression of proteins involved in zinc homeostasis

zinc homeostasis, such as maturation of monocytes and DCs or cytokine expression (Dubben et al. 2010; Kitamura et al. 2006). Furthermore, the altered zinc homeostasis results in an influence of several signaling pathways, but zinc itself does not function as a second messenger.

Examples for each classification of zinc signals occurring in different cell types and signaling pathways are mentioned in the following section and are summarized in Table 10.1.

10.3 Zinc Signal and Innate Immunity

Innate immunity as the first line of defense is a highly important natural protection system against harmful substances, consisting of different cell types such as monocytes/macrophages, granulocytes, dendritic cells (DC), mast cells, and natural killer (NK) cells, as well as countless soluble proteins such as complement proteins, acute-phase proteins, cytokines, and chemokines. It is not highly specific, as is adaptive immunity, but reacts on different antigens in the same way. Its cells are activated by conserved structures in pathogens, called pathogen-associated molecular patterns (PAMPs), that are recognized by conserved receptor-triggering processes such as cytokine production, killing of target cells, or antigen presentation to cells of the adaptive immune system. Compared to the number of processes regulated on the cellular level, only limited numbers of signaling pathways exist. These pathways, depending on the cell type, are induced by different receptors and lead to the expression of cell type-specific genes. Immune cells as well as a myriad of other cell types share these identical pathways, as discussed next.

In an infection, polymorphonuclear leukocytes (PMNs) are the first cells actively entering the infected tissue by following a concentration gradient of chemical messengers, a mechanism known as chemotaxis. It is reported that this and other cellular functions are disturbed by altered zinc levels. Zinc deficiency leads to reduced PMN chemotaxis (Ibs and Rink 2003; Shankar and Prasad 1998), whereas elevated zinc concentrations, about 500 μM, are reported to induce chemotactic activity directly in vitro (Hujanen et al. 1995). PMNs are phagocytes that are essential to destroy pathogens by phagocytosis and generation of the respiratory burst. Zinc deficiency leads to a decreased phagocytosis that can be restored by zinc supplementation (Sheikh et al. 2010). These effects depend on an altered zinc homeostasis.

PMNs kill phagocytosed pathogens through the rapid production and release of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hypochlorous acid. Superoxide anions are produced by NADPH oxidase, which can be inhibited by zinc deficiency as well as by zinc excess (DeCoursey et al. 2003; Chasapis et al. 2012; Hasegawa et al. 2000). Another neutrophil granulocyte function is influenced by altered zinc concentrations, namely, NETosis. NETosis is described as the release of a matrix composed of DNA, chromatin, and granule proteins to capture extracellular bacteria within so-called neutrophil extracellular traps (NETs). NETosis can be induced by protein kinase C (PKC) activator 12-myristate 13-acetate (PMA) and depends on the production of ROS by NADPH oxidase. PMA treatment leads to a fast zinc signal in neutrophil granulocytes that is an essential component of the ROS-dependent signal transduction leading to NET formation. Recently, it was shown that zinc chelation abrogates NET formation in vitro (Hasan et al. 2013).

Similar to PMNs, macrophages also take up pathogens or cellular debris by phagocytosis and kill pathogens by oxidative burst, both of which are zinc dependent. Monocytes produce various cytokines, for example, pro-inflammatory

cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. Therefore, zinc signals are essential and can also be directly induced by stimulation with high extracellular zinc concentrations (Wellinghausen and Rink 1998; Wellinghausen et al. 1997a). Monocytes develop from myeloid precursor cells and can move quickly to sites of infection in the tissues and differentiate into macrophages and dendritic cells to elicit an immune response. The differentiation of monocytes is calcitriol dependent and can be enhanced by zinc sequestration, which indicates a negative regulatory role for this ion (Dubben et al. 2010), depending on an altered zinc homeostasis. After their development, monocytes circulate in the bloodstream, from which they start to migrate into the tissue where the maturation into resident macrophages takes place. Migration from blood into tissues starts by adherence to endothelial cells, which can be augmented by zinc, and stimulation of monocytes with chemoattractant protein-1 (MCP)-1 or PMA induces an intracellular zinc signal promoting adhesion to endothelial cells (Chavakis et al. 1999; Kojima et al. 2007). These effects depend on altered zinc homeostasis.

Monocytes/macrophages and dendritic cells (DC) take up and present antigens to T cells. They coordinate the immune response by producing cytokines in response to a sufficient stimulation that leads to an activation of both cell types, such as lipopolysaccharide (LPS), which is sensed by Toll-like receptor (TLR)-4. Thus, TLR-4 signaling leads to secretion of pro-inflammatory cytokines and maturation and antigen presentation in dendritic cells and macrophages.

In TLR-4 signaling, zinc has a double-edged effect on the cytokine secretion in monocytes (see Fig. 10.1). Herein fast zinc signals that are moderately increased are involved in TLR-4 signal transduction, whereas long-term higher concentrations are inhibitory. A fast zinc signal has been observed within less than 2 min by binding of bacterial LPS to TLR-4 that is involved in the activation of mitogen-activated protein kinase (MAPK) signaling. MAPK activation via zinc can be observed in many cell types. The MAPK extracellular-regulated kinase (ERK) is shown to be activated by zinc treatment of fibroblasts (Hansson 1996), as well as of neurons or neuroblastoma cells (An et al. 2005; Park and Koh 1999). In addition, this effect is also described in the activation of further MAPKs such as p38 and Jun N-terminal kinase (JNK) (An et al. 2005; LaRochelle et al. 2001). Similar observations have been made with other TLR ligands, such as pam3CSK4, insulin, and TNF-α.

Although no direct effect of zinc on MAPK activity has been reported, a fast zinc signal is reported to lead to an inhibition of MAP kinase phosphatases (MKP) that dephosphorylate both tyrosine and threonine residues in activated MAPKs. Thus, indirectly zinc causes inhibition of ERK1/2 and dephosphorylation of p38 in human macrophages (Haase et al. 2008). The zinc-dependent inhibition of MKP is involved in ERK activation during oxidative stress in neurons (Ho et al. 2008) and is one mechanism by which zinc stimulates expression of IL-8 in epithelial cells (Kitamura et al. 2006). However, not all the effects of zinc on MAPK phosphorylation and activation can be explained simply by zinc inhibition of MKP because zinc also affects targets upstream of the MAPKs. For instance, MAPK activation in

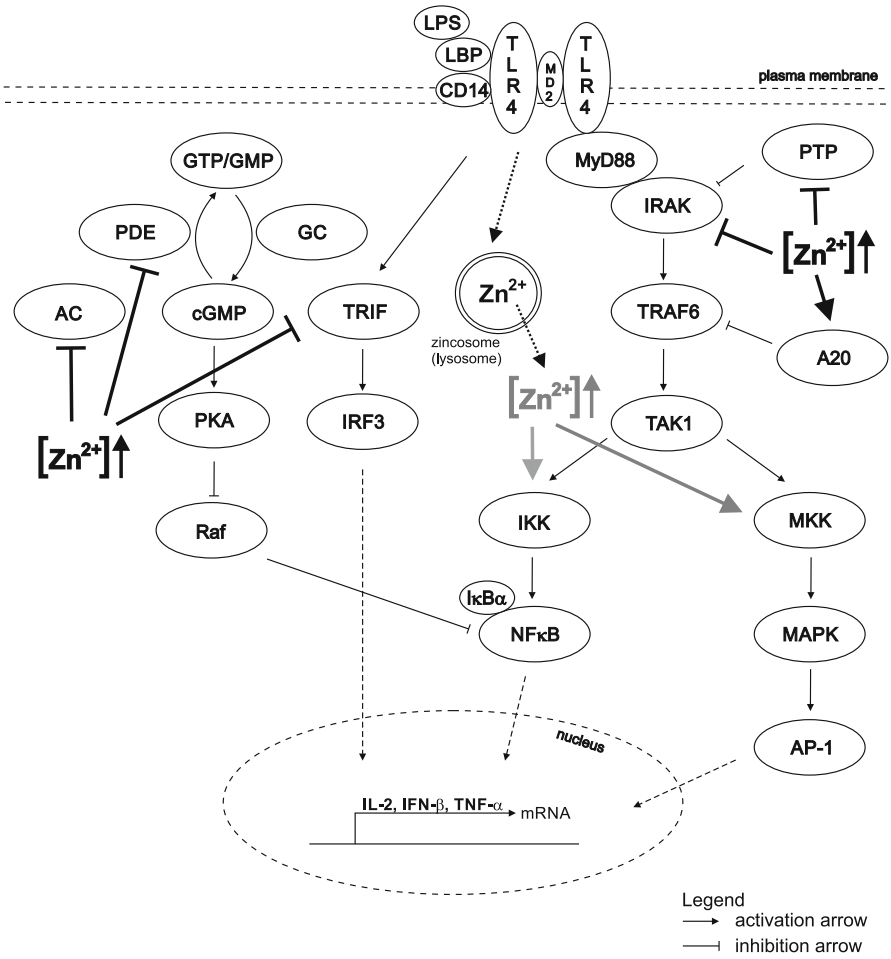

Fig. 10.1 Zinc and Toll-like receptor 4 (*TLR-4*) signaling. Triggering of TLR-4 induces fast zinc release (highlighted in *dotted arrows*) from zincosomes. These physiological fast zinc signals (highlighted in *dark grey*) are required for activation of nuclear factor kappa B (*NF-κB*) and mitogen-activated protein kinase (*MAPK*) signaling (*activation arrow*) leading to translocation (*dashed arrows*) into the nucleus. Long-term higher concentrations of zinc, resulting from the use of ionophores in experimental procedures (highlighted in *bold black*), inhibit TLR-4 signaling (*inhibition arrow*). Suggested mechanisms are a direct inhibition of interleukin-1 receptor-associated kinase (*IRAK*), an upregulation of *A20* that removes the activating ubiquitination of tumor necrosis factor receptor-associated factor 6 (*TRAF6*), an inhibition of cyclic nucleotide phosphodiesterases (*PDE*) or an inhibition of TIR-domain-containing adapter-inducing interferon-β (*TRIF*). Inhibition of PDE leads to a rise in cyclic guanosine monophosphate (*cGMP*) and cross-activation of protein kinase A (*PKA*), resulting in Raf inhibition and thereby TLR-mediated NF-κB activation. Furthermore, increased intracellular zinc concentrations inhibit adenylate cyclase (*AC*). *IKK* I kappa B kinase, *IRF3* interferon regulatory factor 3, *MKK* MAPK kinase, *PKA* protein kinase A, *PTP* protein tyrosine phosphatases, *AP-1* activator protein 1, *GMP* guanosine monophosphate, *GTP* guanosine trisphosphate

airway epithelial cells also depends on a long-time zinc effect on tyrosine phosphorylation upstream of ERK (Wu et al. 2002).

However, the effect of zinc on MAPKs is not always agonistic. Low concentrations of zinc are shown to activate ERK in rat glioma cells, whereas higher concentrations reduced the activating phosphorylation (Haase and Maret 2005). One potent molecular mechanism could be that the zinc transporter (ZnT)-1 homologue CDF-1 mediates zinc efflux, leading to an activation of Ras/Raf/MEK/ERK pathway (Bruinsma et al. 2002). Here zinc may promote the inhibitory phosphorylation of kinase suppressor of Ras (KSR). KSR works as a scaffolding protein that stabilizes the interaction of Ras, Raf, and MEK (Yoder et al. 2004). Another molecular mechanism could be CDF/ZnT-1 binding to Raf-1, promoting its biological activity, which is inhibited by zinc (Jirakulaporn and Muslin 2004). Therefore, an altered zinc homeostasis influences the signaling pathways and the threshold of activation.

Also, extended concentrations of zinc inhibit TLR-4 signaling. In this connection the first suggested mechanism is a direct inhibition of the interleukin-1 receptor-associated kinase (IRAK). Second, the late zinc signal leads to upregulation of mRNA of the cytoplasmatic zinc-finger protein A20 (Prasad et al. 2004) that removes the activating ubiquitination of tumor necrosis factor receptor-associated factor 6 (TRAF6), which could result in disturbed TLR-4 signaling. In TLR signaling, TRAF6 ubiquitination leads to proteasome-independent activation of the transforming growth factor beta-activated kinase (TAK)1, which in turn phosphorylates IκB kinases (IKKs) and MAPK kinases (MKKs) (Deng et al. 2000; Wang et al. 2001). A20 is induced by pro-inflammatory stimuli and is involved in the termination of TLR-induced activity of NF-κB and pro-inflammatory gene expression in macrophages (Boone et al. 2004). Moreover, it deubiquitinates TRAF6, inactivating this protein. Therefore, upregulation of A20 by zinc leads to diminished NF-κB and MAPK activation in response to TLR stimulation. Third, the inhibition of the cyclic nucleotide phosphodiesterases (PDE) via a fast zinc signal is named, which results in an increase in cyclic guanosine monophosphate (cGMP) synthesized by guanylate cyclases (GCs). This effect is caused by cross-activation of protein kinase A (PKA) by elevated cGMP concentrations. In human monocytes, degradation of cyclic nucleotides is mediated by PDEs 1, 3, and 4, that are all inhibited by zinc (von Bulow et al. 2005), which leads to inhibitory phosphorylation of Raf-1, resulting in its inactivation. Because Raf-1 is necessary for TLR-4 mediated NF-κB activation, however, the zinc/cGMP/PKA/Raf pathway blocks the activation of this transcription factor and thereby transcription of genes for pro-inflammatory cytokines (von Bulow et al. 2007). In addition to PDEs, adenylate cyclase (AC) is inhibited by the late zinc signal, leading to a reduced cAMP level, whereas the synthesis of cGMP by soluble GC is unaffected. So far, two different mechanism for zinc inhibition of AC have been presented: zinc may influence enzymatic activity by altering the conformation of AC (Klein et al. 2004) or, alternatively, by inhibiting the activation of AC via the heterotrimeric G-protein alpha-subunit (Gao et al. 2005).

Moreover, during a longer timescale zinc affects the response of DCs to LPS. Treatment of murine DCs with LPS leads to a reduction of free intracellular zinc, based on a TIR-domain-containing adaptor protein inducing IFN-β (TRIF)-dependent change in the expression pattern of several zinc transporters. This step involves a reduction of Zrt-like, Irt-like Protein (Zip) 6 and 10 gene expression, and upregulates expression of zinc transporter (ZnT)-1, -4, and -6. The reduction in free zinc is important for maturation of DCs (Kitamura et al. 2006), but also chelation of zinc influences TLR-4 signaling in macrophages. Stimulation with the zinc chelator TPEN augments TLR-4-mediated production of IFN-β and subsequent synthesis of inducible NO synthase and production of NO. The effect is based on zinc acting as a negative regulator of the TRIF pathway via reducing IFN regulatory factor 3 activation (Brieger et al. 2013).

Zinc is involved in the regulation of LPS signaling in cells of the innate immune system, but effects depending on zinc are not limited to the response to LPS. Also, a role for free zinc in regulation of protein kinase C (PKC) activity during monocyte chemoattractant protein (MCP)-1-induced adhesion is known (Kojima et al. 2007). In this context, which other receptors depend on zinc for cellular signaling need investigation. In vivo, zinc deficiency will impair the function of monocytes, in particular their ability to mount an adequate cytokine response during infection, but because zinc is equally involved in negative regulation of the same pathways, a disturbance of zinc homeostasis could also affect the limitation of pro-inflammatory cytokine production, leading to overproduction. Although this seems contradictory, it is supported by experimental evidence. For instance, in the elderly reduced spontaneous cytokine production is observed by long-term zinc supplementation, but simultaneously an improved cytokine response to PAMPs occurs (Kahmann et al. 2008).

NK cells play also a major role in immunity against infections and tumor development. This subset of lymphocytes mediates the killing of either infected or transformed cells, using a variety of different receptors. They act mainly by two different mechanisms: the first is based on the availability of major histocompatibility complex class I (MHC-I) molecules or other cell-surface proteins on target cells screened by NK cells. The absence of those molecules indicates an attempted evasion of T-cell immune surveillance. The second mechanism is antibody-dependent cell-mediated cytotoxicity (ADCC), which is based on the recognition of antibodies that are bound to the target cell surface. In both cases, target cells are killed by release of the content of cytotoxic granules, such as perforin and granzyme B.

Here, zinc is involved in the recognition of MHC-I on target cells, mainly human leukocyte antigen C, by p58 killer cell inhibitory receptors on NK cells for inhibition of the killing activity (Rajagopalan et al. 1995; Rajagopalan and Long 1998). In general, the NK cell number as well as activity is dependent on the serum zinc level. The lytic activity of NK cells is decreased by zinc deficiency, probably beause of decreased stimulation from T cells via the cytokine IL-2. Furthermore, the relative number of precursors of cytolytic cells is decreased during zinc deficiency as well as the lytic activity of NK cells itself (Prasad 2000), but moderate

zinc supplementation increases differentiation of $CD34^+$ progenitors toward NK cells and their cytotoxic activity (Muzzioli et al. 2007). However, merely the inhibitory signal is zinc dependent, whereas the human leukocyte antigen C interaction and positive signals do not require zinc. Thus, zinc is needed to maintain the normal function of NK cells, and zinc deficiency may result in functional loss and evoke nonspecific killing.

To date intracellular zinc signals have not been investigated in NK cells. However, lines of evidence connect major signaling pathways in NK cells to zinc. A multitude of different receptors are required for fine-tuning of NK cell activity (Yokoyama and Plougastel 2003). A common feature of many of these receptors is the presence of characteristic tyrosine phosphorylation sites that either activate (immunoreceptor tyrosine-based activation motif, ITAM) or inhibit (immunoreceptor tyrosine-based inhibitory motif, ITIM) NK cell function. In this respect, one feature of zinc is of particular importance: its ability to inhibit protein tyrosine phosphatases (PTPs) by a fast zinc signal (Brautigan et al. 1981). This inhibition seems to be a common feature of PTPs, in which zinc interacts with the highly conserved catalytic domain, possibly by binding to the active site cysteine (Haase and Maret 2005). Notably, a nucleophile attack by the catalytically active cysteine residue is a shared feature of PTPs and MKPs, another target of zinc that has already been mentioned. On the other hand, serine/threonine phosphatases have a dinuclear metal center in which water acts as the nucleophile (Barford et al. 1998), and so far there are no reports about a specific inhibition of these enzymes by zinc. Several PTPs are inhibited by low concentrations of zinc, including T-cell PTP with an half-maximal inhibitory concentration (IC_{50}) of 200 nM (Maret et al. 1999), and PTP1B and SHP-1, with an IC_{50} of 17 and 93 nM, respectively (Haase and Maret 2003). These values are close to the concentration range of physiological levels of free zinc and presume a partial inhibition of PTPs by free zinc even at basal levels. These effects therefore depend on the extracellular zinc concentrations and are not zinc signals.

Cell-surface antibody receptors, called Fc receptors, allow different immune cells to utilize antibodies for pathogen recognition, as in mast cells. The triggering of the high-affinity IgE-specific FcεR in mast cells induces degranulation. Notably, the secretory mast cell granules are rich in zinc that is released into the cellular environment together with a variety of immunological mediators (Gustafson 1967; Ho et al. 2004). The relationship between activation of FcRs and zinc status has been investigated in mast cells, but so far the results are undetermined. Measurements with fluorescent probes show a reduction of free zinc in response to treatments that induce degranulation, including the triggering of FcεRs (Ho et al. 2004). Another study (Yamasaki et al. 2007) reports that the crosslinking of FcεRI induces a release of free zinc from the perinuclear area, including the endoplasmic reticulum in mast cells. This phenomenon is known as zinc wave (see Chap. 5), which depends on calcium influx and MAPK.

This discrepancy might be the result of the use of different fluorophores in the two studies, detecting different pools of cellular zinc. Although the decrease of zinc is attributed to a loss of granule zinc, the increase seems to be cytosolic, originating

from the perinuclear region (Yamasaki et al. 2007). Furthermore, treatment with the membrane-permeable zinc chelator TPEN (*N*,*N*,*N*′,*N*′-tetrakis-(2-pyridyl-methyl) ethylenediamine) activates NF-κB. TNF-α-induced NF-κB activation is blocked by treatment with zinc/pyrithione (Ho et al. 2004). A later study states a blockade of NF-κB activation in response to FcεR crosslinking by TPEN administration, and this effect can be reversed by zinc treatment (Kabu et al. 2006). Thus, it remains to be analyzed under which circumstances zinc inhibits NF-κB and caspase-3 activity, or is involved in the activation of NF-κB, ERK, JNK, and PKCβI, and is thereby required for degranulation and cytokine production in mast cells in vitro as well as allergic reactions in vivo (Yamasaki et al. 2007).

Despite the fact that the innate immunity is the first stage in response of the immune system, observations relating to the influence of zinc signals are also associated with the function of the adaptive immunity.

10.4 Zinc Signals and Adaptive Immunity

The influence of zinc has also been identified regarding the two parts of adaptive immunity, humoral (B cells) and cellular immunity (T cells). B and T cells are produced as precursors and educated to recognize their specific antigen in the thymus (T cells) or bone marrow (B cells). The resulting naïve lymphocytes differentiate after antigen contact into effector cells and memory cells, in which the latter are the basis for immunological memory and the stronger reaction to a known antigen as a secondary response. Zinc is highly important, especially concerning the development of T cells and B cells (Osati-Ashtiani et al. 1998).

T cells are effector cells as well as important cells for the regulation of the specific immune system. Zinc not only influences NK cell-mediated killing, as already mentioned, it also affects the activity of cytolytic T cells (Mingari et al. 1998). Here the relative amount of $CD8^+$ $CD73^+$ T cells is decreased during zinc deficiency (Prasad 2000). These cells are predominantly precursors of cytotoxic T cells ($CD8^+$), and CD73 is needed for antigen recognition and proliferation as well as cytolytic process generation (Beck et al. 1997). Furthermore, zinc is involved in the development of T cells, because zinc deficiency results in various T-cell defects manifested in thymus atrophy and lymphopenia (King et al. 2005).

Zinc acts as an essential cofactor for various enzymes, such as the thymic hormone thymulin (a nonapeptide). Thymulin is a hormone that is produced by the thymus, released by thymic epithelial cells (Dardenne et al. 1984), and induces markers of differentiation in immature T cells (Saha et al. 1995). Besides these intrathymic functions on thymocytes and immature T cells, thymulin acts on mature T cells in the periphery. It also modulates cytokine release by peripheral blood mononuclear cells (PBMC) and proliferation of $CD8^+$ T cells in combination with interleukin (IL)-2 (Coto et al. 1992). Therefore, zinc influences immature and mature T cells through the activation of thymulin. As a consequence of zinc deficiency, T-cell proliferation decreases after mitogen stimulation (Dowd

et al. 1986), whereas zinc supplementation is able to reverse zinc deficiency-induced changes in the thymus and on peripheral cells; this can also be observed in patients with acquired immunodeficiency syndrome (Mocchegiani et al. 1995). In contrast to other lymphocyte populations, a direct effect of zinc on T cells can be observed. For instance, zinc induces blast transformation in human lymphocytes (Berger and Skinner 1974; Sood et al. 1999), and ensures the expression of the high-affinity receptor for IL-2 on mature T cells (Tanaka 1989), one effect resulting in decreased proliferation of T cells in zinc deficiency (Crea 1990). Moreover, IL-2, the soluble IL-2 receptor (sIL-2R), and IFN-γ can be induced by high concentrations of zinc in human PBMC (Driessen et al. 1994; Wellinghausen et al. 1997b).

In addition, zinc deficiency results in disturbed polarization of mature T cells. Zinc deprivation inhibits the polarization into Th1 cells and therefore changes the Th1/Th2 ratio toward Th2 cells, leading to unbalanced cell-mediated immune responses (Prasad 2000). It is observed that the production of IFN-γ and IL-2 (products of Th1 cells) is decreased during zinc deficiency, whereas production of IL-4, IL-6, and IL-10 (products of Th2 cells) is not affected. Accordingly, the risk for infections and Th2-driven allergies is increased. Furthermore, the functional impairment of T cell-mediated responses during zinc deficiency favors the development of autoimmune diseases (Honscheid et al. 2009). Consistently, both zinc deficiency, and increased zinc levels influence T-cell function. Increasing zinc levels during T-cell activation are postulated to calibrate T-cell receptor (TCR) signaling, leading to T-cell responses following suboptimal stimuli (Yu et al. 2011). This effect depends on a fast zinc signal. In contrast, physiological zinc levels exceeding zinc concentrations, ranging from 50 to 100 μM, inhibit T-cell activity as demonstrated by suppressed IL-1β-stimulated IFN-γ expression (Wellinghausen et al. 1997b). Furthermore, induction and stabilization of regulatory T cells (Treg) can be observed in zinc-supplemented mixed lymphocyte cultures (MLC), leading to a reduced secretion of IFN-γ (Rosenkranz et al., unpublished data), as a result of altered zinc homeostasis.

In contrast to T-cell stimulation, T-cell inhibition by an excess of zinc can also be observed in vivo, leading to the assumption that T-cell activity is critically regulated by the zinc concentration. This criticality may be a reason why some autoimmune diseases with a T-cell pathology, such as rheumatoid arthritis, are associated with moderate zinc deficiency. Some clinical trials show a reduction of the pain score with zinc supplementation, leading to a presumption that zinc deficiency increases allo- or autoreactivity, whereas it is inhibited by high zinc dosages.

Important molecular zinc targets responsible for the aforementioned zinc effects comprise receptor proteins, kinases, phosphatases, caspases, and transcription factors, which can be activated or inactivated by zinc (Haase and Rink 2009b). Furthermore, it functions as a second messenger in signal transduction, contributing to its immunomodulating capacity (Haase et al. 2008).

In T cells, fast zinc signals have been observed in response to TCR or interleukin-2 receptor (IL-2R) stimulation (Kaltenberg et al. 2010; Yu et al. 2011). Regarding the TCR, the signal seems not to be dependent on direct

activation of this receptor. Initiation of signaling from the TCR occurs by specific interaction with an antigen-loaded MHC molecule on the surface of a neighboring antigen-presenting cell (APC) by formation of a functional immunological synapse (see Fig. 10.2), and this results in an immediate influx of zinc from the extracellular environment through the transporter Zip 6 after T-cell stimulation (Yu et al. 2011). In contrast, triggering of IL-2R results in a release of zinc from lysosomes within 2 min after T-cell stimulation (Kaltenberg et al. 2010). Thus, an altered intracellular zinc level is induced, leading to an influence of dual-specificity phosphatases (DUSP) or protein phosphatase 2 (PP2A), for example, by a fast zinc signal, as well as signal transduction and protein expression by late zinc signals, such as diminished dephosphorylation of MEK and ERK or elevated transcription of Zip 6, leading to an increased MT expression, which supports T-cell proliferation and is of particular importance for T-cell survival and expansion in the elderly (Kaltenberg et al. 2010; Yu et al. 2011; Lee et al. 2008).

Concerning the TCR, the zinc signal influences assembly of the TCR-activating complex by reduced recruitment of SHP-1 to the TCR activation complex and thus increased activation of the lymphocyte protein tyrosine kinase (Lck). TCR has no intrinsic kinase activity and depends on the Src-family tyrosine kinase, Lck, for signal transduction. Lck is one of the first kinases activated, is essential for phosphorylation of the ten ITAM motifs of the T-cell antigen receptor-signaling complex, and augments phosphorylation of the kinase ZAP70 (Palacios and Weiss 2004). The activation of ZAP70 phosphorylates downstream targets that activate MAPK pathways and cause T-cell activation.

Lck expression is upregulated in T cells of zinc-deficient mice (Lepage et al. 1999; Moore et al. 2001), and additional evidence also suggests direct regulation of Lck and sustained calcium influx (Yu et al. 2011). Zinc-provoked Lck activation occurs by linking two distinct protein interface sites. The N-terminal region of Lck is bound to the intracellular domains of the membrane proteins CD4 and CD8 (Huse et al. 1998; Lin et al. 1998). In a so-called "zinc clasp structure," this interaction is stabilized by a zinc ion that is bound to two cysteine residues from each protein at the interface site between Lck and CD4/CD8 (Kim et al. 2003). Because CD4 and CD8 also bind to MHC with their extracellular domains, they are thus recruited in close proximity of the TCR-signaling complex, and this brings Lck close to its substrates.

Another zinc-dependent interface site is necessary for the homodimerization of Lck. Therefore, two zinc ions at the dimer interface of the SH3 domains stabilize the complex (Romir et al. 2007). The activation of Lck is a complex event, depending on two tyrosine phosphorylation sites. The first is tyrosine 394 situated in the so-called activation loop. It is transphosphorylated between Lck molecules upon activation, increasing kinase activity (Palacios and Weiss 2004; Brautigan et al. 1981). Lck activation is caused by the zinc-induced homodimerization by a fast zinc signal, bringing two Lck molecules into close proximity. The second phosphorylation occurs in the C-terminal negative regulatory site at tyrosine 505. According to phosphorylation, the kinase assumes a closed, inactive conformation. The transmembrane PTP CD45 selectively dephosphorylates tyrosine 505, thus

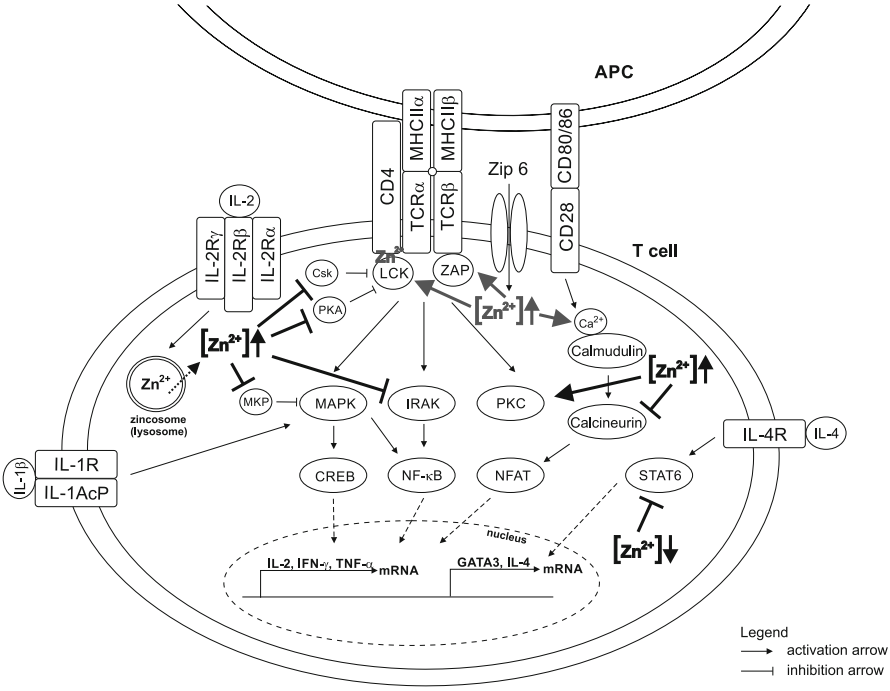

Fig. 10.2 Zinc and T-cell signaling. Overview of T-cell receptor (TCR) signaling, interleukin-1 receptor (*IL-1R*), IL-2R, and IL-4R signaling in T cells. A direct induced zinc signal via Zip 6, by APC-mediated T-cell activation (highlighted in *dark grey*), is known to activate TCR signaling, by augmented ZAP phosphorylation, sustained Ca^{2+} influx, and inducing the active TCR signaling complex by binding of lymphocyte-specific protein tyrosine kinase *LCK*. IL-2R triggering in experiments leads to a zinc release (*dotted arrows*) from zincosomes, resulting in an increased intracellular zinc concentration (highlighted in *bold black*). Zinc mediates the inhibition (*inhibition arrow*) of signaling via interleukin-1 receptor-associated kinase (*IRAK*), or c-src tyrosine kinase (Csk)/protein kinase A (PKA)-mediated inhibition LCK in TCR signaling, but induces (*activation arrow*) zeta-chain (TCR)-associated protein kinase (ZAP), and protein kinase C (PKC) activity, mitogen-activated protein kinase (MAPK) signaling, and nuclear factor kappa B (NF-κB) phosphorylation. Increased intracellular zinc concentrations lead to an inhibition of calcineurin (CN) and avoid translocation into the nucleus (*dashed arrows*) of nuclear factor of activated T cells (NFAT). Besides zinc signals, zinc deficiency also influences T-cell signaling, i.e., IL-4R signaling. Zinc deficiency leads to a decreased phosphorylation of signal transducer and activator of transcription 6 (STAT6). *MKP* MAP kinase phosphatase, *CREB* cyclic adenosine monophosphate response element-binding protein

keeping Lck in a primed state. TCR signaling and Lck phosphorylation are regulated by numerous PTPs (Mustelin and Tasken 2003) that are all potential targets for zinc inhibition. Consequently, it cannot be predicted if this would result in preferential dephosphorylation of an activating or inactivating tyrosine by zinc in vivo. Zinc can activate Lck directly in the absence of phosphatases, most likely as a result of dimerization. Zinc-dependent homodimerization as well as

heterodimerization are specific for Lck, because the cysteine residues that form both intermolecular zinc-binding sites are not present in other Src kinase family members but are unique for this kinase (Romir et al. 2007; Kim et al. 2003).

An inhibition of TCR signaling and T-cell activation results from phosphorylation of the inactivating tyrosine 505 of Lck via c-src tyrosine kinase COOH-terminal Srk kinase (Csk) (Chow et al. 1993). Zinc can interfere with these events in several ways. First, the fast zinc signal leads to an inhibition of Csk by reversible binding of zinc that is mediated by substituting for Mg^{2+} at one of its binding sites (Klein et al. 2002). Second, the activation of Csk in T cells can be observed via phosphorylation by PKA (Vang et al. 2001). Although zinc has no direct influence on PKA activity, it can inhibit AC transcription by a late zinc signal and thereby the formation of the PKA activator cAMP. Furthermore, also fast zinc signals influence cAMP by inhibition of PDE that can block degradation of cyclic nucleotides, leading to activation of PKA (von Bulow et al. 2007). Inhibition of Csk and AC promotes TCR signaling whereas PDE inhibition antagonizes it. The outcome on TCR signaling resulting from the modulation of this pathway by zinc in vivo remains to be investigated, however.

In response to TCR stimulation, another transcription factor, nuclear factor of activated T cells (NFAT), mediates the expression of many genes, such as IL-2. In resting cells, NFAT proteins are constitutively phosphorylated and are found in the cytoplasm. In response to TCR/CD28-mediated calcium signaling, NFAT is dephosphorylated by calcineurin (CN), a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, and translocates into the nucleus (Macian 2005). Iron and zinc are essential cofactors for the catalytic domain of CN, containing a Fe^{2+}-Zn^{2+} binuclear center, but merely an inhibition of CN by zinc is described. Also, in vitro experiments show an inhibition at physiologically relevant zinc concentrations, ranging from 10 nM to 10 μM (Huang et al. 2008; Takahashi et al. 2003). One of the kinases that keeps CN in its phosphorylated, inactive state in resting cells is phosphatidyl inositol-3-kinase (PI3K) which is a negative regulator of glycogen synthase kinase-3-beta (GSK-3β). Therefore, augmented PI3K activity can abrogate NFAT activity (Macian 2005).

Increased GSK-3β phosphorylation via a pathway involving PI3K is known wherein zinc acts through the PI3K pathway in various cell types (Eom et al. 2001; LaRochelle et al. 2001; Tang and Shay 2001). One possible explanation for this mechanism is increased enzyme degradation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Wu et al. 2003). A later study examining the IL-2-induced PI3K/Akt signaling pathway could not verify these results but showed an inhibition of PTEN (Plum et al. 2014). Here it has been indicated that late zinc signals seem to be necessary for the regulation the PI3K/Akt pathway, because under zinc deficiency the IL-2-induced Akt phosphorylation is diminished. Late zinc signals are known to upregulate phosphorylation of Akt at Ser473 and inhibit PTEN at sub-nanomolar concentrations (IC_{50} 0.59 nM). This inhibition seems to be mediated by binding of zinc ions at its cysteine thiol at position 124 (cys124), which is essential for the catalytic activity of PTEN (Plum et al.). Thus, a modulation of this pathway occurs upstream of Akt, but

downstream of Jak1, because Stat5 signaling, which is also downstream of Jak1, is not influenced by zinc.

PTEN in general function as a dephosphorylase of phosphatidylinositol 3,4,5-trisphosphate, which is a product of PI3K, mediating the activation of PDK-1/Akt/GSK-3β. These results suggest a comparable activation of PI3K via zinc occurring in T cells. Inhibition of CN by zinc results in NFAT inactivation and reduction of TCR-mediated transcription simultaneously, whereas activation of PI3K signaling acts agonistically.

In addition to TCR signaling, zinc signals also affects signals originating from the IL-1 receptor. High zinc concentrations of about 100 μM inhibit IL-1-β-stimulated IFN-γ production in primary human T cells and IL-1-dependent proliferation of murine T cells. Zinc incubation leads to reduced activity of IL-1 receptor-associated kinase (IRAK), which is a central kinase in the signaling pathways downstream of the IL-1 receptor. Zinc supplementation to zinc-deficient T cells has also regulatory effects on cytokine production. Zinc supplementation leads to a fast rise in intracellular zinc levels, that is, zinc flux, resulting from increased expression of cell membrane-located zinc transporters Zip 10 and Zip 12 because of the former zinc deficiency condition. Thus, IL-2 production and IL-2 mRNA expression are highly increased after IL-1β stimulation, concerning phosphorylation of MAPK p38, and NF-κB subunit p65 (Daaboul et al. 2012).

Moreover, the IRAK family of serine/threonine kinases is also involved in other pathways. IRAK4 participates in TCR-mediated NF-κB activation (Suzuki et al. 2006), although this observation is debated (Kawagoe et al. 2007). Thus, zinc can inhibit two central signaling pathways (TCR and IL-1 receptor) that are essential in the activation of T cells, by its effect on IRAK, making this a major target for negative regulation of T cells by zinc supplementation. However, the role of IRAK is not limited to merely T cells. The high rate of similarity between TLR-4 and IL-1 receptor signal transduction suggests IRAK inhibition for another mechanism by which zinc negatively influences TLR-4 signaling.

Zinc as a mediator of T-cell signal transduction has been known since the first reports of a potential role of this ion in signaling. Its interaction with PKC is identified as the biochemical basis of these observations (Csermely and Somogyi 1989). The PKC family are serine/threonine kinases that comprise several isoforms. They are differentiated into classical PKCs, which are activated by cofactors such as Ca^{2+} and diacylglycerol, novel PKCs, which bind diacylglycerol but no Ca^{2+}, and atypical PKCs, which do not interact with either cofactor. Each isoform has specific roles in the regulation of cellular functions; for example, in T cells, PKCθ is involved in the activation of several transcription factors in response to TCR/CD28 stimulation. In addition, a role for PKCα in proliferation and IL-2 production of T cells is known. Furthermore, several PKC isoforms are involved in survival of B cells, pre-B cell development, and induction of tolerance toward self-antigens (Tan and Parker 2003).

PKCs have four conserved domains (C1–C4) in common. The diacylglycerol binding C1 domain in the N-terminal regulatory part of PKCβ1 contains two homologous regions with six cysteines and two histidines, forming a total of four

Cys_3His zinc-binding motifs (Hubbard et al. 1991). No information about a differential effect of zinc on the different isoforms is available, and varying forms of C1 domains are present in conventional, novel, and atypical PKCs, indicating zinc binding to all known PKC isoforms (Tan and Parker 2003).

Late zinc signals affect multiple steps during PKC activation, for example, augmented PKC kinase activity, increased affinity to phorbol esters, and enhanced binding to the cytoskeleton and plasma membrane (Forbes et al. 1990; Wellinghausen et al. 1997b). Inhibition of these events can be mediated by membrane-permeable zinc chelators, such as TPEN. PKC itself can be a source for zinc release; thus, interaction between PKC and zinc is not limited to an effect of zinc on the PKC activation. PKC activation by lipid second messengers or thiol oxidation leads to measurable zinc release from the regulatory domain (Knapp and Klann 2000; Korichneva et al. 2002). In addition, PKC regulates the intracellular free zinc concentration and distribution. In T cells, phorbol ester treatment leads to a fast zinc signal, resulting in redistribution of zinc from the nucleus and mitochondria to the cytosol and microsomes (Csermely et al. 1987). Furthermore, treatment with phorbol esters such as PMA leads to an increase of free zinc in human T cells, whereas PHA stimulation, for example, leads to calcium increase (Haase et al. 2008), and the phorbolester-induced differentiation of HL-60 cells into macrophages comprises a transitional increase of nuclear zinc depending on PKCβ (Glesne et al. 2006).

In general, a forecast of zinc effects on T cells is impossible because of the high number of zinc-related signaling pathways. Several studies concerning zinc status and T-cell function in vivo stated an increase of the delayed-type hypersensitivity reaction upon correction of zinc deficiency (Haase et al. 2006b). On the other hand, zinc supplementation diminishes the allogeneic reaction in the MLCs (Faber et al. 2004) and stabilizes regulatory T-cell function (Rosenkranz et al., unpublished data), indicating that zinc may have multiple, opposing functions, depending on its concentration and certainly also on the interaction with multiple other environmental factors.

B cells represent the main cells of humoral immunity and differentiate to antibody-producing plasma cells after stimulation. Nearly all the zinc-regulated signaling pathways already discussed are also important in B cells, such as tyrosine phosphorylation, PKC, MAPK, and activation of the transcription factors NFAT and NF-κB. In contrast to many of the cell types discussed earlier, mature B-cell proliferation and function are not as dependent on the organisms' zinc status as it is in T cells. Therefore, the influence of zinc deficiency on B cells is not comparable to the situation of T cells (Fraker and Telford 1997). Zinc itself seems to have no direct influence on the activity of B cells (Crea 1990). However, zinc-deficient patients, such as elderly and hemodialysis patients, show a reduced response to vaccination (Cakman et al. 1996; Bonomini et al. 1993). Rather, lymphopoiesis and pre-B-cell development are mainly affected by zinc deprivation in vivo (Fraker and King 2004). Zinc deficiency causes a greater loss of lymphoid tissue compared to other tissues, and thus T-cell development is impaired as well as that of B cells (Keen and Gershwin 1990). B cells and their precursors (especially pre-B cells and

immature B cells) are reduced in absolute number during zinc deficiency, but changes among mature B cells are only slight.

During development, lymphocytes are sensitive for apoptosis signals because of positive and negative selection mechanisms in the primary lymphoid organs, whereas mature B and T cells are apoptosis resistant, and therefore inactivated (known as "anergy") in case of autoreactivity. A strict selection guarantees functionality and avoids autoreactive cells by eliminating the majority of newly formed cells by apoptosis. Zinc deficiency increases the rate of apoptosis within the B-cell population (Fraker et al. 2000) and leads to cell depletion. In contrast to zinc deficiency, low zinc levels have no influence on the cell-cycle status of precursor B cells and only modest influence on cycling pro-B cells (King and Fraker 2000). Thus, there are fewer naïve B cells during zinc deficiency that can react on neoantigens. Taking into account that the number of T cells is reduced during zinc deficiency also, and that most antigens are T-cell dependent, it is probable that in a state of zinc deficiency the body is unable to respond with antibody production in response to neoantigens. This assumption is consistent with findings showing a disturbed antibody production by B cells during zinc depletion (DePasquale-Jardieu and Fraker 1984). In accordance with this, a late zinc signal is essential for Stat6 phosphorylation via IL-4 induction, because Stat6 phosphorylation is diminished by zinc deficiency in B cells as well as in T cells (Gruber et al. 2013). Thus, T-cell reactivity is impaired, and B-cell activity and antibody production are affected as well, because IL-4 promotes the activation of early B cells and the immunoglobulin class switch toward IgE, and thereby further antibody specification, leading to a higher risk of parasitic infection (Kopf et al. 1993). Furthermore, zinc signals are important for IL-6-induced Stat3 phosphorylation, increased by zinc deficiency. IL-6 is responsible for the activation and final differentiation of B cells into plasma cells, and IL-6 overproduction is associated with autoantibody production. Many diseases, such as rheumatoid arthritis (RA) and plasma cell neoplasias, accompany reduced serum zinc levels, indicating potential co-effects of IL-6 overproduction and enhanced susceptibility of B cells because of zinc deficiency. This finding indicates that strict regulation is necessary to keep the immune system balanced (Gruber et al. 2013).

In addition, studies reveal that antibodies as a response to T-cell-dependent antigens are more sensitive to zinc deficiency than antibody production in response to T-cell-independent antigens (Moulder and Steward 1989). For example, zinc-deficient mice show reduced antibody recall responses to antigens for which they were previously immunized. This effect has been observed in T-cell-independent as well as in T-cell-dependent systems. Thus, immunological memory also seems to be influenced by zinc (Fraker et al. 1986). However, mature B cells are more resistant to zinc deficiency because of the high Bcl2 level. B-cell memory is less affected than the primary response (Fraker et al. 2000).

Late zinc signals influence several regulatory proteins, such as those from the Bcl/Bax family (Truong-Tran et al. 2001) and furthermore several aspects of apoptotic signal transduction. In this connection, zinc directly regulates the acidity of enzymes in the apoptotic cascade. First, the calcium-dependent endonuclease,

which mediates DNA fragmentation, is inhibited by zinc. However, this target is beyond the point of no return for programmed cell death, and inhibition could explain suppression of DNA fragmentation during apoptosis but not the effect on cellular survival.

Another important group of enzymes in apoptosis are cysteine-aspartic acid proteases, also known as caspases. They form a cascade to transduce initial apoptotic signals to the effector enzymes that mediate the organized cellular destruction which is characteristic of programmed cell death. In this process, inactive pro-caspases are activated by proteolytic cleavage. An additional regulatory mechanism is shown by inhibition of caspases-3, -6, and -8 by application of low micromolar zinc concentrations (Stennicke and Salvesen 1997). A half-maximal inhibitory concentration for caspase-3 was found below 10 nM (Maret et al. 1999). This value is clearly in the physiological range of free intracellular zinc, leading to the suggestion that endogenous zinc can inhibit caspase-3. Furthermore, recent studies showed a blockade of caspase-3-dependent apoptosis occurring in a rat heterotrophic heart transplant model, leading to increased allograft survival (Kown et al. 2000, 2002).

It is interesting that the function of mature B cells, which utilize the same signaling pathway as other immune cells, seems to be affected by zinc to a lesser magnitude. Even the reduced antibody production during zinc deficiency is based on reduced B-cell numbers whereas it is unaffected on a per-cell basis (Cook-Mills and Fraker 1993), which indicates an effect on cellular development rather than on function. One reason might be a difference in zinc homeostasis, making mature B cells less susceptible to conditions of limited zinc availability. Although B cells are highly susceptible to apoptosis during development, and zinc is one factor that influences these signals, mature B cells can tolerate comparable conditions by changes in zinc-regulating proteins, but also by changing the expression patterns of several other factors that regulate the cellular responsiveness to apoptotic signals.

In summary, nearly all immune cells are directly affected by zinc, especially by zinc deficiency, indicating that zinc is a main regulator of cellular function and signal transduction. However, the majority of these effects are characterized by investigations at isolated signaling pathways in vitro. Thus, further efforts must elucidate the in vivo situation, that is, which effects are actually physiologically relevant.

References

Allen JI, Perri RT, McClain CJ, Kay NE (1983) Alterations in human natural killer cell activity and monocyte cytotoxicity induced by zinc deficiency. J Lab Clin Med 102(4):577–589

An WL, Bjorkdahl C, Liu R, Cowburn RF, Winblad B, Pei JJ (2005) Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3beta in SH-SY5Y neuroblastoma cells. J Neurochem 92(5):1104–1115. doi:10.1111/j.1471-4159.2004.02948.x

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5(1):196–201. doi:10.1021/pr050361j

Andreini C, Bertini I, Rosato A (2009) Metalloproteomes: a bioinformatic approach. Acc Chem Res 42(10):1471–1479. doi:10.1021/ar900015x

Aydemir TB, Liuzzi JP, McClellan S, Cousins RJ (2009) Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells. J Leukoc Biol 86 (2):337–348. doi:10.1189/jlb.1208759

Barford D, Das AK, Egloff MP (1998) The structure and mechanism of protein phosphatases: insights into catalysis and regulation. Annu Rev Biophys Biomol Struct 27:133–164. doi:10.1146/annurev.biophys.27.1.133

Beck FW, Kaplan J, Fine N, Handschu W, Prasad AS (1997) Decreased expression of CD73 (ecto-5′-nucleotidase) in the CD8+ subset is associated with zinc deficiency in human patients. J Lab Clin Med 130(2):147–156

Bentley PJ (1992) Influx of zinc by channel catfish (*Ictalurus punctatus*): uptake from external environmental solutions. Comp Biochem Physiol C Comp Pharmacol Toxicol 101(2):215–217

Berger NA, Skinner AM (1974) Characterization of lymphocyte transformation induced by zinc ions. J Cell Biol 61(1):45–55

Beyersmann D, Haase H (2001) Functions of zinc in signaling, proliferation and differentiation of mammalian cells. Biometals 14(3–4):331–341

Bonomini M, Di Paolo B, De Risio F, Niri L, Klinkmann H, Ivanovich P, Albertazzi A (1993) Effects of zinc supplementation in chronic haemodialysis patients. Nephrol Dial Transplant 8 (10):1166–1168

Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, Tsui C, Hurley P, Chien M, Chai S, Hitotsumatsu O, McNally E, Pickart C, Ma A (2004) The ubiquitin-modifying enzyme A20 is required for termination of toll-like receptor responses. Nat Immunol 5(10):1052–1060. doi:10.1038/ni1110

Brautigan DL, Bornstein P, Gallis B (1981) Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn. J Biol Chem 256(13):6519–6522

Briefel RR, Bialostosky K, Kennedy-Stephenson J, McDowell MA, Ervin RB, Wright JD (2000) Zinc intake of the U.S. population: findings from the third national health and nutrition examination survey, 1988–1994. J Nutr 130(5S suppl):1367s–1373s

Brieger A, Rink L (2010) Zink und Immunfunktion. Ernährung Medizin 25:156–160

Brieger A, Rink L, Haase H (2013) Differential regulation of TLR-dependent MyD88 and TRIF signaling pathways by free zinc ions. J Immunol 191(4):1808–1817. doi:10.4049/jimmunol.1301261

Bruinsma JJ, Jirakulaporn T, Muslin AJ, Kornfeld K (2002) Zinc ions and cation diffusion facilitator proteins regulate Ras-mediated signaling. Dev Cell 2(5):567–578

Cakman I, Rohwer J, Schutz RM, Kirchner H, Rink L (1996) Dysregulation between TH1 and TH2 T cell subpopulations in the elderly. Mech Ageing Dev 87(3):197–209

Cavdar AO, Arcasoy A, Cin S, Babacan E, Gozdasoglu S (1983) Geophagia in Turkey: iron and zinc deficiency, iron and zinc absorption studies and response to treatment with zinc in geophagia cases. Prog Clin Biol Res 129:71–97

Chasapis CT, Loutsidou AC, Spiliopoulou CA, Stefanidou ME (2012) Zinc and human health: an update. Arch Toxicol 86(4):521–534. doi:10.1007/s00204-011-0775-1

Chavakis T, May AE, Preissner KT, Kanse SM (1999) Molecular mechanisms of zinc-dependent leukocyte adhesion involving the urokinase receptor and beta2-integrins. Blood 93 (9):2976–2983

Chow LM, Fournel M, Davidson D, Veillette A (1993) Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50csk. Nature (Lond) 365(6442):156–160. doi:10.1038/365156a0

Cook-Mills JM, Fraker PJ (1993) Functional capacity of the residual lymphocytes from zinc-deficient adult mice. Br J Nutr 69(3):835–848

Coto JA, Hadden EM, Sauro M, Zorn N, Hadden JW (1992) Interleukin 1 regulates secretion of zinc-thymulin by human thymic epithelial cells and its action on T-lymphocyte proliferation and nuclear protein kinase C. Proc Natl Acad Sci USA 89(16):7752–7756

Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. J Biol Chem 281(34):24085–24089. doi:10.1074/jbc.R600011200

Crea A (1990) Zinc et système immunitaire. Ann Med Interne (Paris) 141:447–451

Csermely P, Somogyi J (1989) Zinc as a possible mediator of signal transduction in T lymphocytes. Acta Physiol Hung 74(2):195–199

Csermely P, Gueth S, Somogyi J (1987) The tumor promoter tetradecanoyl-phorbol-acetate (TPA) elicits the redistribution of zinc in subcellular fractions of rabbit thymocytes measured by X-ray fluorescence. Biochem Biophys Res Commun 144(2):863–868

Csermely P, Szamel M, Resch K, Somogyi J (1988) Zinc can increase the activity of protein kinase C and contributes to its binding to plasma membranes in T lymphocytes. J Biol Chem 263 (14):6487–6490

Daaboul D, Rosenkranz E, Uciechowski P, Rink L (2012) Repletion of zinc in zinc-deficient cells strongly up-regulates IL-1beta-induced IL-2 production in T-cells. Metallomics 4 (10):1088–1097. doi:10.1039/c2mt20118f

Dardenne M, Savino W, Wade S, Kaiserlian D, Lemonnier D, Bach JF (1984) In vivo and in vitro studies of thymulin in marginally zinc-deficient mice. Eur J Immunol 14(5):454–458. doi:10.1002/eji.1830140513

DeCoursey TE, Morgan D, Cherny VV (2003) The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. Nature (Lond) 422(6931):531–534. doi:10.1038/nature01523

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103(2):351–361

DePasquale-Jardieu P, Fraker PJ (1984) Interference in the development of a secondary immune response in mice by zinc deprivation: persistence of effects. J Nutr 114(10):1762–1769

Dong XP, Cheng X, Mills E, Delling M, Wang F, Kurz T, Xu H (2008) The type IV mucolipidosis-associated protein TRPML1 is an endolysosomal iron release channel. Nature (Lond) 455 (7215):992–996. doi:10.1038/nature07311

Dowd PS, Kelleher J, Guillou PJ (1986) T-lymphocyte subsets and interleukin-2 production in zinc-deficient rats. Br J Nutr 55(1):59–69

Driessen C, Hirv K, Rink L, Kirchner H (1994) Induction of cytokines by zinc ions in human peripheral blood mononuclear cells and separated monocytes. Lymphokine Cytokine Res 13 (1):15–20

Dubben S, Honscheid A, Winkler K, Rink L, Haase H (2010) Cellular zinc homeostasis is a regulator in monocyte differentiation of HL-60 cells by 1 alpha,25-dihydroxyvitamin D3. J Leukoc Biol 87(5):833–844. doi:10.1189/jlb.0409241

Eide DJ (2006) Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta 1763 (7):711–722. doi:10.1016/j.bbamcr.2006.03.005

Eom SJ, Kim EY, Lee JE, Kang HJ, Shim J, Kim SU, Gwag BJ, Choi EJ (2001) Zn(2+) induces stimulation of the c-Jun N-terminal kinase signaling pathway through phosphoinositide 3-Kinase. Mol Pharmacol 59(5):981–986

Faber C, Gabriel P, Ibs KH, Rink L (2004) Zinc in pharmacological doses suppresses allogeneic reaction without affecting the antigenic response. Bone Marrow Transplant 33(12):1241–1246. doi:10.1038/sj.bmt.1704509

Failla ML, van de Veerdonk M, Morgan WT, Smith JC Jr (1982) Characterization of zinc-binding proteins of plasma in familial hyperzincemia. J Lab Clin Med 100(6):943–952

Fessatou S, Fagerhol MK, Roth J, Stamoulakatou A, Kitra V, Hadarean M, Paleologos G, Chandrinou H, Sampson B, Papassotiriou I (2005) Severe anemia and neutropenia associated with hyperzincemia and hypercalprotectinemia. J Pediatr Hematol Oncol 27(9):477–480

Forbes IJ, Zalewski PD, Giannakis C, Petkoff HS, Cowled PA (1990) Interaction between protein kinase C and regulatory ligand is enhanced by a chelatable pool of cellular zinc. Biochim Biophys Acta 1053(2-3):113–117

Fosmire GJ (1990) Zinc toxicity. Am J Clin Nutr 51(2):225–227

Fraker PJ, King LE (2004) Reprogramming of the immune system during zinc deficiency. Annu Rev Nutr 24:277–298. doi:10.1146/annurev.nutr.24.012003.132454

Fraker PJ, Telford WG (1997) A reappraisal of the role of zinc in life and death decisions of cells. Proc Soc Exp Biol Med 215(3):229–236

Fraker PJ, Gershwin ME, Good RA, Prasad A (1986) Interrelationships between zinc and immune function. Fed Proc 45(5):1474–1479

Fraker PJ, King LE, Laakko T, Vollmer TL (2000) The dynamic link between the integrity of the immune system and zinc status. J Nutr 130(5S suppl):1399s–1406s

Gao X, Du Z, Patel TB (2005) Copper and zinc inhibit G-alphas function: a nucleotide-free state of G-alphas induced by Cu^{2+} and Zn^{2+}. J Biol Chem 280(4):2579–2586. doi:10.1074/jbc.M409791200

Glesne D, Vogt S, Maser J, Legnini D, Huberman E (2006) Regulatory properties and cellular redistribution of zinc during macrophage differentiation of human leukemia cells. J Struct Biol 155(1):2–11. doi:10.1016/j.jsb.2005.09.012

Gruber K, Maywald M, Rosenkranz E, Haase H, Plumakers B, Rink L (2013) Zinc deficiency adversely influences interleukin-4 and interleukin-6 signaling. J Biol Regul Homeost Agents 27(3):661–671

Gustafson GT (1967) Heavy metals in rat mast cell granules. Lab Invest 17(6):588–598

Gyulkhandanyan AV, Lee SC, Bikopoulos G, Dai F, Wheeler MB (2006) The Zn^{2+}-transporting pathways in pancreatic beta-cells: a role for the L-type voltage-gated Ca^{2+} channel. J Biol Chem 281(14):9361–9372. doi:10.1074/jbc.M508542200

Haase H, Maret W (2003) Intracellular zinc fluctuations modulate protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Exp Cell Res 291(2):289–298

Haase H, Maret W (2005) Fluctuations of cellular, available zinc modulate insulin signaling via inhibition of protein tyrosine phosphatases. J Trace Elem Med Biol 19(1):37–42. doi:10.1016/j.jtemb.2005.02.004

Haase H, Maret W (2010) The regular and signaling functions of zinc in human cellular physiology. In: Koropatnik J, Zalups RK (eds) Cellular and molecular biology of metals. Taylor and Francis. London, pp. 179–210

Haase H, Rink L (2009a) Functional significance of zinc-related signaling pathways in immune cells. Annu Rev Nutr 29:133–152. doi:10.1146/annurev-nutr-080508-141119

Haase H, Rink L (2009b) The immune system and the impact of zinc during aging. Immun Ageing 6:9. doi:10.1186/1742-4933-6-9

Haase H, Rink L (2010) Das essentielle Spurenelement Zink. Biol Unserer Zeit 40:314–321

Haase H, Hebel S, Engelhardt G, Rink L (2006a) Flow cytometric measurement of labile zinc in peripheral blood mononuclear cells. Anal Biochem 352(2):222–230. doi:10.1016/j.ab.2006.02.009

Haase H, Mocchegiani E, Rink L (2006b) Correlation between zinc status and immune function in the elderly. Biogerontology 7(5-6):421–428. doi:10.1007/s10522-006-9057-3

Haase H, Ober-Blobaum JL, Engelhardt G, Hebel S, Heit A, Heine H, Rink L (2008) Zinc signals are essential for lipopolysaccharide-induced signal transduction in monocytes. J Immunol 181(9):6491–6502

Hansson A (1996) Extracellular zinc ions induces mitogen-activated protein kinase activity and protein tyrosine phosphorylation in bombesin-sensitive Swiss 3 T3 fibroblasts. Arch Biochem Biophys 328(2):233–238. doi:10.1006/abbi.1996.0168

Hasan R, Rink L, Haase H (2013) Zinc signals in neutrophil granulocytes are required for the formation of neutrophil extracellular traps. Innate Immun 19(3):253–264. doi:10.1177/1753425912458815

Hasegawa H, Suzuki K, Suzuki K, Nakaji S, Sugawara K (2000) Effects of zinc on the reactive oxygen species generating capacity of human neutrophils and on the serum opsonic activity in vitro. Luminescence 15(5):321–327. doi:10.1002/1522-7243(200009/10)15:5<321::aid-bio605>3.0.co;2-o

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(12):7750–7760

Ho Y, Samarasinghe R, Knoch ME, Lewis M, Aizenman E, DeFranco DB (2008) Selective inhibition of mitogen-activated protein kinase phosphatases by zinc accounts for extracellular signal-regulated kinase 1/2-dependent oxidative neuronal cell death. Mol Pharmacol 74 (4):1141–1151. doi:10.1124/mol.108.049064

Hogstrand C, Verbost PM, Bonga SE, Wood CM (1996) Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am J Physiol 270(5 pt 2): R1141–R1147

Honscheid A, Rink L, Haase H (2009) T-lymphocytes: a target for stimulatory and inhibitory effects of zinc ions. Endocr Metab Immune Disord Drug Targets 9(2):132–144

Huang J, Zhang D, Xing W, Ma X, Yin Y, Wei Q, Li G (2008) An approach to assay calcineurin activity and the inhibitory effect of zinc ion. Anal Biochem 375(2):385–387. doi:10.1016/j.ab.2007.12.016

Hubbard SR, Bishop WR, Kirschmeier P, George SJ, Cramer SP, Hendrickson WA (1991) Identification and characterization of zinc binding sites in protein kinase C. Science 254 (5039):1776–1779

Hujanen ES, Seppa ST, Virtanen K (1995) Polymorphonuclear leukocyte chemotaxis induced by zinc, copper and nickel in vitro. Biochim Biophys Acta 1245(2):145–152

Huse M, Eck MJ, Harrison SC (1998) A Zn^{2+} ion links the cytoplasmic tail of CD4 and the N-terminal region of Lck. J Biol Chem 273(30):18729–18733

Ibs KH, Rink L (2003) Zinc-altered immune function. J Nutr 133(5 suppl 1):1452s–1456s

Jia Y, Jeng JM, Sensi SL, Weiss JH (2002) Zn^{2+} currents are mediated by calcium-permeable AMPA/kainate channels in cultured murine hippocampal neurones. J Physiol 543(pt 1):35–48

Jirakulaporn T, Muslin AJ (2004) Cation diffusion facilitator proteins modulate Raf-1 activity. J Biol Chem 279(26):27807–27815. doi:10.1074/jbc.M401210200

Kabu K, Yamasaki S, Kamimura D, Ito Y, Hasegawa A, Sato E, Kitamura H, Nishida K, Hirano T (2006) Zinc is required for Fc epsilon RI-mediated mast cell activation. J Immunol 177 (2):1296–1305

Kahmann L, Uciechowski P, Warmuth S, Plumakers B, Gressner AM, Malavolta M, Mocchegiani E, Rink L (2008) Zinc supplementation in the elderly reduces spontaneous inflammatory cytokine release and restores T cell functions. Rejuvenation Res 11 (1):227–237. doi:10.1089/rej.2007.0613

Kaltenberg J, Plum LM, Ober-Blobaum JL, Honscheid A, Rink L, Haase H (2010) Zinc signals promote IL-2-dependent proliferation of T cells. Eur J Immunol 40(5):1496–1503. doi:10.1002/eji.200939574

Kawagoe T, Sato S, Jung A, Yamamoto M, Matsui K, Kato H, Uematsu S, Takeuchi O, Akira S (2007) Essential role of IRAK-4 protein and its kinase activity in Toll-like receptor-mediated immune responses but not in TCR signaling. J Exp Med 204(5):1013–1024. doi:10.1084/jem.20061523

Keen CL, Gershwin ME (1990) Zinc deficiency and immune function. Annu Rev Nutr 10:415–431. doi:10.1146/annurev.nu.10.070190.002215

Kim PW, Sun ZY, Blacklow SC, Wagner G, Eck MJ (2003) A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. Science 301(5640):1725–1728. doi:10.1126/science.1085643

King LE, Fraker PJ (2000) Variations in the cell cycle status of lymphopoietic and myelopoietic cells created by zinc deficiency. J Infect Dis 182(suppl 1):S16–S22. doi:10.1086/315923

King LE, Frentzel JW, Mann JJ, Fraker PJ (2005) Chronic zinc deficiency in mice disrupted T cell lymphopoiesis and erythropoiesis while B cell lymphopoiesis and myelopoiesis were maintained. J Am Coll Nutr 24(6):494–502

Kitamura H, Morikawa H, Kamon H, Iguchi M, Hojyo S, Fukada T, Yamashita S, Kaisho T, Akira S, Murakami M, Hirano T (2006) Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nat Immunol 7(9):971–977. doi:10.1038/ni1373

Klein C, Sunahara RK, Hudson TY, Heyduk T, Howlett AC (2002) Zinc inhibition of cAMP signaling. J Biol Chem 277(14):11859–11865. doi:10.1074/jbc.M108808200

Klein C, Heyduk T, Sunahara RK (2004) Zinc inhibition of adenylyl cyclase correlates with conformational changes in the enzyme. Cell Signal 16(10):1177–1185. doi:10.1016/j.cellsig.2004.03.008

Knapp LT, Klann E (2000) Superoxide-induced stimulation of protein kinase C via thiol modification and modulation of zinc content. J Biol Chem 275(31):24136–24145. doi:10.1074/jbc.M002043200

Kojima C, Kawakami A, Takei T, Nitta K, Yoshida M (2007) Angiotensin-converting enzyme inhibitor attenuates monocyte adhesion to vascular endothelium through modulation of intracellular zinc. J Pharmacol Exp Ther 323(3):855–860. doi:10.1124/jpet.107.127944

Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G (1993) Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature (Lond) 362(6417):245–248. doi:10.1038/362245a0

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(46):44327–44331. doi:10.1074/jbc.M205634200

Kown MH, Van der Steenhoven T, Blankenberg FG, Hoyt G, Berry GJ, Tait JF, Strauss HW, Robbins RC (2000) Zinc-mediated reduction of apoptosis in cardiac allografts. Circulation 102 (19 suppl 3):III228–III232

Kown MH, van der Steenhoven TJ, Jahncke CL, Mari C, Lijkwan MA, Koransky ML, Blankenberg FG, Strauss HW, Robbins RC (2002) Zinc chloride-mediated reduction of apoptosis as an adjunct immunosuppressive modality in cardiac transplantation. J Heart Lung Transplant 21(3):360–365

Krezel A, Maret W (2007) Dual nanomolar and picomolar Zn(II) binding properties of metallothionein. J Am Chem Soc 129(35):10911–10921. doi:10.1021/ja071979s

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(3):239–240. doi:10.1038/ng913

Laity JH, Andrews GK (2007) Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). Arch Biochem Biophys 463(2):201–210. doi:10.1016/j.abb.2007.03.019

Laura MP, Brieger A, Engelhardt G, Hebel S, Nessel A, Arlt M, Kaltenberg J, Schwaneberg U, Huber M, Rink L, Haase H (2014) PTEN-inhibition by zinc ions augments interleukin-2-mediated Akt phosphorylation. Metallomics 6(7):1277–1287. doi: 10.1039/c3mt00197k

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 (45):41879–41888. doi:10.1074/jbc.M108313200

Lee WW, Cui D, Czesnikiewicz-Guzik M, Vencio RZ, Shmulevich I, Aderem A, Weyand CM, Goronzy JJ (2008) Age-dependent signature of metallothionein expression in primary CD4 T cell responses is due to sustained zinc signaling. Rejuvenation Res 11(6):1001–1011. doi:10.1089/rej.2008.0747

Lepage LM, Giesbrecht JA, Taylor CG (1999) Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr 129(3):620–627

Lin RS, Rodriguez C, Veillette A, Lodish HF (1998) Zinc is essential for binding of p56(lck) to CD4 and CD8alpha. J Biol Chem 273(49):32878–32882

Macian F (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5(6):472–484. doi:10.1038/nri1632

Maret W (2006) Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxid Redox Signal 8(9–10):1419–1441. doi:10.1089/ars.2006.8.1419

Maret W, Jacob C, Vallee BL, Fischer EH (1999) Inhibitory sites in enzymes: zinc removal and reactivation by thionein. Proc Natl Acad Sci USA 96(5):1936–1940

Mills CF (1989) Physiology of zinc: general aspects. In: Mills CF (ed) Zinc in human biology. Springer, London, pp 1–14
Mingari MC, Moretta A, Moretta L (1998) Regulation of KIR expression in human T cells: a safety mechanism that may impair protective T-cell responses. Immunol Today 19(4):153–157
Mocchegiani E, Santarelli L, Muzzioli M, Fabris N (1995) Reversibility of the thymic involution and of age-related peripheral immune dysfunctions by zinc supplementation in old mice. Int J Immunopharmacol 17(9):703–718
Moore JB, Blanchard RK, McCormack WT, Cousins RJ (2001) cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr 131(12):3189–3196
Moulder K, Steward MW (1989) Experimental zinc deficiency: effects on cellular responses and the affinity of humoral antibody. Clin Exp Immunol 77(2):269–274
Mustelin T, Tasken K (2003) Positive and negative regulation of T-cell activation through kinases and phosphatases. Biochem J 371(pt 1):15–27. doi:10.1042/bj20021637
Muzzioli M, Stecconi R, Donnini A, Re F, Provinciali M (2007) Zinc improves the development of human CD34+ cell progenitors towards natural killer cells and induces the expression of GATA-3 transcription factor. Int J Biochem Cell Biol 39(5):955–965. doi:10.1016/j.biocel.2007.01.011
Osati-Ashtiani F, King LE, Fraker PJ (1998) Variance in the resistance of murine early bone marrow B cells to a deficiency in zinc. Immunology 94(1):94–100
Palacios EH, Weiss A (2004) Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene 23(48):7990–8000. doi:10.1038/sj.onc.1208074
Park JA, Koh JY (1999) Induction of an immediate early gene egr-1 by zinc through extracellular signal-regulated kinase activation in cortical culture: its role in zinc-induced neuronal death. J Neurochem 73(2):450–456
Prasad AS (1981) Zinc deficiency and effects of zinc supplementation on sickle cell anemia subjects. Prog Clin Biol Res 55:99–122
Prasad AS (1985) Clinical manifestations of zinc deficiency. Annu Rev Nutr 5:341–363. doi:10.1146/annurev.nu.05.070185.002013
Prasad AS (2000) Effects of zinc deficiency on Th1 and Th2 cytokine shifts. J Infect Dis 182(suppl 1):S62–S68. doi:10.1086/315916
Prasad AS (2002) Zinc deficiency in patients with sickle cell disease. Am J Clin Nutr 75 (2):181–182
Prasad AS (2009) Impact of the discovery of human zinc deficiency on health. J Am Coll Nutr 28 (3):257–265
Prasad AS, Halsted JA, Nadimi M (1961) Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. Am J Med 31:532–546
Prasad AS, Miale A Jr, Farid Z, Sandstead HH, Schulert AR (1963) Zinc metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism, and hypogonadism. J Lab Clin Med 61:537–549
Prasad AS, Bao B, Beck FW, Kucuk O, Sarkar FH (2004) Antioxidant effect of zinc in humans. Free Radic Biol Med 37(8):1182–1190. doi:10.1016/j.freeradbiomed.2004.07.007
Radtke F, Heuchel R, Georgiev O, Hergersberg M, Gariglio M, Dembic Z, Schaffner W (1993) Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. EMBO J 12(4):1355–1362
Ragozzino D, Giovannelli A, Degasperi V, Eusebi F, Grassi F (2000) Zinc permeates mouse muscle ACh receptor channels expressed in BOSC 23 cells and affects channel function. J Physiol 529(pt 1):83–91
Rajagopalan S, Long EO (1998) Zinc bound to the killer cell-inhibitory receptor modulates the negative signal in human NK cells. J Immunol 161(3):1299–1305
Rajagopalan S, Winter CC, Wagtmann N, Long EO (1995) The Ig-related killer cell inhibitory receptor binds zinc and requires zinc for recognition of HLA-C on target cells. J Immunol 155 (9):4143–4146

Rink L, Gabriel P (2000) Zinc and the immune system. Proc Nutr Soc 59(4):541–552
Romir J, Lilie H, Egerer-Sieber C, Bauer F, Sticht H, Muller YA (2007) Crystal structure analysis and solution studies of human Lck-SH3; zinc-induced homodimerization competes with the binding of proline-rich motifs. J Mol Biol 365(5):1417–1428. doi:10.1016/j.jmb.2006.10.058
Saha AR, Hadden EM, Hadden JW (1995) Zinc induces thymulin secretion from human thymic epithelial cells in vitro and augments splenocyte and thymocyte responses in vivo. Int J Immunopharmacol 17(9):729–733
Saito Y, Saito K, Hirano Y, Ikeya K, Suzuki H, Shishikura K, Manno S, Takakuwa Y, Nakagawa K, Iwasa A, Fujikawa S, Moriya M, Mizoguchi N, Golden BE, Osawa M (2002) Hyperzincemia with systemic inflammation: a heritable disorder of calprotectin metabolism with rheumatic manifestations? J Pediatr 140(2):267–269. doi:10.1067/mpd.2002.121699
Sampson B, Kovar IZ, Rauscher A, Fairweather-Tait S, Beattie J, McArdle HJ, Ahmed R, Green C (1997) A case of hyperzincemia with functional zinc depletion: a new disorder? Pediatr Res 42 (2):219–225. doi:10.1203/00006450-199708000-00015
Sandstead HH (1991) Zinc deficiency. A public health problem? Am J Dis Child 145(8):853–859
Shankar AH, Prasad AS (1998) Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr 68(2 suppl):447s–463s
Sheikh A, Shamsuzzaman S, Ahmad SM, Nasrin D, Nahar S, Alam MM, Al Tarique A, Begum YA, Qadri SS, Chowdhury MI, Saha A, Larson CP, Qadri F (2010) Zinc influences innate immune responses in children with enterotoxigenic *Escherichia coli*-induced diarrhea. J Nutr 140(5):1049–1056. doi:10.3945/jn.109.111492
Smith JC, Zeller JA, Brown ED, Ong SC (1976) Elevated plasma zinc: a heritable anomaly. Science 193(4252):496–498
Sood SM, Wu MX, Hill KA, Slattery CW (1999) Characterization of zinc-depleted alanyl-tRNA synthetase from *Escherichia coli*: role of zinc. Arch Biochem Biophys 368(2):380–384. doi:10.1006/abbi.1999.1314
Stennicke HR, Salvesen GS (1997) Biochemical characteristics of caspases-3, -6, -7, and -8. J Biol Chem 272(41):25719–25723
Suzuki N, Suzuki S, Millar DG, Unno M, Hara H, Calzascia T, Yamasaki S, Yokosuka T, Chen NJ, Elford AR, Suzuki J, Takeuchi A, Mirtsos C, Bouchard D, Ohashi PS, Yeh WC, Saito T (2006) A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. Science 311(5769):1927–1932. doi:10.1126/science.1124256
Takahashi K, Akaishi E, Abe Y, Ishikawa R, Tanaka S, Hosaka K, Kubohara Y (2003) Zinc inhibits calcineurin activity in vitro by competing with nickel. Biochem Biophys Res Commun 307(1):64–68
Tan SL, Parker PJ (2003) Emerging and diverse roles of protein kinase C in immune cell signalling. Biochem J 376(Pt 3):545–552. doi:10.1042/bj20031406
Tanaka Y (1989) Zinc inhibits pokeweed mitogen-induced development of immunoglobulin-secreting cells through augmentation of both CD4 and CD8 cells. Int J Immunopharmacol 11(6):673–679
Tang X, Shay NF (2001) Zinc has an insulin-like effect on glucose transport mediated by phosphoinositol-3-kinase and Akt in 3 T3-L1 fibroblasts and adipocytes. J Nutr 131 (5):1414–1420
Truong-Tran AQ, Carter J, Ruffin RE, Zalewski PD (2001) The role of zinc in caspase activation and apoptotic cell death. Biometals 14(3-4):315–330
Vallee BL, Falchuk KH (1993) The biochemical basis of zinc physiology. Physiol Rev 73 (1):79–118
Vang T, Torgersen KM, Sundvold V, Saxena M, Levy FO, Skalhegg BS, Hansson V, Mustelin T, Tasken K (2001) Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. J Exp Med 193(4):497–507
von Bulow V, Rink L, Haase H (2005) Zinc-mediated inhibition of cyclic nucleotide phosphodiesterase activity and expression suppresses TNF-alpha and IL-1 beta production in monocytes by elevation of guanosine 3′,5′-cyclic monophosphate. J Immunol 175(7):4697–4705

von Bulow V, Dubben S, Engelhardt G, Hebel S, Plumakers B, Heine H, Rink L, Haase H (2007) Zinc-dependent suppression of TNF-alpha production is mediated by protein kinase A-induced inhibition of Raf-1, I kappa B kinase beta, and NF-kappa B. J Immunol 179(6):4180–4186

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature (Lond) 412(6844):346–351. doi:10.1038/35085597

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(1):66–73. doi:10.1086/341125

Wellinghausen N, Rink L (1998) The significance of zinc for leukocyte biology. J Leukoc Biol 64 (5):571–577

Wellinghausen N, Fischer A, Kirchner H, Rink L (1996) Interaction of zinc ions with human peripheral blood mononuclear cells. Cell Immunol 171(2):255–261. doi:10.1006/cimm.1996.0201

Wellinghausen N, Kirchner H, Rink L (1997a) The immunobiology of zinc. Immunol Today 18 (11):519–521

Wellinghausen N, Martin M, Rink L (1997b) Zinc inhibits interleukin-1-dependent T cell stimulation. Eur J Immunol 27(10):2529–2535. doi:10.1002/eji.1830271010

WHO (2002) The World Health Report 2002. World Health Organisation, Geneva

Williams RJ (1984) Zinc: what is its role in biology? Endeavour 8(2):65–70

Wu W, Jaspers I, Zhang W, Graves LM, Samet JM (2002) Role of Ras in metal-induced EGF receptor signaling and NF-kappaB activation in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 282(5):L1040–L1048. doi:10.1152/ajplung.00390.2001

Wu W, Wang X, Zhang W, Reed W, Samet JM, Whang YE, Ghio AJ (2003) Zinc-induced PTEN protein degradation through the proteasome pathway in human airway epithelial cells. J Biol Chem 278(30):28258–28263. doi:10.1074/jbc.M303318200

Wuehler SE, Peerson JM, Brown KH (2005) Use of national food balance data to estimate the adequacy of zinc in national food supplies: methodology and regional estimates. Public Health Nutr 8(7):812–819

Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, Kurosaki T, Yamashita S, Tokunaga M, Nishida K, Hirano T (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177(4):637–645. doi:10.1083/jcb.200702081

Yoder JH, Chong H, Guan KL, Han M (2004) Modulation of KSR activity in *Caenorhabditis elegans* by Zn ions, PAR-1 kinase and PP2A phosphatase. EMBO J 23(1):111–119. doi:10.1038/sj.emboj.7600025

Yokoyama WM, Plougastel BF (2003) Immune functions encoded by the natural killer gene complex. Nat Rev Immunol 3(4):304–316. doi:10.1038/nri1055

Yu M, Lee WW, Tomar D, Pryshchep S, Czesnikiewicz-Guzik M, Lamar DL, Li G, Singh K, Tian L, Weyand CM, Goronzy JJ (2011) Regulation of T cell receptor signaling by activation-induced zinc influx. J Exp Med 208(4):775–785. doi:10.1084/jem.20100031

Chapter 11
Zinc Signal in Inflammation

Ming-Jie Liu and Daren L. Knoell

Abstract Zinc (Zn) is essential to maintain normal health, to protect the host from damage, and to repair damage once it has occurred. Inflammation is typically defined as a response to cellular injury that is marked by capillary dilatation, leukocytic infiltration, redness, heat, and pain. Inflammation is designed to ultimately eliminate noxious agents, such as bacterial and viral pathogens, and to repair damaged tissue. The extent of inflammation that occurs following cellular injury must be tightly regulated so that danger is localized, damage is minimized, and recovery occurs expeditiously. Recent advances have established Zn as a critical component of the inflammatory response that is directly coupled to regulation of immune function. Importantly, Zn intake and the capacity to maintain normal Zn levels within the body are critical for proper regulation of immune function, host defense, and the extent of inflammation that occurs. Insufficient Zn levels within the body and particularly within key cells that are involved in host defense increase susceptibility to improper immune function, excessive inflammation, tissue damage, and failure to repair properly. As one leading example, Zn has been shown to directly regulate the extent of inflammation through interaction with the NF-κB signaling pathway, a pathway that is essential for normal immune function. In addition, Zn deficiency promotes chronic, systemic, low-grade inflammation associated with obesity and aging. It is anticipated that continued discovery of the many roles that Zn plays in controlling inflammation will yield meaningful insight for nutritional strategies that prevent or significantly reduce the morbidity and mortality associated with inflammation-based diseases.

Keywords Adipose tissue inflammation • Host defense • Infection • Inflammation • Innate immunity • NF-κB • Obesity • Sepsis • Zinc • Zinc deficiency

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11.1 Zinc and Inflammation-Based Disorders

The word *inflammation* comes from the Latin word "inflammo," meaning "I set alight, I ignite." Inflammation is a programmed biological response to harmful stimuli. For example, infection is caused by virulent pathogens that gain access within our body and inflammation is the ensuing response to the infection. It is an essential component of host defense and considered to be part of our innate immune system that is required for full recovery. Inflammation that occurs subsequent to cellular injury is marked by capillary dilatation, leukocytic infiltration, redness, heat, and pain. The extent of inflammation that occurs following cellular injury must be tightly regulated so that danger is localized, damage is minimized, and recovery occurs expeditiously. If left unchecked, inflammation can become self-perpetuating such that more inflammation is created in response to the existing inflammation, leading to chronic imbalance.

The trace element Zinc (Zn) is one of the most important and second most abundant trace elements in the human body. It has established roles in facilitating some of the most fundamental molecular functions that include catalyzing biochemical reactions, maintaining proper protein structure, and regulating cell signaling. In doing so, it is involved in governing required cellular operations that include transcription, translation, replication, and repair. Zn is essential for proper innate immune function and plays an indispensable role in modulating the host inflammatory response to noxious stimuli. The concomitant presence of low-grade systemic chronic inflammation that is associated with mild Zn deficiency further underscores the importance of Zn in the context of chronic inflammatory diseases. A strong correlation exists between low Zn levels and chronic diseases that include, but are not limited to, cardiometabolic disease, diabetes, Alzheimer's disease, aging, and chronic obesity. Accordingly, Zn status in humans is important in the context of how the host responds to both acute and chronic stimuli that can provoke inflammation.

11.2 Zn and Acute Inflammation-Based Disorders

Nutritional Zn deficiency, which commonly occurs in socioeconomically challenged populations, is a leading cause of newly acquired bacterial pneumonia and diarrhea in children and the elderly (Caulfield et al. 2004). Many of these infections are caused by pathogens that, under normal circumstances, would not cause significant problems to the host. Upon pathogen exposure, Zn deficiency can increase susceptibility to acquiring infection and increase the duration and extent of infection once it has occurred (Black 2003). Importantly, Zn supplementation in these at-risk populations can significantly reduce the risk of developing newly acquired infections and can also improve morbidity and mortality if infection has already occurred (Yakoob et al. 2011).

The long-standing paradigm for understanding the pathophysiology of septic shock, often the result of bacteremia, is centered on a dysfunctional innate immune system, wherein excessive and uncontrolled inflammation leads to direct tissue and organ injury (Cornell et al. 2010; Wynn et al. 2010). Multiple gene expression studies have consistently shown that septic shock is characterized by widespread repression of gene families that either directly participate in Zn homeostasis or directly depend on Zn homeostasis for normal function (Cvijanovich et al. 2008, 2009; Shanley et al. 2007; Wong et al. 2007, 2010). These observations suggest that altered Zn homeostasis may contribute to the pathogenesis associated with septic shock. Indeed, low serum Zn concentrations have been documented in pediatric septic shock, and nonsurvivors have lower serum Zn concentrations compared with survivors (Wong et al. 2007). Low serum Zn concentrations have also been reported in other forms of critical illness in both adults and children (Besecker et al. 2011; Cvijanovich et al. 2009; Heyland et al. 2008). These observations support the notion that Zn supplementation may be a low-cost and effective therapeutic strategy for septic shock (Knoell and Liu 2010). In animal models of sepsis, Zn supplementation confers a survival benefit, and the survival benefit correlates with enhanced bacterial clearance as well as modulation of excessive inflammation (Bao et al. 2010b; Knoell et al. 2009; Nowak et al. 2012). Two recent studies that involve children with either pneumonia or bacteremia in developing countries demonstrated that Zn supplementation significantly reduced infection-related complications, including mortality (Srinivasan et al. 2012; Bhatnagar et al. 2012). A study involving critically ill children in the United States tested the efficacy of oral Zn supplementation, in combination with oral selenium, glutamine, and metoclopramide, as a means of preventing nosocomial infection or sepsis (Carcillo et al. 2012). There was no efficacy in the overall study population for the primary study endpoint (time to development of nosocomial infection/sepsis), but a secondary analysis restricted to patients with baseline immune dysfunction suggested a beneficial effect in reducing nosocomial infections. Clinical trials demonstrate that antioxidant cocktails administered either enterally or parenterally improve patient survival (when evaluated in aggregate: relative risk 0.72, 95 % confidence interval 0.62–0.85, $P < 0.0001$). Zn is one component of the cocktail administered in most of these trials. Importantly, all published trials are limited in scope because of substantial patient heterogeneity. In fact, very few study subjects had sepsis. Further complicating interpretation of findings, Zn was exclusively studied in combination with other micronutrients thereby making it impossible to distinguish its individual impact. In addition, Zn status before critical illness was not determined, so that it was unclear whether Zn deficiency existed as a premorbid condition. Accordingly, Zn supplementation as an adjunctive therapeutic strategy for septic shock remains an intriguing concept that requires further testing.

The common cold, which is caused by a constellation of more than 20 viruses, occurs throughout the world and across all populations. The morbidity and corresponding cost associated with the cold are substantial. Zn is now commonly marketed as a “cure” for the common cold and sold over the counter. The use of Zn is predicated on past studies that have demonstrated that when Zn supplements,

typically in the form of lozenges, are administered within the first 24 h of symptoms, the severity and duration of cold symptoms are significantly reduced (Prasad et al. 2000, 2008; Eby et al. 1984). Further analysis in study subjects revealed a significant decrease in soluble interleukin (IL) receptor antagonist and soluble ICAM-1 plasma levels that indicated a reduction in the extent of inflammation associated with cold symptoms. Similar studies that also aimed at determining whether Zn indeed does prevent the occurrence of a cold have yielded contradictory findings, which may in part be explained by the design of these studies that varied significantly. Taking all studies into consideration, a recent meta-analysis that evaluated only randomized, double-blind, placebo-controlled trials concluded that Zn supplementation can reduce the duration and severity of the common cold when therapy is started within the first 24 h of the onset of a cold (Singh and Das 2011).

11.3 Zn and Chronic Inflammation-Based Disorders

Alcohol use and abuse are highly prevalent within both developed and underdeveloped countries and impose a substantial health burden to society. Alcohol abuse is the third leading cause of preventable death in the United States and is associated with numerous chronic disease states (Mokdad et al. 2000). Importantly, alcohol abuse is also a cause of nutritional deficits, including Zn deficiency. Zn deficiency caused by alcoholism has been shown to increase oxidative stress within vital organs including the lung, thereby causing dysregulation of inflammation, innate immune function, and macrophage phagocytosis, and, hence, bacterial clearance (Joshi et al. 2009). This point is important because alcoholics have a higher incidence of acquiring pneumonia, resulting in increased lung injury and mortality (Mehta et al. 2013). Based on these findings, which are strongly supported by animal models, future studies that involve Zn supplementation in patients that suffer from alcoholism and are prone to life-threatening infection, are warranted.

Aging, and particularly deficits in Zn deficiency in the elderly, is now recognized as a potential cause of the chronic inflammation that is observed in a substantial proportion of the elderly population (Mocchegiani et al. 2012; Kanoni et al. 2010). Aging is associated with gradual impairment of immune function and a corresponding systemic low-grade chronic inflammation (Wilcock and Griffin 2013). Remarkable similarities between the hallmarks of Zn deficiency and aging-related immune dysfunction have been observed. Zn supplementation may improve age-related immune dysfunction and chronic inflammation (Wong and Ho 2012). Further, many age-related diseases, including cancer, cardiovascular disease, type 2 diabetes, osteoporosis, and Alzheimer's disease, are associated with low-grade chronic inflammation. Alzheimer's disease (AD) is a progressive neurodegenerative disease, and chronic inflammation has been shown to be a pathological hallmark of the most vulnerable regions of the AD brain (Griffin 2013). Compared to other vital organs, the brain is highest in Zn content. An evolving

paradigm is that bioavailabile pools of Zn, or a lack thereof, may regulate the immune response and inflammation in the brain: this is based on previous studies that have revealed abnormally low Zn levels within key compartments of the brain (Molina et al. 1998; Kapaki et al. 1989). In support of this concept, a placebo-controlled clinical trial revealed that Zn supplementation stabilized cognitive function whereas the placebo group continued to deteriorate (Maylor et al. 2006). Although it is plausible that alterations in Zn homeostasis in the brain may contribute to AD pathogenesis, the molecular mechanisms that account for these disturbances remain to be identified (Vasto et al. 2008).

Obesity is recognized as a worldwide public health problem (Finucane et al. 2011). In the United States, obesity has become epidemic, and the proportion of obese people is among the highest (more than 30 %) in the world (Flegal et al. 2010). Obesity is a prevalent risk factor for multiple disorders, including insulin resistance, type 2 diabetes, and cardiovascular diseases (Berrington de Gonzalez et al. 2010; Zheng et al. 2011; Lumeng and Saltiel 2011; Ferrante 2007). The obese population chronically consumes energy-dense foods that are rich in fats but lacking in vitamins and micronutrients (Finucane et al. 2011). As a result, these individuals commonly experience micronutrient deficiencies (Garcia et al. 2009). In obese people, decreased dietary Zn intake has been shown to inappropriately alter immune function, lipid metabolism, and insulin production (Costarelli et al. 2010). In contrast, weight loss has been shown to significantly improve metabolic function and increase circulating Zn levels (Voruganti et al. 2010). Adipose tissue has emerged as an active participant in regulating endocrine function through the secretion of adipokines, especially leptin (Ouchi et al. 2011). Accumulating evidence suggests that chronic inflammation, which originates in adipose tissue, plays a crucial role in the pathogenesis of obesity-related metabolic dysfunction (Hotamisligil 2006). Activated M1 macrophages are recruited into adipose tissue and have a central etiological role in the development of chronic tissue inflammation, leading to the secretion of adipokines and cytokines (Chawla et al. 2011; Xu et al. 2003; Weisberg et al. 2003; Ouchi et al. 2011). This secretion is in part the result of liberation of saturated free fatty acids from adipocytes, which bind to macrophage Toll-like receptor-4 (TLR-4) and activate inhibitor of κB kinase-β (IKK-β) and nuclear factor-κB (NF-κB) (Suganami et al. 2007; Arkan et al. 2005). Inhibition of IKK and NF-κB has been proposed as the mechanism whereby antiinflammatory agents such as salicylates reverse insulin resistance (Yin et al. 1998; Yuan et al. 2001). Obese people have lower circulating Zn levels, but it is unknown how and why this happens. Further, their plasma Zn levels are inversely correlated with systemic inflammatory markers (Costarelli et al. 2010; Voruganti et al. 2010). Based on these observations, our group recently reported that Zn deficiency may be a factor that contributes to the development of systemic chronic inflammation associated with obesity through immune dysregulation (Liu et al. 2013a). C57BL/6 mice were randomly assigned to receive either a 60 % high-fat diet or control diet for 6 weeks, followed by further subdivision into Zn-deficient and matched Zn-sufficient groups for 3 additional weeks. Although Zn deficiency did not influence the extent of visceral adiposity,

insulin resistance, or systemic inflammation in the obese mice, Zn deficiency significantly augmented circulating leptin levels and leptin signaling in the liver. Furthermore, Zn deficiency increased the expression of the macrophage-specific markers ADAM8 and CD68, indicating an increase in adipose macrophage infiltration and the exacerbation of tissue inflammation. These findings demonstrate that Zn deficiency contributes to metabolic and immune dysregulation in obesity in vivo.

Obesity is also a significant risk factor for developing diabetes. Zn metabolism is altered in diabetic patients, and the Zn transporter ZnT-8 has recently been identified as an etiological cause. A single-nucleotide polymorphism (SNP) located within the SLC30A8 gene that codes for the protein ZnT-8 in conjunction with SNPs within metallothioneins (MT) have been implicated as a cause of type 2 diabetes (Jansen et al. 2009). Similarly, recent advances using β-cell-specific *Slc30a8* deficiency (ZnT8KO mice) demonstrated that SLC30A8 regulates hepatic insulin clearance and that its genetic defect contributes to the pathogenesis of type 2 diabetes (Tamaki et al. 2013). The pathogenesis of diabetes mellitus is multifactorial, but establishment of this novel pathway through alteration of Zn metabolism and corresponding inflammation has fostered new approaches to the treatment of this disease.

11.4 Zn Deficiency and Immune Disorders

Zn deficiency was first recognized in humans more than 50 years ago and was originally observed in adult males in developing areas of the Middle East (Prasad et al. 1963). Nutritional Zn deficiency results in complex immune disorders, as perhaps best exemplified by a rare autosomal recessive disease called acrodermatitis enteropathica (AE) that is a cause of severe Zn deficiency. It is now known that mutations harbored within the Zn transporter gene *SLC39A4* (ZIP4), which is abundantly expressed in the intestine and is responsible for Zn uptake from the gut, is centrally involved in the pathogenesis of this condition (Kury et al. 2002). Treatment of AE requires lifelong Zn supplementation. Typically, 1 to 3 mg/kg Zn gluconate or sulfate is administered orally each day. Clinical improvement, including restoration of proper immune function, occurs before any significant change in the plasma Zn levels, usually within days to weeks of initiating treatment. Clinically, the serum Zn levels and alkaline phosphatase levels are monitored every 3 to 6 months. As already discussed, Zn deficiency is a leading contributing factor to immune dysfunction, thereby increasing susceptibility to acquiring infectious-based pneumonia, diarrhea, and malaria (Caulfield et al. 2004). Zn supplementation has been reported to prevent or reduce the burden of these infectious diseases. In addition to bacterial infection, Zn is also important in regulating the extent of inflammation in the setting of viral infection. Zn supplementation has been shown to enhance the response to interferon therapy in the setting of intractable chronic hepatitis C (Takagi et al. 2001) and also to

decrease the extent of common debilitating adverse side effects associated with treatment in chronic hepatitis C patients (Ko et al. 2005).

11.5 The Impact of Inflammation on Zn Metabolism

The acute-phase response (APR) to stress, trauma, and infection results in the robust induction of APR proteins in the blood as part of the inflammatory response. The purpose of this response is to help inhibit the growth of pathogens, modulate the extent of inflammation, and activate coagulation. There are also a number of proteins whose concentration is significantly reduced. This group of "negative" acute-phase response proteins includes (but is not limited to) albumin, antithrombin, and transferrin. The rationale for reduced synthesis of these proteins is to divert critical amino acids toward the synthesis of positive APRs. As previously stated, Zn is required for gene transcription and translation of new proteins. Zn homeostasis rapidly and dynamically changes in humans in response to inflammation as an extension of the acute-phase response. The primary and most apparent alteration observed is the rapid decline in blood Zn levels, which is commonly referred to as hypozincemia. Previously it has been shown in a human endotoxemia model that blood cytokine levels increase [tumor necrosis factor (TNF)-α and IL-6] with a concomitant precipitous decrease in blood Zn levels within the first hour following systemic endotoxin injection. Importantly, in these subjects the decrease in Zn levels could not be explained by changes in circulating albumin levels, plasma protein binding, or urinary excretion (Gaetke et al. 1997). Based on this seminal work and that of others, it is now clear that the ensuing APR results in redistribution of Zn into vital organs, such as the liver and lung, as well as immune cells (Liuzzi et al. 2005; Beker Aydemir et al. 2012; Liu et al. 2013b). The rationale behind Zn redistribution during the host response supports the concept of "nutritional immunity," akin to iron deprivation, whereby the body shunts micronutrients away from pathogens following infection, thereby affording a competitive host advantage (Kehl-Fie and Skaar 2010; Liuzzi et al. 2005). Zn redistribution into the liver also facilitates the reprogramming of hepatic protein synthesis in a manner that is beneficial to the host. Our group recently discovered a novel negative feedback loop whereby extracellular Zn, in addition to enhancing protein synthesis, is deliberately shunted into leukocytes and parenchymal cells for Zn to specifically decrease the extent of inflammation as part of the resolution process following systemic infection (Bao et al. 2010b; Knoell et al. 2009; Liu et al. 2013b). Importantly, deficient Zn levels before the onset of infection result in exuberant inflammation, excessive collateral damage in vital organs, and increased mortality. Collectively, these findings indicate that Zn plays multiple pivotal roles during the early stages of the host inflammatory response to potentially overwhelming challenges.

Zn is an essential structural component of many proteins, as perhaps best exemplified by zinc-finger motifs, that is required for normal molecular function

of proteins within and outside cells. Cellular Zn homeostasis is regulated by two families of Zn transporters and also by the Zn-binding proteins known as metallothioneins (MT), a family of cysteine-rich proteins that bind up to seven Zn molecules per protein. In mammals, the Zn transporter proteins are encoded by two solute-linked carrier (SLC) gene families that include 14 SLC39 (aka ZIP) family members and 10 SLC30 (aka ZnT) family members. SLC39 transporters increase cytosolic Zn content by promoting extracellular uptake or release from subcellular organelles, whereas SLC30 transporters function as counter-regulators that decrease intracellular Zn levels (Lichten and Cousins 2009). The MT protein family serves as an intracellular buffer that helps to maintain intracellular Zn levels and participates in the regulation of labile Zn content (Maret 2006).

Protein-bound Zn, which largely includes a pool that is avidly bound to zinc-finger motifs, is typically believed to be less exchangeable, especially during stress and inflammation. In sharp contrast the labile Zn pool, also referred to as bioavailable Zn, is defined as the Zn that is loosely bound and easily exchanged within and outside of the cell, as well as between subcellular organelles. Using Zn-specific intracellular probes it has been shown that the labile Zn pool is observed as a speck-like pattern that is contained within intracellular vesicles, which have been referred to as zincosomes (Haase and Rink 2007). Consistent with the hypozincemia observed following systemic infection or endotoxin challenge, the concentration of labile Zn within cells rapidly increases in response to inflammatory mediators. As one example, it has been shown that labile Zn rapidly accumulates in hepatocytes in an IL-6-dependent manner following a systemic endotoxin challenge. Similarly, our group observed a rapid increase of labile Zn within monocytes in response to endotoxin and bacterial sepsis (Liu et al. 2013b). Other groups observed a similar sequestration of Zn within macrophages in response to intracellular bacteria and fungi (Botella et al. 2011; Subramanian Vignesh et al. 2013). Collectively, these findings indicate that Zn is naturally sequestered in key cells by a variety of diverse pathogens and danger signals as the host mounts a defense response. The labile pool that is rapidly sequestered following systemic insult then becomes critical in transmitting essential signals within the cell through direct and indirect interaction with key signal transduction pathways. In the context of Zn fluctuations or intracellular translocation, mobilization of labile Zn is viewed as a "Zn signal." Numerous examples have recently emerged demonstrating that Zn signals are propagated through Zn binding with regulatory sites on proteins that possess enzymatic activity. Indeed, labile Zn has been shown to regulate the function of many proteins, including kinases and phosphatases, many of which are instrumental in orchestrating the inflammatory response. Depending upon the protein and the environment wherein it resides, Zn has the potential to either inhibit or activate protein function. Direct inhibition of catalytic activity by Zn is often through transient binding to specific amino acids (cysteines or histidines) that reside within reactive sites, as perhaps best exemplified by protein tyrosine phosphatases (PTP) (Haase and Maret 2003), PKC (Nishida et al. 2009), and IKKβ (Liu et al. 2013b).

Changes in cellular labile Zn content are primarily navigated by Zn transporter proteins. A clearer picture has emerged in recent years suggesting two broad classifications of Zn transporters, those that are constitutively expressed for housekeeping functions and those which are induced for specialized function. Clearly it requires an orchestrated effort of both types, working in concert, to maintain balance, particularly in the setting of stress and inflammation. ZIP4 perhaps provides the best example of a constitutively abundant transporter that is required to maintain normal homeostatic function through its capacity to continuously absorb dietary Zn from the intestinal tract. In contrast, other Zn transporters, for example, ZIP8 and ZIP14, are not naturally abundant but are rapidly induced at the onset of danger signals (Besecker et al. 2008; Liuzzi et al. 2005). The function of these Zn transporter proteins is often transcriptionally regulated following insult and subsequent activation of transcription factors and corresponding upstream kinase pathways (Liu et al. 2013b; Haase and Rink 2009). In addition to modulation of gene expression, Zn transporters can be controlled through posttranscriptional regulation. The best example of this has been shown through CK2-dependent phosphorylation of ZIP7, thereby allowing Zn release from the endoplasmic reticulum as a key component of cell proliferation (Taylor et al. 2012). How subsequent downstream Zn-dependent signaling occurs in this setting remains to be resolved. In the setting of inflammation, Zn metabolism can also be modulated through alternative mechanisms. For example, the metal-response element-binding transcription factor-1 (MTF-1) functions as a cellular Zn sensor that coordinates the expression of genes involved in Zn homeostasis as well as protection against metal toxicity and oxidative stress (Laity and Andrews 2007). When Zn begins to accumulate in the cytosol, MTF-1 senses increased metal load because the Zn-finger domain of MTF-1 directly (and reversibly) binds to Zn. This metalloregulatory protein then adopts a DNA-binding conformation and translocates into the nucleus, where it binds to metal-response elements in specific gene promoter regions of target genes that lead to transcriptional activation. MTF-1 function is believed to be essential in regulating Zn homeostasis (Botella et al. 2011). Although substantial progress has been made, a full account of the dynamic redistribution of Zn that occurs particularly at the subcellular level in response to danger signals requires further investigation. With the continued development of new and improved Zn sensors, it is anticipated that many new discoveries in this emerging area will become possible.

11.6 Zn Signaling in Response to Systemic Infection

Inflammation is a primordial response designed to protect the host against pathogen invasion or body injury (Takeuchi and Akira 2010). Upon recognition of pathogen-associated molecular patterns, the Toll-like receptor (TLR) pathway becomes activated in immune cells that include monocytes, macrophages, and dendritic cells (Kawai and Akira 2010). TLR signaling initiates recruitment of adaptor molecules such as TRIF, TIRAP, and MyD88 (Takeuchi and Akira 2010). In

turn, the inflammatory signal is transmitted coordinately through a series of molecular events that involve the IRAK family, TRAF6 and TAK1, leading to activation of IκB kinase (IKK) and mitogen-activated protein kinases (MAPKs) (Johnson and Lapadat 2002; Hayden and Ghosh 2008). Activation of the IKK complex, which includes IKKα, IKKβ, and NEMO, results in IκB phosphorylation and degradation, thereby allowing phosphorylated NF-κB dimers to translocate into the nucleus and bind κB sites located within target gene promoters to activate transcription (Hayden and Ghosh 2008). NF-κB regulates an array of inflammatory genes, including cytokines, chemokines, and adhesion molecules, and plays a central role in inflammation.

Coordination of the initial host response to infection through regulation of the NF-κB pathway must be tightly regulated to maintain proper immune balance, thereby maximizing host defense while simultaneously minimizing collateral damage (Liew et al. 2005). To achieve this precise balance, multiple counter-regulatory elements have evolved within these pathways, including but not limited to IκBα (Chiao et al. 1994), MyD88s (Burns et al. 2003), IRAKM (Kobayashi et al. 2002), and A20 (Boone et al. 2004). The expression and function of many of these negative regulators, including IκBα, A20, MyD88s, and IRAKM, are themselves activated by TLR ligands and thus constitute classic negative regulatory feedback loops that ensure attenuation of the TLR response in a threshold-dependent manner (Ruland 2011). The negative feedback pathways have evolved to control the extent of innate immune activation and the resolution of inflammation (Ruland 2011).

Nutritional Zn deficiency in a mouse model of multibacterial sepsis (using a technique called cecal ligation and puncture) results in augmentation of the initial pro-inflammatory response, increased bacterial burden, increased vital organ damage, and higher mortality (Liu et al. 2013b; Bao et al. 2010b; Knoell et al. 2009). Further, prophylactic Zn supplementation reduces bacterial load and improves survival (Nowak et al. 2012). These studies that use relevant animal models are consistent with clinical studies conducted on critically ill and septic humans. (Wong et al. 2007; Heyland et al. 2008; Besecker et al. 2011; Cvijanovich et al. 2009). Collectively, these findings strongly support the hypothesis that Zn intake, Zn metabolism, and Zn signaling are essential for proper host defense and innate immune function.

Although much remains to be learned regarding the key signaling pathways and molecules at play with Zn in the setting of sepsis, new findings have emerged indicating that Zn metabolism and immune function are directly coupled to Zn transporter function. In particular, it was discovered that ZIP8 functions as a critical negative feedback regulator that directly couples Zn metabolism to the regulation of innate immunity (Fig. 11.1). At the onset of inflammation, NF-κB directly activates the expression of ZIP8, which then localizes to the plasma membrane, thereby mediating Zn uptake. The new pool of Zn that enters the cytosol via ZIP8 then goes on to inhibit IKKβ kinase activity by binding to a specific coordination site located within the kinase domain of this protein, thereby attenuating the pro-inflammatory response. This concept is also supported by past observations demonstrating that Zn has the capacity to directly interact with IKK (Jeon et al. 2000). Consistent with

these findings, fetal fibroblasts obtained from ZIP8 transgenic hypomorphic mice exhibit decreased Zn uptake and an increased inflammatory response in response to TNF-α or IL-1β, thereby confirming the role of ZIP8 as a negative regulator of the NF-κB pathway (Liu et al. 2013b) (Galvez-Peralta et al. 2012). Consistent with this, the redistribution of Zn into the liver during the acute-phase response was significantly decreased in ZIP14 (*Slc39a14*$^{-/-}$) knockout mice in response to lipopolysaccharide (LPS)-induced endotoxemia (Beker Aydemir et al. 2012). This finding is intriguing when taking into account that ZIP8 and ZIP14 genes emerged in land animals from a single gene in sea animals approximately 420 million years ago. This evolutionary divergence supports the notion that specialized Zn transporter function(s) may have evolved as a consequence of new environmental pressures capable of provoking inflammation (Girijashanker et al. 2008). These findings also provide novel mechanistic insight that links Zn metabolism via specialized Zn transporters to innate immune function, and in so doing enriches the list of regulatory mechanisms that control NF-κB and TLR signaling. Three other mechanisms have been reported that may also explain the inhibitory effects of Zn on inflammation (Fig. 11.1). A hypothetical model suggests that increased expression of A20, which is a de-ubiquitinase and negative regulator of NF-κB, is modulated by Zn (Prasad et al. 2011). Although provocative, there is yet no direct experimental evidence demonstrating that Zn directly regulates the activity of A20. A second mechanism demonstrating the inhibitory effect of Zn on NF-κB has shown that Zn is an inhibitor of cyclic nucleotide phosphodiesterase (PDE). The inhibition of PDE leads to elevation of the cyclic nucleotide cGMP, which actives PKA and subsequently inhibits NF-κB (von Bulow et al. 2007). In a similar fashion, Zn has also been shown to inhibit PMA-mediated PKC translocation to the plasma membrane through binding to a Zn finger-like motif found in PKC. When this occurs, NF-κB activity is indirectly inhibited in mast cells (Nishida et al. 2009). Although some of the latter findings require confirmation in validated in vivo settings, taken together these findings demonstrate that Zn-mediated regulation of the host response to inflammation is complex and that Zn may possess the capacity to interact at multiple points within individual pathways.

The capacity for Zn, in concert with ZIP8, to inhibit NF-κB activation is conserved across different cell types that directly control the innate immune response and include both immune (monocytes and macrophages) and nonimmune cells (lung epithelia). However, ZIP8 is not expressed by every cell in the body. The regulatory function of Zn in coordinating the inflammatory response across all cells within the body is presumed to be broad in scope, requiring the activity of many proteins. Further, there exist an impressive number of conserved Zn transporter proteins in mammals, which would imply that there are many yet to be identified specialized functions for Zn transporters in human health and disease. Consistent with this, loss of the Zn exporter *Slc30a5* (Znt5) in mast cells resulted in increased labile Zn and suppressed NF-κB signaling in response to FcεRI stimulation (Nishida et al. 2009).

Additional pathways have been shown to be modulated by Zn and therefore may also play important roles in controlling inflammation. As one example, Zn has been

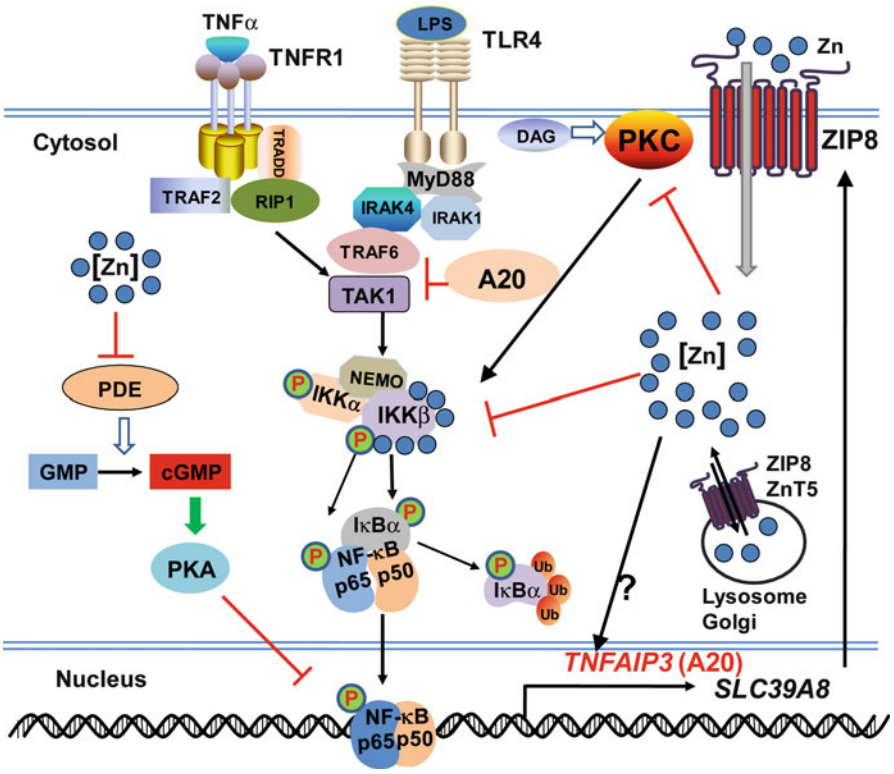

Fig. 11.1 Zn signaling pathways in the Toll-like receptor (*TLR*)-mediated inflammatory response. Zn inhibits the TLR and *NF-κB* pathway through interaction with IKK, A20, PDE-cGMP-PKA, and PKC. *IKK* I kappa B kinase, *PDE* phosphodiesterase, *PKA* protein kinase A, *cGMP* cyclic guanosine monophosphate, *PKC* protein kinase C, [*Zn*] labile Zn pool

shown to be involved in the activation of multiple signaling pathways that are important in cell activation following Toll-like receptor (TLR) stimulation. TLRs constitute a family of proteins with a key role in the innate immune system. TLRs are single, membrane-spanning, noncatalytic receptors, usually expressed in sentinel cells such as macrophages and dendritic cells, that recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers they are recognized by TLRs, which activate immune cell responses. In particular, activation of TLR4 triggers Zn-mediated signaling in a MyD88-dependent manner in monocytes. This signal is transmitted through the MAPK pathway signaling and ultimately results in phosphorylation of IKKα/β, thereby modulating NF-κB signaling (Haase et al. 2008). In contrast, the same Zn signal also results in inhibition of TRIF-mediated activation of IRF3. Based on these observations, one can conclude that labile Zn can simultaneously affect TLR signaling at multiple levels and within different pathways (Brieger et al. 2013). In addition to TLR-mediated MAPK signaling, Zn, or a lack thereof, has recently

been shown to modify the JAK-STAT3 pathway, thereby modulating the acute-phase response (work from our group, in review). In the setting of sepsis, Zn deficiency increased the acute-phase response through alteration of JAK-STAT signaling in the liver and significantly enhanced the production of acute-phase reactants including serum amyloid A. Upon inspection of whole exome expression profiles and subsequent computational pathway analysis, we identified that Zn deficiency significantly increased JAK-STAT3 signaling in hepatocytes whereas Zn supplementation reversed this effect. The detailed molecular mechanism(s) that fully account for these observations remain under investigation.

Oxygen radicals are important mediators of the inflammatory response, and excessive radical production is known to be a cause of multiple inflammatory-based disorders. Reactive oxygen species (ROS) are produced within cells as part of the host defense and are vital to maintain proper immune function to eliminate invading pathogens (Fang 1997), with an essential role in the intracellular killing of microorganisms by macrophages (Subramanian Vignesh et al. 2013). Increased cellular accumulation of ROS is counterbalanced by antioxidant molecules, thereby preventing excessive damage to the host. The ability of Zn to inhibit oxidative processes and reduce ROS formation is well established (Puertollano et al. 2011). Although Zn is not considered a direct antioxidant molecule, it possesses antioxidant properties through its ability to protect protein sulfhydryl groups or reduce hydroxide formation from H_2O_2 through the antagonism of redox-active transition metals, such as iron and copper (Powell 2000). Zn sequestration from eukaryotic cells or subcellular organelles results in the increased production of ROS. For example, Zn deprivation from phagosomes in activated macrophages elevated intraphagosomal ROS generation by NADPH oxidase and enhanced the clearance of the intracellular pathogen *Histoplasma capsulatum* (Subramanian Vignesh et al. 2013). In a clinical trial involving the impact of Zn supplementation in healthy elderly subjects, those taking supplemental Zn exhibited a higher plasma antioxidant index (represented by ascorbate equivalent units) with a corresponding decrease in oxidative stress markers, as well as the cytokine concentrations in plasma, including CRP, IL-6, macrophage chemo-attractant protein 1 (MCP-1), and vascular endothelial cell adhesion molecule 1 (VCAM-1) (Bao et al. 2010a). Based on these findings and many others, the role of Zn as an antioxidant has great potential to prevent or treat inflammatory-based diseases and warrants considerably more study.

11.7 Zn Signals in Immune Cells

Zn deficiency compromises the function of immune cells that are directly involved in regulating the extent of inflammation, including leukocytes (Haase and Rink 2009). It is also important to recognize that Zn deficiency also alters the phenotype of nonimmune cell types (for example, epithelia, enodothelia, hepatocytes, and fibroblasts) that are also integral components of the inflammatory response. Zn

deficiency alters the function of multiple immune cells, including monocytes, neutrophil granulocytes, natural killer (NK), and T- and B cells, and therefore Zn is critical to the function of both the innate and adaptive immune systems. It is important to recognize that Zn signaling and trafficking are highly dependent on the cell type and the specific environment in which cell activation occurs. In recent years, new data have emerged that have increasingly proven that Zn transporters have very specific functions within cells that comprise both the innate and adaptive immune systems. In the setting of inflammation, there remains much to be known when considering the similarities and differences in Zn metabolism that exist between different immune cell types.

As one example, Zn supplementation has been shown to inhibit the secretion of pro-inflammatory cytokines by primary human monocytes in response to stimulation with LPS (von Bulow et al. 2005). Similarly, Zn protects animals from LPS-induced liver injury (Zhou et al. 2004). Diverse results have been observed in other cell types whereby LPS-mediated TLR4 stimulation of dendritic cells decreased cytosolic free Zn through the suppression of ZIP6. The downmodulation of intracellular labile Zn was an instrumental component of dendritic cell activation. Accordingly, Zn supplementation or overexpression of the Zn transporter ZIP6 inhibited LPS-induced upregulation of major histocompatibility complex class II and costimulatory molecules (Kitamura et al. 2006). In T cells, it was revealed that ZIP8 possesses alternative functions when compared to monocytes and macrophages. In particular, T-cell ($CD3^+$) activation upregulated ZIP8, which then translocated to the lysosome membrane, promoting labile Zn release, which resulted in the increase of IFN-γ production (Aydemir et al. 2009). TCR activation in $CD4^+$ T cells is also known to induce Zn influx through ZIP6, resulting in augmentation of TCR signaling (Yu et al. 2011). In mast cells, FcR-mediated activation induced a burst of free Zn in the cytosol, which then enhanced cytokine secretion (Yamasaki et al. 2007); and in macrophages, a robust increase of free Zn following release from MT during *Mycobacterium tuberculosis* infection resulted in Zn accumulation within the mycobacterial phagosome to presumably toxify this compartment to prevent bacterial growth (Botella et al. 2011). Similarly, recent advances that involve Zn function in macrophages in response to the fungus *H. capsulatum* have revealed the importance of intracellular Zn trafficking in this setting. In particular, Zn trafficking and sequestration are regulated by the Zn transporters *Slc30a4* (ZnT4) and *Slc30a7* (ZnT7) within the Golgi apparatus (Subramanian Vignesh et al. 2013). Fewer studies have examined the sustained effects of Zn or Zn deficiency in vivo. In a ground-breaking study conducted years ago it was revealed that the thymus is the most sensitive primary tissue to chronic Zn deficiency. By day 50 of suboptimal Zn intake, the thymus had atrophied by 36 % with significant loss of pre-T cells via apoptosis such that T-cell lymphopoiesis was disrupted. Significant reductions were also noted in the erythropoietic population by day 50. Conversely, the marrow maintained myelopoiesis and B cell lymphopoiesis during this time. Importantly, the changes observed in gene expression for cytokines, DNA repair enzymes, Zn transporters, and signaling

molecules indicated that cells of the immune system attempt to adapt to the stress of suboptimal Zn (Fraker and King 2004; King et al. 2005).

From this constellation of observations, a clearer picture has emerged that supports the concept that Zn trafficking and metabolism are unique to specific immune cell types, guided by specific Zn transporters reserved for specialized function. Further, Zn-mediated responses to diverse danger signals that provoke the primordial inflammatory response occur through distinct mechanisms which are not shared by all cell types. In other words, Zn metabolism, in response to danger signals, does not adhere to a "one size fits all" policy but rather is customized to accommodate unique circumstances under a variety of conditions, presumably to better serve the host.

11.8 Concluding Remarks

Exciting advances in the field of Zn biology have substantially improved our understanding of the role of Zn and its metabolism with respect to immune function and the inflammatory response to danger signals. Clearly, Zn is absolutely required to support normal immune function. Changes in Zn concentration, particularly suboptimal concentrations, have profound influence on how the host responds to danger signals that provoke inflammation through activation of the innate immune system. Similarly, substantial progress has been made in demonstrating that Zn supplementation, particularly within Zn-deficient populations, improves immune function and helps to prevent the incidence and extent of inflammatory-based disease. For many years it was not clear exactly how Zn mediates its beneficial effects; however, it is now becoming much better defined that adequate amounts of Zn are required to help "buffer" the host inflammatory response in virtually all cells involved in immune function. When sufficient amounts of Zn are present, the host response is optimal and danger is minimized, affording a greater chance for full recovery from the insult. When Zn content within and outside of cells is insufficient, alteration of the host response occurs through numerous changes in vital cell-signaling pathways. These effects are in large part the result of changes in Zn metabolism, via transporters, which subsequently alter the manner by which Zn can directly interact with target proteins that alter cellular phenotype. The impact of these changes can be quite substantial because these subtle molecular events influence virtually all the most fundamentally important cellular processes, which include transcription, translation, replication, and cellular repair. Importantly, prolonged Zn deficiency, caused by insufficient intake or inherent deficits in Zn metabolism, alters the acute inflammatory response but also creates an environment that prevents normal repair, thereby paving a pathway for chronic inflammation. Multiple examples whereby Zn deficiency perpetuates a viscous cycle of unresolved inflammation leading to chronic disease states have now emerged. The implications of these consequences are enormous when considering that Zn deficiency is highly frequent within undeveloped and developed countries.

Continued advances in state-of the art technologies will help to further establish the role of Zn in inflammatory-based disease. Clearly, the mosaic created so far has revealed that Zn metabolism in mammals is highly sophisticated. Continued effort must be placed on translational studies that bridge basic and clinical science so that fundamental discoveries can have meaningful impact for patients who may benefit from Zn supplementation. The cost of Zn treatment is highly affordable, and its safety profile is very favorable. Based on this, one can envision an expansion of Zn supplementation strategies whereby we prevent the incidence of acute and chronic inflammatory diseases across a broader range of susceptible populations.

References

Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M (2005) IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med 11(2):191–198. doi:10.1038/nm1185

Aydemir TB, Liuzzi JP, McClellan S, Cousins RJ (2009) Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells. J Leukoc Biol 86(2):337–348. doi:10.1189/jlb.1208759

Bao B, Prasad AS, Beck FW, Fitzgerald JT, Snell D, Bao GW, Singh T, Cardozo LJ (2010a) Zinc decreases C-reactive protein, lipid peroxidation, and inflammatory cytokines in elderly subjects: a potential implication of zinc as an atheroprotective agent. Am J Clin Nutr 91(6):1634–1641. doi:10.3945/ajcn.2009.28836

Bao S, Liu MJ, Lee B, Besecker B, Lai JP, Guttridge DC, Knoell DL (2010b) Zinc modulates the innate immune response in vivo to polymicrobial sepsis through regulation of NF-kappaB. Am J Physiol Lung Cell Mol Physiol 298(6):L744–L754. doi:10.1152/ajplung.00368.2009

Beker Aydemir T, Chang SM, Guthrie GJ, Maki AB, Ryu MS, Karabiyik A, Cousins RJ (2012) Zinc transporter ZIP14 functions in hepatic zinc, iron and glucose homeostasis during the innate immune response (endotoxemia). PLoS One 7(10):e48679. doi:10.1371/journal.pone.0048679

Berrington de Gonzalez A, Hartge P, Cerhan JR, Flint AJ, Hannan L, MacInnis RJ, Moore SC, Tobias GS, Anton-Culver H, Freeman LB, Beeson WL, Clipp SL, English DR, Folsom AR, Freedman DM, Giles G, Hakansson N, Henderson KD, Hoffman-Bolton J, Hoppin JA, Koenig KL, Lee IM, Linet MS, Park Y, Pocobelli G, Schatzkin A, Sesso HD, Weiderpass E, Willcox BJ, Wolk A, Zeleniuch-Jacquotte A, Willett WC, Thun MJ (2010) Body-mass index and mortality among 1.46 million white adults. N Engl J Med 363(23):2211–2219. doi:10.1056/NEJMoa1000367

Besecker B, Bao S, Bohacova B, Papp A, Sadee W, Knoell DL (2008) The human zinc transporter SLC39A8 (Zip8) is critical in zinc-mediated cytoprotection in lung epithelia. Am J Physiol Lung Cell Mol Physiol 294(6):L1127–L1136. doi:10.1152/ajplung.00057.2008

Besecker BY, Exline MC, Hollyfield J, Phillips G, Disilvestro RA, Wewers MD, Knoell DL (2011) A comparison of zinc metabolism, inflammation, and disease severity in critically ill infected and noninfected adults early after intensive care unit admission. Am J Clin Nutr 93(6):1356–1364. doi:10.3945/ajcn.110.008417

Bhatnagar S, Wadhwa N, Aneja S, Lodha R, Kabra SK, Natchu UC, Sommerfelt H, Dutta AK, Chandra J, Rath B, Sharma M, Sharma VK, Kumari M, Strand TA (2012) Zinc as adjunct treatment in infants aged between 7 and 120 days with probable serious bacterial infection: a randomised, double-blind, placebo-controlled trial. Lancet 379(9831):2072–2078. doi:10.1016/S0140-6736(12)60477-2

Black RE (2003) Zinc deficiency, infectious disease and mortality in the developing world. J Nutr 133(5 suppl 1):1485S–1489S

Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, Tsui C, Hurley P, Chien M, Chai S, Hitotsumatsu O, McNally E, Pickart C, Ma A (2004) The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol 5(10):1052–1060. doi:10.1038/ni1110

Botella H, Peyron P, Levillain F, Poincloux R, Poquet Y, Brandli I, Wang C, Tailleux L, Tilleul S, Charriere GM, Waddell SJ, Foti M, Lugo-Villarino G, Gao Q, Maridonneau-Parini I, Butcher PD, Castagnoli PR, Gicquel B, de Chastellier C, Neyrolles O (2011) Mycobacterial p(1)-type ATPases mediate resistance to zinc poisoning in human macrophages. Cell Host Microbe 10 (3):248–259. doi:10.1016/j.chom.2011.08.006

Brieger A, Rink L, Haase H (2013) Differential regulation of TLR-dependent MyD88 and TRIF signaling pathways by free zinc ions. J Immunol 191(4):1808–1817. doi:10.4049/jimmunol.1301261

Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, Tschopp J (2003) Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J Exp Med 197(2):263–268

Carcillo JA, Dean JM, Holubkov R, Berger J, Meert KL, Anand KJ, Zimmerman J, Newth CJ, Harrison R, Burr J, Willson DF, Nicholson C (2012) The randomized comparative pediatric critical illness stress-induced immune suppression (CRISIS) prevention trial. Pediatr Crit Care Med 13(2):165–173. doi:10.1097/PCC.0b013e31823896ae

Caulfield LE, de Onis M, Blossner M, Black RE (2004) Undernutrition as an underlying cause of child deaths associated with diarrhea, pneumonia, malaria, and measles. Am J Clin Nutr 80 (1):193–198

Chawla A, Nguyen KD, Goh YP (2011) Macrophage-mediated inflammation in metabolic disease. Nat Rev Immunol 11(11):738–749. doi:10.1038/nri3071

Chiao PJ, Miyamoto S, Verma IM (1994) Autoregulation of I kappa B alpha activity. Proc Natl Acad Sci USA 91(1):28–32

Cornell TT, Wynn J, Shanley TP, Wheeler DS, Wong HR (2010) Mechanisms and regulation of the gene-expression response to sepsis. Pediatrics 125(6):1248–1258. doi:10.1542/peds.2009-3274

Costarelli L, Muti E, Malavolta M, Cipriano C, Giacconi R, Tesei S, Piacenza F, Pierpaoli S, Gasparini N, Faloia E, Tirabassi G, Boscaro M, Polito A, Mauro B, Maiani F, Raguzzini A, Marcellini F, Giuli C, Papa R, Emanuelli M, Lattanzio F, Mocchegiani E (2010) Distinctive modulation of inflammatory and metabolic parameters in relation to zinc nutritional status in adult overweight/obese subjects. J Nutr Biochem 21(5):432–437. doi:10.1016/j.jnutbio.2009.02.001

Cvijanovich N, Shanley TP, Lin R, Allen GL, Thomas NJ, Checchia P, Anas N, Freishtat RJ, Monaco M, Odoms K, Sakthivel B, Wong HR (2008) Validating the genomic signature of pediatric septic shock. Physiol Genomics 34(1):127–134. doi:10.1152/physiolgenomics.00025.2008

Cvijanovich NZ, King JC, Flori HR, Gildengorin G, Wong HR (2009) Zinc homeostasis in pediatric critical illness. Pediatr Crit Care Med 10(1):29–34. doi:10.1097/PCC.0b013e31819371ce

Eby GA, Davis DR, Halcomb WW (1984) Reduction in duration of common colds by zinc gluconate lozenges in a double-blind study. Antimicrob Agents Chemother 25(1):20–24

Fang FC (1997) Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest 99(12):2818–2825. doi:10.1172/JCI119473

Ferrante AW Jr (2007) Obesity-induced inflammation: a metabolic dialogue in the language of inflammation. J Intern Med 262(4):408–414. doi:10.1111/j.1365-2796.2007.01852.x

Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, Singh GM, Gutierrez HR, Lu Y, Bahalim AN, Farzadfar F, Riley LM, Ezzati M (2011) National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and

epidemiological studies with 960 country-years and 9.1 million participants. Lancet 377 (9765):557–567. doi:10.1016/S0140-6736(10)62037-5
Flegal KM, Carroll MD, Ogden CL, Curtin LR (2010) Prevalence and trends in obesity among US adults, 1999–2008. JAMA 303(3):235–241. doi:10.1001/jama.2009.2014
Fraker PJ, King LE (2004) Reprogramming of the immune system during zinc deficiency. Annu Rev Nutr 24:277–298. doi:10.1146/annurev.nutr.24.012003.132454
Gaetke LM, McClain CJ, Talwalkar RT, Shedlofsky SI (1997) Effects of endotoxin on zinc metabolism in human volunteers. Am J Physiol 272(6 pt 1):E952–E956
Galvez-Peralta M, He L, Jorge-Nebert LF, Wang B, Miller ML, Eppert BL, Afton S, Nebert DW (2012) ZIP8 zinc transporter: indispensable role for both multiple-organ organogenesis and hematopoiesis in utero. PLoS One 7(5):e36055. doi:10.1371/journal.pone.0036055
Garcia OP, Long KZ, Rosado JL (2009) Impact of micronutrient deficiencies on obesity. Nutr Rev 67(10):559–572. doi:10.1111/j.1753-4887.2009.00228.x
Girijashanker K, He L, Soleimani M, Reed JM, Li H, Liu Z, Wang B, Dalton TP, Nebert DW (2008) Slc39a14 gene encodes ZIP14, a metal/bicarbonate symporter: similarities to the ZIP8 transporter. Mol Pharmacol 73(5):1413–1423. doi:10.1124/mol.107.043588
Griffin WS (2013) Neuroinflammatory cytokine signaling and Alzheimer's disease. N Engl J Med 368(8):770–771. doi:10.1056/NEJMcibr1214546
Haase H, Maret W (2003) Intracellular zinc fluctuations modulate protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Exp Cell Res 291(2):289–298
Haase H, Rink L (2007) Signal transduction in monocytes: the role of zinc ions. Biometals 20 (3-4):579–585. doi:10.1007/s10534-006-9029-8
Haase H, Rink L (2009) Functional significance of zinc-related signaling pathways in immune cells. Annu Rev Nutr 29:133–152. doi:10.1146/annurev-nutr-080508-141119
Haase H, Ober-Blobaum JL, Engelhardt G, Hebel S, Heit A, Heine H, Rink L (2008) Zinc signals are essential for lipopolysaccharide-induced signal transduction in monocytes. J Immunol 181 (9):6491–6502
Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132(3):344–362. doi:10.1016/j.cell.2008.01.020
Heyland DK, Jones N, Cvijanovich NZ, Wong H (2008) Zinc supplementation in critically ill patients: a key pharmaconutrient? JPEN J Parenter Enteral Nutr 32(5):509–519. doi:10.1177/0148607108322402
Hotamisligil GS (2006) Inflammation and metabolic disorders. Nature (Lond) 444(7121):860–867. doi:10.1038/nature05485
Jansen J, Karges W, Rink L (2009) Zinc and diabetes–clinical links and molecular mechanisms. J Nutr Biochem 20(6):399–417. doi:10.1016/j.jnutbio.2009.01.009
Jeon KI, Jeong JY, Jue DM (2000) Thiol-reactive metal compounds inhibit NF-kappa B activation by blocking I kappa B kinase. J Immunol 164(11):5981–5989
Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298(5600):1911–1912. doi:10.1126/science.1072682
Joshi PC, Mehta A, Jabber WS, Fan X, Guidot DM (2009) Zinc deficiency mediates alcohol-induced alveolar epithelial and macrophage dysfunction in rats. Am J Respir Cell Mol Biol 41 (2):207–216. doi:10.1165/rcmb.2008-0209OC
Kanoni S, Dedoussis GV, Herbein G, Fulop T, Varin A, Jajte J, Rink L, Monti D, Mariani E, Malavolta M, Giacconi R, Marcellini F, Mocchegiani E (2010) Assessment of gene-nutrient interactions on inflammatory status of the elderly with the use of a zinc diet score: ZINCAGE study. J Nutr Biochem 21(6):526–531. doi:10.1016/j.jnutbio.2009.02.011
Kapaki E, Segditsa J, Papageorgiou C (1989) Zinc, copper and magnesium concentration in serum and CSF of patients with neurological disorders. Acta Neurol Scand 79(5):373–378
Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11(5):373–384. doi:10.1038/ni.1863
Kehl-Fie TE, Skaar EP (2010) Nutritional immunity beyond iron: a role for manganese and zinc. Curr Opin Chem Biol 14(2):218–224. doi:10.1016/j.cbpa.2009.11.008

King LE, Frentzel JW, Mann JJ, Fraker PJ (2005) Chronic zinc deficiency in mice disrupted T cell lymphopoiesis and erythropoiesis while B cell lymphopoiesis and myelopoiesis were maintained. J Am Coll Nutr 24(6):494–502

Kitamura H, Morikawa H, Kamon H, Iguchi M, Hojyo S, Fukada T, Yamashita S, Kaisho T, Akira S, Murakami M, Hirano T (2006) Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nat Immunol 7(9):971–977. doi:10.1038/ni1373

Knoell DL, Liu MJ (2010) Impact of zinc metabolism on innate immune function in the setting of sepsis. Int J Vitam Nutr Res 80(4-5):271–277. doi:10.1024/0300-9831/a000034

Knoell DL, Julian MW, Bao S, Besecker B, Macre JE, Leikauf GD, DiSilvestro RA, Crouser ED (2009) Zinc deficiency increases organ damage and mortality in a murine model of polymicrobial sepsis. Crit Care Med 37(4):1380–1388. doi:10.1097/CCM.0b013e31819cefe4

Ko WS, Guo CH, Hsu GS, Chiou YL, Yeh MS, Yaun SR (2005) The effect of zinc supplementation on the treatment of chronic hepatitis C patients with interferon and ribavirin. Clin Biochem 38(7):614–620. doi:10.1016/j.clinbiochem.2005.04.003

Kobayashi K, Hernandez LD, Galan JE, Janeway CA Jr, Medzhitov R, Flavell RA (2002) IRAK-M is a negative regulator of Toll-like receptor signaling. Cell 110(2):191–202

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(3):239–240. doi:10.1038/ng913

Laity JH, Andrews GK (2007) Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). Arch Biochem Biophys 463(2):201–210. doi:10.1016/j.abb.2007.03.019

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr 29:153–176. doi:10.1146/annurev-nutr-033009-083312

Liew FY, Xu D, Brint EK, O'Neill LA (2005) Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 5(6):446–458. doi:10.1038/nri1630

Liu M-J, Bao S, Bolin ER, Burris DL, Xu X, Sun Q, Killilea DW, Shen Q, Ziouzenkova O, Belury MA (2013a) Zinc deficiency augments leptin production and exacerbates macrophage infiltration into adipose tissue in mice fed a high-fat diet. J Nutr 143(7):1036–1045

Liu MJ, Bao S, Galvez-Peralta M, Pyle CJ, Rudawsky AC, Pavlovicz RE, Killilea DW, Li C, Nebert DW, Wewers MD, Knoell DL (2013b) ZIP8 regulates host defense through zinc-mediated inhibition of NF-kappaB. Cell Rep 3(2):386–400. doi:10.1016/j.celrep.2013.01.009

Liuzzi JP, Lichten LA, Rivera S, Blanchard RK, Aydemir TB, Knutson MD, Ganz T, Cousins RJ (2005) Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc Natl Acad Sci USA 102(19):6843–6848. doi:10.1073/pnas.0502257102

Lumeng CN, Saltiel AR (2011) Inflammatory links between obesity and metabolic disease. J Clin Invest 121(6):2111–2117. doi:10.1172/JCI57132

Maret W (2006) Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxid Redox Signal 8(9–10):1419–1441. doi:10.1089/ars.2006.8.1419

Maylor EA, Simpson EE, Secker DL, Meunier N, Andriollo-Sanchez M, Polito A, Stewart-Knox B, McConville C, O'Connor JM, Coudray C (2006) Effects of zinc supplementation on cognitive function in healthy middle-aged and older adults: the ZENITH study. Br J Nutr 96 (4):752–760

Mehta AJ, Yeligar SM, Elon L, Brown LA, Guidot DM (2013) Alcoholism causes alveolar macrophage zinc deficiency and immune dysfunction. Am J Respir Crit Care Med 188 (6):716–723. doi:10.1164/rccm.201301-0061OC

Mocchegiani E, Costarelli L, Giacconi R, Piacenza F, Basso A, Malavolta M (2012) Micronutrient (Zn, Cu, Fe)–gene interactions in ageing and inflammatory age-related diseases: implications for treatments. Ageing Res Rev 11(2):297–319. doi:10.1016/j.arr.2012.01.004

Mokdad AH, Serdula MK, Dietz WH, Bowman BA, Marks JS, Koplan JP (2000) The continuing epidemic of obesity in the United States. JAMA 284(13):1650–1651

Molina JA, Jimenez-Jimenez FJ, Aguilar MV, Meseguer I, Mateos-Vega CJ, Gonzalez-Munoz MJ, de Bustos F, Porta J, Orti-Pareja M, Zurdo M, Barrios E, Martinez-Para MC (1998) Cerebrospinal fluid levels of transition metals in patients with Alzheimer's disease. J Neural Transm 105(4-5):479–488

Nishida K, Hasegawa A, Nakae S, Oboki K, Saito H, Yamasaki S, Hirano T (2009) Zinc transporter Znt5/Slc30a5 is required for the mast cell-mediated delayed-type allergic reaction but not the immediate-type reaction. J Exp Med 206(6):1351–1364. doi:10.1084/jem.20082533

Nowak JE, Harmon K, Caldwell CC, Wong HR (2012) Prophylactic zinc supplementation reduces bacterial load and improves survival in a murine model of sepsis. Pediatr Crit Care Med 13(5): e323–e329. doi:10.1097/PCC.0b013e31824fbd90

Ouchi N, Parker JL, Lugus JJ, Walsh K (2011) Adipokines in inflammation and metabolic disease. Nat Rev Immunol 11(2):85–97. doi:10.1038/nri2921

Powell SR (2000) The antioxidant properties of zinc. J Nutr 130(5S suppl):1447S–1454S

Prasad AS, Miale A Jr, Farid Z, Sandstead HH, Schulert AR (1963) Zinc metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism, and hypognadism. J Lab Clin Med 61:537–549

Prasad AS, Fitzgerald JT, Bao B, Beck FW, Chandrasekar PH (2000) Duration of symptoms and plasma cytokine levels in patients with the common cold treated with zinc acetate. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 133(4):245–252

Prasad AS, Beck FW, Bao B, Snell D, Fitzgerald JT (2008) Duration and severity of symptoms and levels of plasma interleukin-1 receptor antagonist, soluble tumor necrosis factor receptor, and adhesion molecules in patients with common cold treated with zinc acetate. J Infect Dis 197 (6):795–802. doi:10.1086/528803

Prasad AS, Bao B, Beck FW, Sarkar FH (2011) Zinc-suppressed inflammatory cytokines by induction of A20-mediated inhibition of nuclear factor-kappaB. Nutrition 27(7-8):816–823. doi:10.1016/j.nut.2010.08.010

Puertollano MA, Puertollano E, de Cienfuegos GA, de Pablo MA (2011) Dietary antioxidants: immunity and host defense. Curr Top Med Chem 11(14):1752–1766

Ruland J (2011) Return to homeostasis: downregulation of NF-kappaB responses. Nat Immunol 12 (8):709–714. doi:10.1038/ni.2055

Shanley TP, Cvijanovich N, Lin R, Allen GL, Thomas NJ, Doctor A, Kalyanaraman M, Tofil NM, Penfil S, Monaco M, Odoms K, Barnes M, Sakthivel B, Aronow BJ, Wong HR (2007) Genome-level longitudinal expression of signaling pathways and gene networks in pediatric septic shock. Mol Med 13(9-10):495–508. doi:10.2119/2007-00065.Shanley

Singh M, Das RR (2011) Zinc for the common cold. Cochrane Database Syst Rev 2, CD001364. doi:10.1002/14651858.CD001364.pub3

Srinivasan MG, Ndeezi G, Mboijana CK, Kiguli S, Bimenya GS, Nankabirwa V, Tumwine JK (2012) Zinc adjunct therapy reduces case fatality in severe childhood pneumonia: a randomized double blind placebo-controlled trial. BMC Med 10:14. doi:10.1186/1741-7015-10-14

Subramanian Vignesh K, Landero Figueroa JA, Porollo A, Caruso JA, Deepe GS Jr (2013) Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival. Immunity 39(4):697–710. doi:10.1016/j.immuni.2013.09.006

Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, Aoe S, Kamei Y, Ogawa Y (2007) Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol 27(1):84–91. doi:10.1161/01.ATV.0000251608.09329.9a

Takagi H, Nagamine T, Abe T, Takayama H, Sato K, Otsuka T, Kakizaki S, Hashimoto Y, Matsumoto T, Kojima A, Takezawa J, Suzuki K, Sato S, Mori M (2001) Zinc supplementation enhances the response to interferon therapy in patients with chronic hepatitis C. J Viral Hepat 8 (5):367–371

Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. Cell 140(6):805–820. doi:10.1016/j.cell.2010.01.022

Tamaki M, Fujitani Y, Hara A, Uchida T, Tamura Y, Takeno K, Kawaguchi M, Watanabe T, Ogihara T, Fukunaka A, Shimizu T, Mita T, Kanazawa A, Imaizumi MO, Abe T, Kiyonari H, Hojyo S, Fukada T, Kawauchi T, Nagamatsu S, Hirano T, Kawamori R, Watada H (2013) The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. J Clin Invest 123(10):4513–4524. doi:10.1172/JCI68807

Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P (2012) Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal 5(210): ra11. doi:10.1126/scisignal.2002585

Vasto S, Candore G, Listi F, Balistreri CR, Colonna-Romano G, Malavolta M, Lio D, Nuzzo D, Mocchegiani E, Di Bona D, Caruso C (2008) Inflammation, genes and zinc in Alzheimer's disease. Brain Res Rev 58(1):96–105. doi:10.1016/j.brainresrev.2007.12.001

von Bulow V, Rink L, Haase H (2005) Zinc-mediated inhibition of cyclic nucleotide phosphodiesterase activity and expression suppresses TNF-alpha and IL-1 beta production in monocytes by elevation of guanosine 3′,5′-cyclic monophosphate. J Immunol 175(7):4697–4705

von Bulow V, Dubben S, Engelhardt G, Hebel S, Plumakers B, Heine H, Rink L, Haase H (2007) Zinc-dependent suppression of TNF-alpha production is mediated by protein kinase A-induced inhibition of Raf-1, I kappa B kinase beta, and NF-kappa B. J Immunol 179(6):4180–4186

Voruganti VS, Cai G, Klohe DM, Jordan KC, Lane MA, Freeland-Graves JH (2010) Short-term weight loss in overweight/obese low-income women improves plasma zinc and metabolic syndrome risk factors. J Trace Elem Med Biol. doi:10.1016/j.jtemb.2010.05.001

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr (2003) Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112(12):1796–1808. doi:10.1172/JCI19246

Wilcock DM, Griffin WS (2013) Down's syndrome, neuroinflammation, and Alzheimer neuropathogenesis. J Neuroinflamm 10:84. doi:10.1186/1742-2094-10-84

Wong CP, Ho E (2012) Zinc and its role in age-related inflammation and immune dysfunction. Mol Nutr Food Res 56(1):77–87. doi:10.1002/mnfr.201100511

Wong HR, Shanley TP, Sakthivel B, Cvijanovich N, Lin R, Allen GL, Thomas NJ, Doctor A, Kalyanaraman M, Tofil NM, Penfil S, Monaco M, Tagavilla MA, Odoms K, Dunsmore K, Barnes M, Aronow BJ (2007) Genome-level expression profiles in pediatric septic shock indicate a role for altered zinc homeostasis in poor outcome. Physiol Genomics 30(2):146–155. doi:10.1152/physiolgenomics.00024.2007

Wong HR, Freishtat RJ, Monaco M, Odoms K, Shanley TP (2010) Leukocyte subset-derived genomewide expression profiles in pediatric septic shock. Pediatr Crit Care Med 11(3):349–355. doi:10.1097/PCC.0b013e3181c519b4

Wynn J, Cornell TT, Wong HR, Shanley TP, Wheeler DS (2010) The host response to sepsis and developmental impact. Pediatrics 125(5):1031–1041. doi:10.1542/peds.2009-3301

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112(12):1821–1830. doi:10.1172/JCI19451

Yakoob MY, Theodoratou E, Jabeen A, Imdad A, Eisele TP, Ferguson J, Jhass A, Rudan I, Campbell H, Black RE, Bhutta ZA (2011) Preventive zinc supplementation in developing countries: impact on mortality and morbidity due to diarrhea, pneumonia and malaria. BMC Public Health 11(suppl 3):S23. doi:10.1186/1471-2458-11-S3-S23

Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, Kurosaki T, Yamashita S, Tokunaga M, Nishida K, Hirano T (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177(4):637–645. doi:10.1083/jcb.200702081

Yin MJ, Yamamoto Y, Gaynor RB (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature (Lond) 396(6706):77–80. doi:10.1038/23948

Yu M, Lee WW, Tomar D, Pryshchep S, Czesnikiewicz-Guzik M, Lamar DL, Li G, Singh K, Tian L, Weyand CM, Goronzy JJ (2011) Regulation of T cell receptor signaling by activation-induced zinc influx. J Exp Med 208(4):775–785. doi:10.1084/jem.20100031

Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of IKKbeta. Science 293(5535):1673–1677. doi:10.1126/science.1061620

Zheng W, McLerran DF, Rolland B, Zhang X, Inoue M, Matsuo K, He J, Gupta PC, Ramadas K, Tsugane S, Irie F, Tamakoshi A, Gao YT, Wang R, Shu XO, Tsuji I, Kuriyama S, Tanaka H, Satoh H, Chen CJ, Yuan JM, Yoo KY, Ahsan H, Pan WH, Gu D, Pednekar MS, Sauvaget C, Sasazuki S, Sairenchi T, Yang G, Xiang YB, Nagai M, Suzuki T, Nishino Y, You SL, Koh WP, Park SK, Chen Y, Shen CY, Thornquist M, Feng Z, Kang D, Boffetta P, Potter JD (2011) Association between body-mass index and risk of death in more than 1 million Asians. N Engl J Med 364(8):719–729. doi:10.1056/NEJMoa1010679

Zhou Z, Wang L, Song Z, Saari JT, McClain CJ, Kang YJ (2004) Abrogation of nuclear factor-kappaB activation is involved in zinc inhibition of lipopolysaccharide-induced tumor necrosis factor-alpha production and liver injury. Am J Pathol 164(5):1547–1556

Chapter 12
Zinc Signal in Growth Control and Bone Diseases

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Abstract Zinc (Zn) deficiency syndrome, discovered only half a century ago, first showed the physiological significance of this trace mineral. More recent studies have shown that a disturbance in Zn homeostasis in mammals is primarily characterized by growth retardation, accompanied by impaired bone metabolism, and Zn is now recognized as an indispensable metal required for systemic growth. Zn homeostasis is controlled by a family of Zn transporters, transmembrane proteins that regulate the intracellular availability of Zn. Recent studies using Zn transporter knockout mice and human genetic data have revealed a novel role for Zn as a signaling factor that affects multiple cellular processes, impacting numerous biological and physiological functions. These studies have also shed light on the mechanisms by which Zn regulates mammalian systemic growth. In this chapter, we present an overview of the roles of Zn and Zn transporters in regulating systemic growth and bone metabolism, focusing on the role of Zn as a signaling molecule that fine-tunes multiple intracellular signaling pathways.

Keywords Bone • Ehlers–Danlos syndrome • Growth • Osteogenesis imperfecta • Signal transduction • Transporter • Zinc

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12.1 Zinc and Systemic Growth

Zinc (Zn) is an essential micronutrient that is indispensable for the cellular activities of all organisms (Broadley et al. 2007; Prasad 2008; Sugarman 1983). The total human body content of Zn, 2 to 4 g (Rink and Gabriel 2000), is primarily located in the bone, muscle, brain, and liver (Wapnir and Chemical Rubber Company 1990), where it supports the structural conformation and catalytic functions of enzymes and transcription factors. Recent in silico studies predicted that approximately 2,800 proteins (corresponding to 10 % of the human proteome) are potential Zn-binding proteins (Andreini et al. 2006), consistent with an important role for Zn in human biological functions. Zn deficiency occurs when Zn levels are insufficient to meet the physiological requirements of organisms, resulting in their abnormal growth. An estimated 2 billion people in the developing world suffer from various diseases associated with Zn deficiency (Prasad 1995). In children, it causes growth retardation, delayed sexual maturation, increased susceptibility to infection, and diarrhea, resulting in the death of approximately 800,000 children per year in the world (Hambidge and Krebs 2007).

Somatic growth is primarily regulated by the endocrine factors growth hormone (GH) and insulin-like growth factor-I (IGF-I), whose main sources are the pituitary gland and liver, respectively (MacDonald 2000; Nilsson et al. 2005; Procter et al. 1998). Circulating GH stimulates the liver to produce IGF-I, which in turn binds to the IGF receptor (IGFR) to promote somatogenic cell proliferation via IGFR-mediated tyrosine kinase activation (De Meyts et al. 1994). GH and IGF-I also regulate longitudinal bone growth by controlling the endochondral ossification process, a well-organized sequence of events involving chondrocyte differentiation at the growth plate (Kronenberg 2003). Thus, the GH–IGF axis plays a critical role in systemic growth.

Zn deficiency causes a marked reduction in circulating GH and IGF-I concentrations (MacDonald 2000; Roth and Kirchgessner 1994; Underwood 1996), leading to dwarfism, shortened growth plate width (Rossi et al. 2001), and decreased food intake (Giugliano and Millward 1984). The pituitary gland has a relatively high Zn content, which has been shown to promote hormone function (Henkin 1976). Several studies have demonstrated that Zn deficiency leads to impaired GH secretion, resulting in low concentrations of circulating GH (Root et al. 1979; Roth and Kirchgessner 1994). Interestingly, when Zn-deprived rats are treated with exogenous GH, the stunted growth is not corrected despite the increased circulating levels of GH, whereas Zn treatment effectively reverses the growth impairment (Prasad et al. 1969). Similar findings are obtained in Zn-deficient animals treated with IGF-I, in which the growth retardation phenotype persists despite increased serum IGF-I levels (MacDonald 2000). Intriguingly, Zn deficiency leads to reduced food intake, suggesting that inadequate dietary protein or energy intake could contribute to the reduced growth. However, the decreased levels of IGF-I in Zn-deficient rats are not overcome with a high protein-containing diet but are corrected with a low-protein plus Zn-containing diet (Cossack 1986). Furthermore,

force-feeding rats a Zn-depleted diet results in decreased circulating IGF-I levels, whereas a Zn-adequate diet containing a similar level of dietary energy intake promotes normal growth (Roth and Kirchgessner 1994). Moreover, in humans, Zn deficiency-induced serum IGF-I reduction was shown to occur independently of the total energy intake (Cossack 1991). Thus, Zn deficiency affects circulating GH and IGF levels, although the administration of exogenous GH or IGF-I does not correct Zn deficiency-associated growth defects.

The GH–IGF axis is also known to positively regulate bone growth (Ohlsson et al. 1998). Similar results to those just described were observed in the bone growth of Zn-deficient rats (Cha and Rojhani 1997). Hypophysectomized Zn-deficient rats implanted with GH-containing miniosmotic pumps in the hindlimb were found to be hyporesponsive to exogenous GH, resulting in poor growth of the tibial epiphyseal cartilage. This finding indicated that the impaired bone growth in Zn-deficient rats is resistant to GH treatment, and was consistent with an earlier report indicating that Zn-deficient rats are hyporesponsive to GH, resulting in reduced levels of circulating IGF-I (Oner et al. 1984). Thus, it is likely that the failure of GH to stimulate somatic growth under Zn-deficient conditions is caused by the limited availability of intracellular Zn, which is independent of the GH-IGF status. Given that Zn increases IGF-I synthesis (Yamaguchi and Hashizume 1994) and potentiates IGF-I-mediated actions (Matsui and Yamaguchi 1995), these findings collectively suggest that intracellular Zn affects the hormone- or growth factor-mediated signaling cascade(s) required for somatic growth.

12.2 Zn Transporters in Growth and Bone Homeostasis

Zn homeostasis is tightly controlled by the coordinated actions of Zn transporters and metallothioneins (Fig. 12.1) (Fukada and Kambe 2011; Gaither and Eide 2001; Kambe et al. 2004). Mammalian Zn transporters are divided into two major families, SLC39s/ZIPs and SLC30s/ZnTs, which have opposing roles in the regulation of intracellular Zn levels. ZIPs increase cytoplasmic Zn by promoting Zn transport from extracellular fluid or intracellular organelles into the cytoplasm; ZnTs reduce cytoplasmic Zn by promoting Zn transport from the cytoplasm into the extracellular space or intracellular organelles (Fukada and Kambe 2011). Although the molecular mechanisms by which Zn transporter's functions are largely unknown, sequence analysis of these transporters does not suggest that they transport Zn using ATP-dependent processes. Instead they are postulated to promote Zn transport by facilitated diffusion, secondary active transport, or by symport (see Chap. 3).

Molecular and genetic studies in rodents and humans have revealed important roles of Zn transporters in physiological regulatory mechanisms, including growth control (Table 12.1). Mice with a targeted disruption of ZIP1, ZIP2, or ZIP3 show no overt abnormalities under dietary Zn-adequate conditions, but under Zn-limited conditions during pregnancy, the embryos from single (*Zip1*, *Zip2*, *or Zip3*)- and double (*Zip1*/*Zip2* or *Zip1*/*Zip3*)-knockout mice display abnormal development

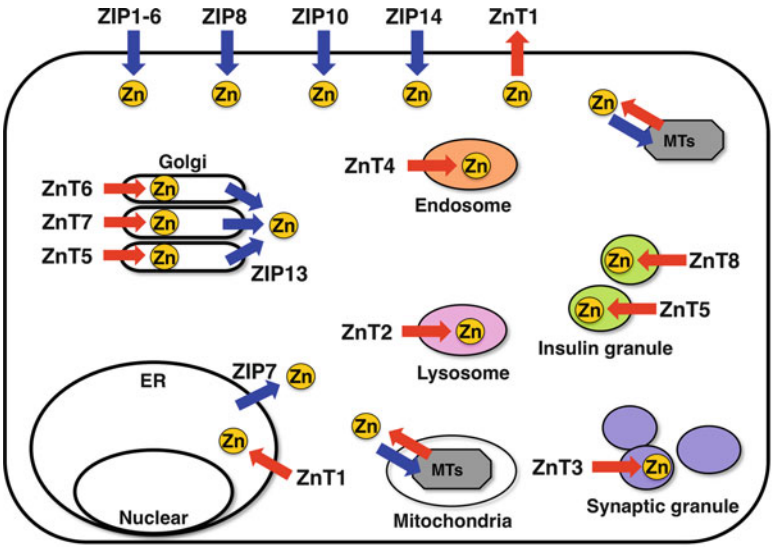

Fig. 12.1 Subcellular localization of Zn transporters and metallothioneins. Localization and potential functions of Zn transporters from the SLC39/ZIP (*blue*) and SLC30/ZnT (*red*) families, and intracellular metallothioneins (MTs), based on currently available information. *Arrows* show the predicted direction of Zn mobilization. *ER* endoplasmic reticulum

compared to those from wild-type mice. In addition, *Zip8* hypomorphic and *ZnT1*-null mice exhibit embryonic lethality (Andrews et al. 2004; Galvez-Peralta et al. 2012; Wang et al. 2011). These studies suggest that Zn is a critical nutritional factor for normal embryonic development. Furthermore, *Lethal milk* mutant mice, which have a loss-of-function mutation of *ZnT4*, produce Zn-deficient milk (Huang and Gitschier 1997), and a similar abnormality is observed in patients with a *ZNT2* loss-of-function mutation (Chowanadisai et al. 2006). These findings clearly demonstrate that Zn is crucial for postnatal growth, indicating that Zn transporters in the mammary gland are required for the transfer of Zn into milk for lactation (Fig. 12.2a). Similarly, Zn ingested through dietary intake or breast milk is absorbed through the action of several intestinal Zn transporters (Wang and Zhou 2010). Human ZIP4, encoded by the *SLC39A4* gene, is expressed on the apical membrane of polarized enterocytes in the intestine and plays a crucial role in intestinal Zn transport (Fig. 12.2b). Loss-of-function mutations in this gene cause a rare and fatal recessive disorder called acrodermatitis enteropathica (AE) (Kury et al. 2002; Wang et al. 2002). Mouse ZIP4 is essential for early embryonic development, in which it is postulated to transfer Zn via the visceral yolk sac into the embryo, and is later required for the uptake of dietary Zn (Dufner-Beattie et al. 2007). Once absorbed, Zn is transported through the blood to cells in various tissues. Zn transporters on the cell surface such as ZIP14 play crucial roles in intracellular Zn accumulation (Fig. 12.2c), and Zn transporters on the membranes of intracellular organelles, such as ZIP13, contribute to Zn distribution within cells

Table 12.1 Genetic evidence for the biological significance of Zn transporters

Gene symbol	Protein name	Mutation type	Phenotypes and disorders	References
SLC39A1	ZIP1	KO	Abnormal embryonic development	Dufner-Beattie et al. (2006)
SLC39A2	ZIP2	KO	Abnormal embryonic development	Peters et al. (2007)
SLC39A3	ZIP3	KO	Abnormal embryonic and T-cell development	Dufner-Beattie et al. (2005)
SLC39A4	ZIP4	KO	Embryonic lethal	Dufner-Beattie et al. (2007), Kury et al. (2002), Wang et al. (2002)
		Mutation	Acrodermatitis enteropathica	
SLC39A5	ZIP5	Mutation	Autosome dominant non-syndromic high myopia	Guo et al. (2014)
SLC39A8	ZIP8	Mutation	Impaired multiorgan organogenesis and hematopoiesis	Galvez-Peralta et al. (2012), Kim et al. (2014), Liu et al. (2013)
		KO	Abnormal innate immune function	
			Osteoarthritis	
SLC39A10	ZIP10	KO	Abnormal early B-cell development	Miyai et al (2014), Hojyo et al (2014)
			Impaired humoral immune response	
SLC39A13	ZIP13	KO	Connective tissue dysplasia	Fukada et al. (2008), Giunta et al. (2008)
		Mutation	Spondylocheiro dysplastic Ehlers–Danlos syndrome	
SLC39A14	ZnT14	KO	Growth retardation and impaired gluconeogenesis	Hojyo et al. (2011)
SLC30A1	ZnT1	KO	Embryonic lethal	(Andrews et al. 2004; Bruinsma et al. 2002)
			Abnormal vulva formation	
SLC30A2	ZnT2	Mutation	Low Zn in milk	Chowanadisai et al. (2006), Itsumura et al. (2013)
SLC30A3	ZnT3	KO	Prone to seizures	Adlard et al. (2010), Cole et al. (1999)
			Alzheimer's disease-like	
SLC30A4	ZnT4	Mutation	*lethal milk*: *lm*	Huang and Gitschier (1997)
			Low Zn in milk	
SLC30A5	ZnT5	KO	Growth retardation, osteopenia, hypodontia, and male-specific cardiac death	Inoue et al. (2002), Nishida et al. (2009)
			Impaired mast cell functions	

(continued)

Table 12.1 (continued)

Gene symbol	Protein name	Mutation type	Phenotypes and disorders	References
SLC30A7	ZnT7	KO	Reduced body fat accumulation	Huang et al. (2012), Huang et al. (2007)
			Susceptible to diet-induced glucose intolerance and insulin resistance	
SLC30A8	ZnT8	KO	Type 2 diabetes mellitus	Lemaire et al. (2009), Nicolson et al. (2009), Tamaki et al. (2013), Wenzlau et al. (2007)
		SNP	Type 1 and 2 diabetes mellitus	
SLC30A10	ZnT10	Mutation	Parkinsonism, Dystonia, Hypermanganesemia, Polycythemia, Chronic Liver Disease	Quadri et al. (2012), Tuschl et al. (2012)

(Fig. 12.2d). Thus, the coordinated actions of the various Zn transporters are important for Zn uptake and maintenance throughout the body (Wang and Zhou 2010), and are indispensable for normal growth control in mammals.

12.3 Zn Signaling and the Zn Signal Axis

Zn has been known to primarily function as a cofactor for cellular proteins, nucleic acids, carbohydrates, and lipids. Enzymes with a Zn atom in their reactive center are widespread in metabolism, such as alcohol dehydrogenase in humans (Maret 2013). More than 300 proteins contain Zn-interacting regions such as zinc-finger motifs, RING fingers, or LIM domains, which influence cellular responses by coordinating ions. However, several reports have recently demonstrated that the Zn mobilized by Zn transporters acts as a signaling molecule similar to calcium and cAMP. This "Zn signal" (see Chaps. 2 and 3) regulates a variety of intracellular signaling pathways (Fukada et al. 2011b; Maret 2011), including growth factor- (Haase and Maret 2003), antigen receptor- (Hojyo et al. 2014; Nishida et al. 2009), apoptotic pathway (Miyai et al. 2014), and JAK-STAT-mediated pathways (Kitabayashi et al. 2010; Yamashita et al. 2004).

These recent reports have also led to an increased understanding of the physiological significance of Zn. For example, the Zn transporter ZIP6 induces the epithelial mesenchymal transition (EMT) in the zebrafish gastrula organizer by regulating the nuclear translocation of SNAIL, a transcriptional suppressor of *E-cadherin*. This finding suggests that the Zn signal depends on the induction of Zn transporter expression to regulate the early developmental process. In addition, mice deficient in *Zip13*, *Zip14*, *ZnT5*, or *ZnT7* exhibit impaired growth (Fukada et al. 2008; Hojyo et al. 2011; Huang et al. 2007; Inoue et al. 2002), and ZnT5 is

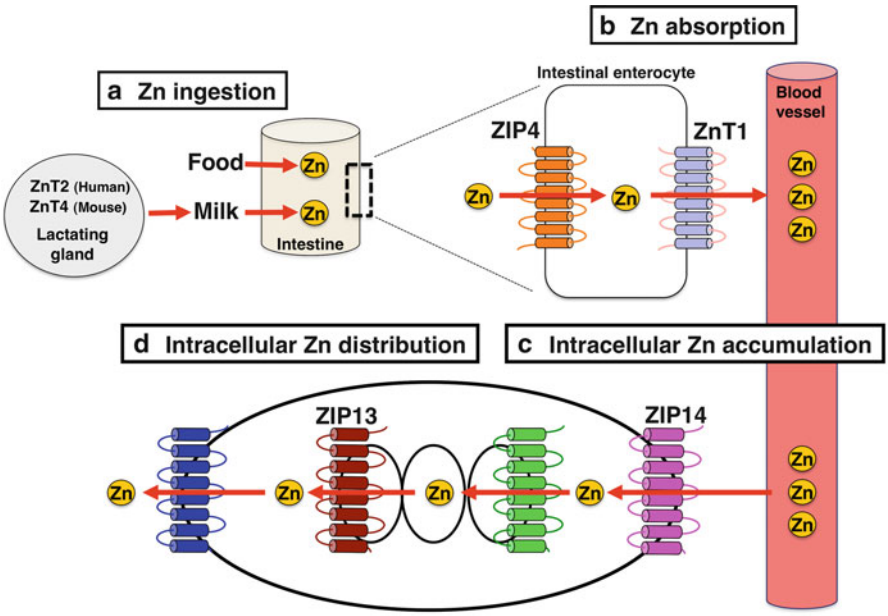

Fig. 12.2 Zn homeostasis mediated by Zn transporters is essential for the control of normal mammalian physiology. Appropriate levels of Zn are ingested from the daily diet by adults, or from breast milk during lactation. **a** ZnT2 (Chowanadisai et al. 2006) or ZnT4 (Huang and Gitschier 1997) in the mammary gland transports Zn into milk for lactation in both humans and mice, respectively. **b** Zn absorption through intestinal Zn transporters such as ZIP4 (Dufner-Beattie et al. 2007; Wang et al. 2002; Weaver et al. 2007) and ZnT1 (Wang et al. 2009) is the critical event determining Zn levels in the body. **c** Zn is transported into peripheral or somatic cells by ZIP14 (Hojyo et al. 2011) or other ZIPs to maintain intracellular Zn levels. **d** Intracellular Zn is then delivered to organelles by intracellular Zn transporters such as ZIP13 (Fukada et al. 2008) and ZnT5 (Inoue et al. 2002), which fine-tune the intracellular distribution of Zn. Each step is critical for the maintenance of mammalian health; a disruption in any one of them can cause disorders associated with Zn deficiency, including growth retardation, even in the presence of normal serum Zn levels (Fukada et al. 2008; Hojyo et al. 2011)

also involved in the allergic response through mast cell activation (Nishida et al. 2009) (See Chap. 5). Collectively, these reports strongly suggest that Zn transporters act not only to maintain Zn homeostasis but also to provide signaling factors to control a variety of cellular functions. Importantly, each individual Zn transporter defines a signal transduction cascade that regulates specific target molecules to elicit cellular effects. Within these pathways, the Zn regulatory system is referred to as the "Zn signal axis" (Fukada et al. 2013) (Fig. 12.3).

In the following section, we introduce the physiological significance of Zn signaling and two Zn signal axes that are involved in systemic growth and bone metabolism.

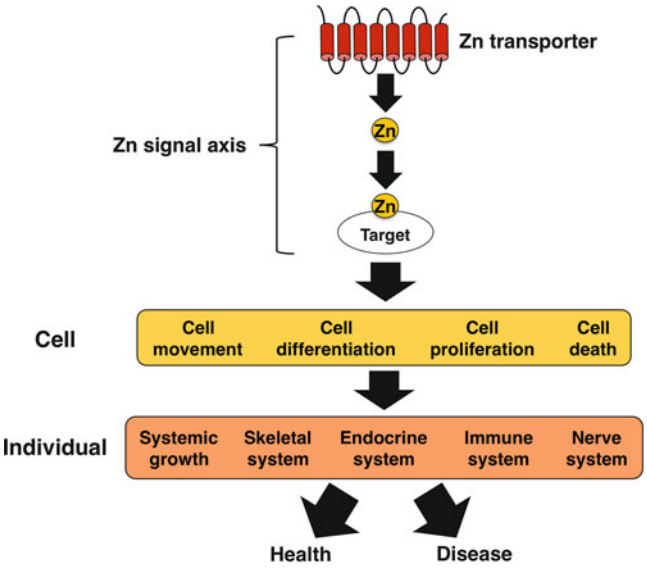

Fig. 12.3 Different "Zn signal axes" affect distinct signaling pathways to promote and control cellular functions. Each Zn signal axis targets a specific molecule. This mode of Zn action exerts a variety of cellular functions by selectively regulating distinct signaling molecules. Therefore, the disruption of a Zn signal axis can lead to pathogenesis in the absence of redundant machinery

12.4 Zn Signals in Growth and Bone Metabolism

The Zn concentration is relatively high in bone, cartilage, and teeth (Bergman and Soremark 1968), and Zn has been proposed to stimulate bone formation and mineralization (Yamaguchi and Gao 1998). As just described, Zn is absorbed in the intestine, circulates in the blood, and is transported to cells in the peripheral tissues. However, the mechanism by which the Zn signal regulates systemic growth and bone metabolism is currently unclear. Because systemic growth is controlled by the endocrine system, it is hypothesized that the Zn signal plays pivotal roles in the regulation of endocrine signaling.

The analysis of ZIP13 and ZIP14 knockout mice (Fukada et al. 2008; Hojyo et al. 2011) revealed that these transporters control Zn signal axes that selectively regulate the intracellular signaling cascades required for bone metabolism and systemic growth. Defects in either the ZIP13 or ZIP14-mediated axis lead to impaired bone homeostasis or growth, and the specific signaling cascades of both axes are nonredundant.

12.4.1 ZIP13-Mediated Zn Signaling Controls Bone and Connective Tissue Formation

ZIP13, encoded by the *Slc39a13* gene, is a homodimeric intracellular Zn transporter that plays a critical role in the development of hard and connective tissues (Bin et al. 2011, 2014; Fukada and Kambe 2011; Fukada et al. 2008).

12.4.1.1 Abnormal Formation of Bone, Skin, and Teeth in *Zip13*-KO Mice

Zip13-KO mice exhibit multiple phenotypes, including growth retardation, progressive kyphosis, abnormal bone and teeth formation, increased skin fragility, and reduced levels of corneal stromal collagen at 3 to 4 weeks of age (Fig. 12.4a). Endochondral ossification is a process required for the proper development of long bones and vertebrate growth (Kronenberg 2003). At the growth plate of long bones, resting chondrocytes differentiate into proliferative chondrocytes with a columnar morphology, and then stop dividing, followed by maturation into pre-hypertrophic and then hypertrophic chondrocytes. *Zip13*-KO mice also exhibit a significant reduction in the length of their long bones. The hypertrophic chondrocytes in their growth plates are rarely observed; instead, irregularly organized proliferative chondrocytes are present (Fig. 12.4a). Furthermore, *Zip13*-KO mice exhibit reduced osteoblast activity, consistent with the observed osteopenia (Fig. 12.4a), whereas the osteoclast activity appears normal. These findings indicate that ZIP13 controls the normal differentiation and function of chondrocytes and osteoblasts during the growth phase of long bones.

Zip13-KO mice also display odontological morphological aberrations, including the abnormal development of incisor teeth, including deformity, malocclusion, and breakage, the reduced formation of molar root dentine, and the reduced volume of both the mandible and the alveolar bone, although the molar crowns are morphologically unchanged (Fig. 12.4b). Thus, the *Zip13*-KO-associated abnormalities appear to occur selectively in tissues of mesenchymal origin (Fukada et al. 2011a; Fukada et al. 2008). Collectively, ZIP13 has indispensable roles for the proper development of both hard and connective tissues such as bone, teeth, and skin.

12.4.1.2 ZIP13 and Human Disease

The phenotypes of *Zip13*-KO mice are reminiscent of human Ehlers–Danlos syndrome (EDS), a group of genetic recessive disorders that affect connective tissue development (Steinmann and Royce 2002; Steinmann et al. 2002), and of osteogenesis imperfecta (OI), which affects bone and teeth formation (Rauch and Glorieux 2004) (Fig. 12.4c). Patients with spondylocheiro dysplastic EDS (SCD-EDS) display short stature, downslanting palpebral fissures, hypodontia, fragile skin, osteopenia,

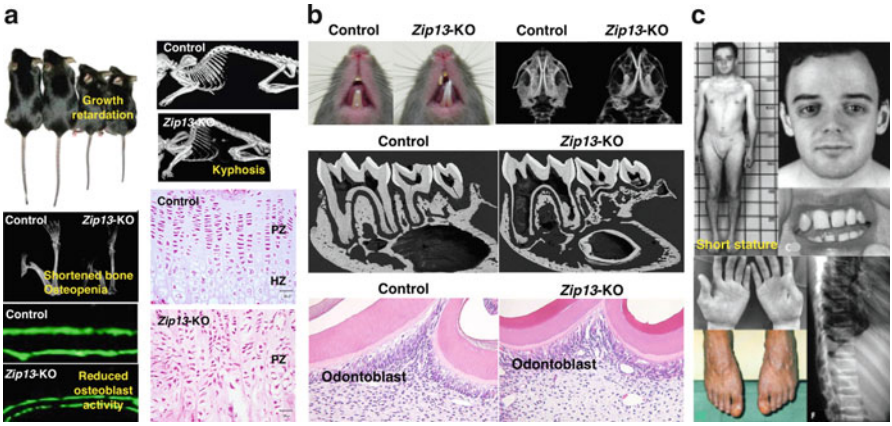

Fig. 12.4 ZIP13 plays a role in regulating systemic growth and bone homeostasis. **a** Growth retardation, kyphosis, osteopenia, shortened long bones, reduced osteoblast activity, and abnormal cartilage development in the *Zip13*-KO mouse. *PZ* proliferative zone; *HZ* hypertrophic zone. **b** Abnormal teeth and craniofacial development in the *Zip13*-KO mouse. *Zip13*-KO mice develop abnormal incisor teeth, and exhibit reduced molar root dentin and mandible bone volume. Abnormal arrangement of odontoblasts is observed. **c** Clinical features of an SCD-EDS patient possessing the ZIP13/SLC39A13 mutation, including short stature, anti-mongoloid eye slant with lack of periorbital tissue, missing upper lateral incisors, wrinkled skin, severe varicosity, and vertebral flattening with sclerosis of the vertebral endplates

flattened or biconcave vertebral bodies with flaky irregularity of the endplates, mild dysplastic changes at the metaphyses of the long bones and phalanges, bluish or greyish sclerae, and varicose veins (Fig. 12.4c). DNA analysis revealed that these patients possess a homozygous loss-of-function point mutation in *ZIP13* involving a G-to-A transition at nucleotide 221, resulting in a mutant ZIP13 with a G64D substitution (Fukada et al. 2008; Bin et al. 2014). Furthermore, another EDS-associated loss-of-function mutation in *ZIP13* was identified as a deletion encompassing nucleotides 483 to 491 (FLA amino acids deletion: ZIP13 ΔFLA) by a genome-wide SNP scan and sequence analysis (Giunta et al. 2008), clearly confirming the importance of ZIP13 for growth and bone homeostasis in humans.

12.4.1.3 Regulation of the BMP/TGF-β Signaling Pathway by ZIP13

Consistent with the multiple defects observed in *Zip13*-KO mice, ZIP13 was found to be expressed in osteoblasts, proliferative chondrocytes, odontoblasts, and skin fibroblasts. Both primary osteoblasts and chondrocytes isolated from *Zip13*-KO mice show impaired gene marker expression, indicating that ZIP13 affects their differentiation and function. Microarray analysis indicated that the BMP/TGF-β signaling cascade is significantly dysregulated in *Zip13*-KO cells. Indeed, BMP4-induced *Msx2* gene transcription is reduced in ZIP13-deficient primary osteoblasts, and this phenotype is reversed by the introduction of wild-type ZIP13, but not by

the G64D mutant ZIP13, demonstrating that the G64D substitution results in loss of function. Further analysis revealed that the SMAD phosphorylation levels are unchanged, but the nuclear translocation of SMAD is disrupted in BMP4-treated *Zip13*-KO osteoblasts (Fukada et al. 2008). BMP and TGF-β are coordinated local paracrine regulators of bone formation and elongation, and of systemic growth (Akhurst 2004; Cao and Chen 2005; Cobourne and Sharpe 2003). Human genetic studies also support the pivotal role of these regulators in hard and connective tissue development (Akhurst 2004; Mizuguchi et al. 2004). Collectively, these findings provide evidence of a functional link between ZIP13-mediated Zn signaling and BMP/TGF-β-mediated growth regulation.

A series of biochemical analyses revealed that ZIP13 possesses eight putative transmembrane domains and unique hydrophilic regions at both its N- and C-termini, which face the luminal side of the Golgi, and that ZIP13 forms a homodimer and contributes to Zn influx from the Golgi to the cytosol (Bin et al. 2011). We also demonstrated the molecular pathogenic mechanism of SCD-EDS caused by two different mutant ZIP13 proteins, G64D and ΔFLA. Both mutant proteins are susceptible to rapid degradation via the valosin-containing protein (VCP)-linked ubiquitin proteasome pathway without the effects on dimer-formation (Bin et al. 2014). The inhibition of degradation pathways rescued the protein expression levels, resulting in improved intracellular Zn homeostasis. Together, these findings suggest that intracellular Zn levels and its distribution regulated by ZIP13 are critical for the normal control of mammalian bone and connective tissue development as well as systemic growth control.

12.4.2 ZIP14-Mediated Zn Signaling Controls Bone Formation

ZIP14, encoded by the *Slc39a14* gene, is a cell membrane-localized Zn transporter that plays a critical role in regulating systemic growth and bone metabolism (Hojyo et al. 2011).

12.4.2.1 Abnormal Bone Formation in *Zip14*-KO Mice

Zip14-KO mice exhibit dwarfism, torticollis (wry neck), scoliosis, osteopenia, and shortened long bones (Fig. 12.5a). The finding that *Zip14* mRNA is highly expressed in bone tissues (Fig. 12.5b) is consistent with the *Zip14*-KO phenotype and suggests that ZIP14 plays a role in bone formation. In particular, ZIP14 was found to be expressed in each of the zones of the epiphyseal growth plate during endochondral ossification, at relatively high levels in the proliferative zone (PZ), and at minimal levels in the hypertrophic zone (HZ) (Fig. 12.5b). *Zip14*-deficient mice exhibit growth retardation accompanied by a shortened proliferative zone and

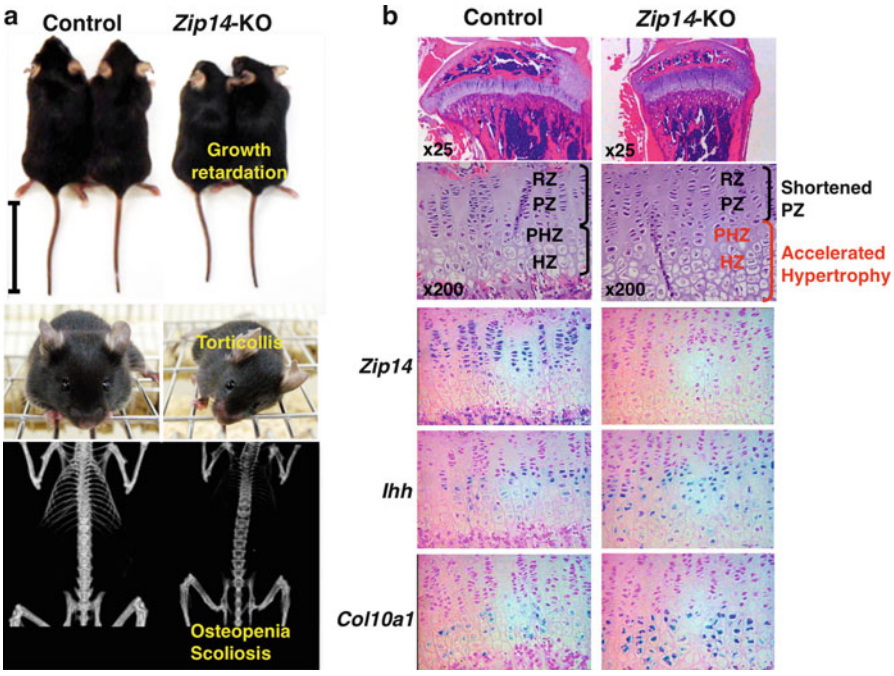

Fig. 12.5 ZIP14 has a role in regulating systemic growth and bone homeostasis. **a** Dwarfism, torticollis, osteopenia, and scoliosis in *Zip14*-KO mice. *Bar* 5 cm. **b** Abnormal chondrocyte differentiation in the growth plate of *Zip14*-KO mice. Hematoxylin and eosin (H&E) staining and in situ hybridization analysis for *Zip14*, *Ihh*, and *Col10a1* in the growth plates from control and *Zip14*-KO mice

an extended hypertrophic zone, resulting in impaired bone elongation. Consistent with this observation, expression of *type II Collagen* (*Col2a1*), an RZ and PZ marker, was decreased, whereas the expressions of *Indian hedgehog* (*Ihh*), a pre-hypertrophic zone (PHZ) marker, and *type X Collagen* (*Col10a1*), an HZ marker, were both enhanced, indicating accelerated hypertrophy at the *Zip14*-KO growth plate (Fig. 12.5b).

The morphology of the *Zip14*-KO growth plate is a partial phenocopy of that in mice with a chondrocyte-specific deletion of *parathyroid hormone 1 receptor* (*Pth1r*) (Chung et al. 1998; Kobayashi et al. 2002; Kronenberg 2006). Parathyroid hormone-related peptide (PTHrP) binds to PTH1R and stimulates the phosphorylation of cAMP response element-binding protein (CREB) by the nuclear-translocated catalytic alpha-subunit of protein kinase A (PKA-Cα), followed by the phosphorylated CREB (p-CREB)-mediated induction of *c-fos* (Guo et al. 1995). Interestingly, PTHrP-induced *c-fos* transcription is significantly reduced in the *Zip14*-KO chondrocytes. The level of p-CREB and the nuclear translocation of PKA-Cα in response to PTHrP treatment are also reduced in the *Zip14*-KO chondrocytes (Hojyo et al. 2011), suggesting a functional interaction between ZIP14- and PTHrP-regulated signal transduction.

12.4.2.2 Regulation of the PTHrP Signaling Pathway by ZIP14

PTHrP binding to PTH1R, a G protein-coupled receptor, leads to adenylyl cyclase (AC) activation, resulting in high levels of cAMP that in turn activate PKA by binding to its regulatory subunit (Ionescu et al. 2001). To identify the defective component in PTH1R signaling in *Zip14*-KO cells, we evaluated AC activity by measuring cAMP levels. The basal cAMP level was significantly reduced in *Zip14*-KO chondrocytes, and even after treatment with either PTHrP or the AC activator forskolin (FSK), the cAMP levels were lower than in control chondrocytes, while the treatment-induced fold increase in cAMP levels was comparable between the control and *Zip14*-KO chondrocytes. These findings indicated that ligand-mediated AC activation was intact in the *Zip14*-KO cells, and that ZIP14 is required to maintain the basal level of cAMP in chondrocytes. Phosphodiesterase (PDE), a critical regulator of cyclic nucleotide signaling, degrades cAMP and cyclic guanosine monophosphate (cGMP) (Jeon et al. 2005). *Zip14*-KO chondrocytes show enhanced PDE activity and reduced cAMP levels, both of which are corrected by treatment with a PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), consistent with an earlier finding that Zn inhibits cAMP-specific PDE activity (Percival et al. 1997; von Bulow et al. 2005). Taken together, these results suggest that ZIP14 regulates the PTH1R-cAMP-CREB pathway by affecting Zn homeostasis.

In fact, the Zn levels are significantly decreased in proliferative but not in hypertrophic chondrocytes at the *Zip14*-KO growth plate, consistent with the high *Zip14* expression levels in the PZ. We confirmed that ZIP14 is localized to the plasma membrane, as previously reported (Liuzzi et al. 2005). Furthermore, we found that Zn treatment reduces the cAMP-specific PDE activity and elevates the cAMP levels in *Zip14*-KO chondrocytes, and that the ectopic expression of ZIP14 in KO cells rescues the intracellular Zn and cAMP levels.

12.4.2.3 Regulation of the GH-IGF-I Axis by ZIP14

Zip14-KO mice show phenotypic similarity to GH-mutant mice and human cases of GH deficiency (Procter et al. 1998). The GPCR growth hormone-releasing hormone receptor (GHRHR) acts on pituitary somatotroph cells to produce GH through the cAMP-CREB pathway (Mayo et al. 1995; Struthers et al. 1991), suggesting that ZIP14 may be involved in GHRHR-mediated GH production. Indeed, we found that the pituitary gland expresses *Zip14*, and that the levels of pituitary cAMP and Zn are decreased in *Zip14*-KO mice. Intriguingly, bolus administration of GHRH increases the plasma GH levels in control mice but has little effect in the *Zip14*-KO mice. In addition, a similar defect in *Gh* induction is observed in the *Zip14*-KO mice in response to fasting, during which GHRHR-mediated GH induction is known to occur (Sakharova et al. 2008). *Zip14*-KO mice show reduced serum IGF-I and hepatic *Igf-I* expression levels, which may be explained by impaired GH production, because their *growth hormone receptor* (*Ghr*) expression levels are

similar to wild-type levels. These observations suggest that ZIP14 is involved in the signaling of GHRHR, a GPCR in the pituitary gland. Collectively, these results clearly demonstrate that ZIP14 not only acts as a positive GPCR regulator in the endochondral ossification process, but also functions to regulate GH production, resulting in the concomitant regulation of vertebrate systemic growth. This conclusion may explain why Zn deficiency causes reductions in serum GH and IGF-I levels (MacDonald 2000).

12.5 Conclusions and Future Perspectives

Zn transporter-mediated Zn signaling plays essential roles in the control of mammalian growth. Mice lacking ZIP13 or ZIP14 exhibit stunted growth, accompanied by impaired bone growth. Both transporters are preferentially expressed in the PZ of the growth plate during endochondral ossification (Fukada et al. 2008; Hojyo et al. 2011; Inoue et al. 2002), and the absence of either transporter alters the morphology of the PZ and HZ. However, it should be noted that the *Zip13*- and *Zip14*-KO mice exhibit opposing alterations in growth plate morphologies; the loss of ZIP13 results in the formation of an elongated PZ and a shortened HZ, whereas the loss of ZIP14 leads to a shortened PZ and an elongated HZ. Consistent with these morphological differences, ZIP13 and ZIP14 play distinct roles in the regulation of growth-related signaling cascades. For example, ZIP13 regulates SMAD nuclear translocation during BMP/TGF-β signaling, whereas ZIP14 regulates PDE activity, to alter GPCR-mediated signaling. These transporters also exhibit different localizations: ZIP13 is localized to the Golgi and controls intracellular Zn distribution, whereas ZIP14 is expressed on the cell membrane and controls intracellular Zn accumulation (Fukada et al. 2008; Fukada and Kambe 2011; Hojyo et al. 2011; Inoue et al. 2002). Thus, the intracellular Zn status is controlled by the localization of each Zn transporter, and their coordinated functions ultimately impact mammalian growth. These findings support the concept that various "Zn signal axes" act in different signaling pathways to promote systemic growth (Fig. 12.3).

Currently, a four-step model of Zn homeostasis is postulated for the mediation of mammalian growth: (1) ZnT2 (Chowanadisai et al. 2006) and ZnT4 (Huang and Gitschier 1997) contribute to Zn ingestion into the body through breast milk and daily diet (Fig. 12.2a); (2) Zn is absorbed by intestinal transporters such as ZIP4 (Dufner-Beattie et al. 2007) and ZnT1 (Wang et al. 2009) (Fig. 12.2b); (3) intracellular Zn accumulates and is maintained through Zn influx via cell-surface Zn transporters such as ZIP14 (Hojyo et al. 2011) (Fig. 12.2c); and (4) intracellular Zn is distributed by intracellular Zn transporters such as ZIP13 (Fukada et al. 2008) and ZnT5 (Inoue et al. 2002) (Fig. 12.2d), leading to the fine-tuning of cellular functions. Thus, the unique Zn signal axes generated by the coordinated Zn transporter functions are essential for the proper control of mammalian systemic growth and regeneration. The disruption of each Zn signaling axis can affect pathogenesis in the absence of redundant machinery.

Many aspects of Zn signaling and its effects on growth regulation and bone homeostasis remain to be investigated. Zn may also have additional uncharacterized functions and interactions with other involved signaling factors (Akhurst 2004; Cao and Chen 2005; Nilsson et al. 2005; Procter et al. 1998) that impact bone homeostasis and body growth. Other areas of interest include the molecular basis of the specificity of the Zn signal axes (Fig. 12.3) and the downstream functions of Zn as a signaling molecule, following its mobilization by Zn transporters, as discussed in other chapters. Further molecular and genetics-based studies on Zn transporters are required to address these issues and to expand our knowledge about Zn signaling.

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References

Adlard PA, Parncutt JM, Finkelstein DI, Bush AI (2010) Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease? J Neurosci 30:1631–1636

Akhurst RJ (2004) TGF beta signaling in health and disease. Nat Genet 36:790–792

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196–201

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

Bergman B, Soremark R (1968) Autoradiographic studies on the distribution of zinc-65 in mice. J Nutr 94:6–12

Bin BH, Fukada T, Hosaka T, Yamasaki S, Ohashi W, Hojyo S, Miyai T, Nishida K, Yokoyama S, Hirano T (2011) Biochemical characterization of human ZIP13 protein: a homo-dimerized zinc transporter involved in the Spondylocheiro dysplastic Ehlers–Danlos syndrome. J Biol Chem 286:40255–40265

Bin B-H et al (2014) Molecular pathogenesis of spondylocheirodysplastic Ehlers–Danlos syndrome caused by mutant ZIP13 proteins. EMBO Mol Med 6:1028–1042

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A (2007) Zinc in plants. New Phytol 173:677–702

Bruinsma JJ, Jirakulaporn T, Muslin AJ, Kornfeld K (2002) Zinc ions and cation diffusion facilitator proteins regulate Ras-mediated signaling. Dev Cell 2:567–578

Cao X, Chen D (2005) The BMP signaling and in vivo bone formation. Gene (Amst) 357:1–8

Cha MC, Rojhani A (1997) Zinc deficiency inhibits the direct growth effect of growth hormone on the tibia of hypophysectomized rats. Biol Trace Elem Res 59:99–111

Chowanadisai W, Lonnerdal B, Kelleher SL (2006) Identification of a mutation in SLC30A2 (ZnT-2) in women with low milk zinc concentration that results in transient neonatal zinc deficiency. J Biol Chem 281:39699–39707

Chung UI, Lanske B, Lee K, Li E, Kronenberg H (1998) The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. Proc Natl Acad Sci USA 95:13030–13035

Cobourne MT, Sharpe PT (2003) Tooth and jaw: molecular mechanisms of patterning in the first branchial arch. Arch Oral Biol 48:1–14

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

Cossack ZT (1986) Somatomedin-C and zinc status in rats as affected by Zn, protein and food intake. Br J Nutr 56:163–169

Cossack ZT (1991) Decline in somatomedin-C (insulin-like growth factor-1) with experimentally induced zinc deficiency in human subjects. Clin Nutr 10:284–291

De Meyts P, Wallach B, Christoffersen CT, Urso B, Gronskov K, Latus LJ, Yakushiji F, Ilondo MM, Shymko RM (1994) The insulin-like growth factor-I receptor. Structure, ligand-binding mechanism and signal transduction. Horm Res 42:152–169

Dufner-Beattie J, Huang ZL, Geiser J, Xu W, Andrews GK (2005) Generation and characterization of mice lacking the zinc uptake transporter ZIP3. Mol Cell Biol 25:5607–5615

Dufner-Beattie J, Huang ZL, Geiser J, Xu W, Andrews GK (2006) Mouse ZIP1 and ZIP3 genes together are essential for adaptation to dietary zinc deficiency during pregnancy. Genesis 44:239–251

Dufner-Beattie J, Weaver BP, Geiser J, Bilgen M, Larson M, Xu W, Andrews GK (2007) The mouse acrodermatitis enteropathica gene Slc39a4 (Zip4) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency. Hum Mol Genet 16:1391–1399

Fukada T, Kambe T (2011) Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics 3:662–674

Fukada T, Civic N, Furuichi T, Shimoda S, Mishima K, Higashiyama H, Idaira Y, Asada Y, Kitamura H, Yamasaki S et al (2008) The zinc transporter SLC39A13/ZIP13 is required for connective tissue development; its involvement in BMP/TGF-beta signaling pathways. PLoS One 3:e3642

Fukada T, Asada Y, Mishima K, Shimoda S, Saito I (2011a) Slc39a13/Zip13: a crucial zinc transporter involved in tooth development and inherited disorders. J Oral Biosci 53:1–12

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011b) Zinc homeostasis and signaling in health and diseases: zinc signaling. J Biol Inorg Chem 16:1123–1134

Fukada T, Hojyo S, Furuichi T (2013) Zinc signal: a new player in osteobiology. J Bone Miner Metab 31:129–135

Gaither LA, Eide DJ (2001) Eukaryotic zinc transporters and their regulation. Biometals 14:251–270

Galvez-Peralta M, He L, Jorge-Nebert LF, Wang B, Miller ML, Eppert BL, Afton S, Nebert DW (2012) ZIP8 zinc transporter: indispensable role for both multiple-organ organogenesis and hematopoiesis in utero. PLoS One 7:e36055

Giugliano R, Millward DJ (1984) Growth and zinc homeostasis in the severely Zn-deficient rat. Br J Nutr 52:545–560

Giunta C, Elcioglu NH, Albrecht B, Eich G, Chambaz C, Janecke AR, Yeowell H, Weis M, Eyre DR, Kraenzlin M et al (2008) Spondylocheiro dysplastic form of the Ehlers–Danlos syndrome–an autosomal-recessive entity caused by mutations in the zinc transporter gene SLC39A13. Am J Hum Genet 82:1290–1305

Guo H et al (2014) SLC39A5 mutations interfering with the BMP/TGF-β pathway in non-syndromic high myopia. J Med Genet 51:518–525

Guo J, Iida-Klein A, Huang X, Abou-Samra AB, Segre GV, Bringhurst FR (1995) Parathyroid hormone (PTH)/PTH-related peptide receptor density modulates activation of phospholipase C and phosphate transport by PTH in LLC-PK1 cells. Endocrinology 136:3884–3891

Haase H, Maret W (2003) Intracellular zinc fluctuations modulate protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Exp Cell Res 291:289–298

Hambidge KM, Krebs NF (2007) Zinc deficiency: a special challenge. J Nutr 137:1101–1105

Henkin RI (1976) Trace metals in endocrinology. Med Clin N Am 60:779–797

Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin BH, Koseki H, Hirano T (2011) The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. PLoS One 6:e18059

Hojyo S et al (2014) Zinc transporter SLC39A10/ZIP10 controls humoral immunity by modulating B cell receptor signal strength. Proc Natl Acad Sci U S A 111:11786–11791
Huang L, Gitschier J (1997) A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat Genet 17:292–297
Huang L, Yu YY, Kirschke CP, Gertz ER, Lloyd KK (2007) Znt7 (Slc30a7)-deficient mice display reduced body zinc status and body fat accumulation. J Biol Chem 282:37053–37063
Huang L, Kirschke CP, Lay YA, Levy LB, Lamirande DE, Zhang PH (2012) Znt7-null mice are more susceptible to diet-induced glucose intolerance and insulin resistance. J Biol Chem 287:33883–33896
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
Ionescu AM, Schwarz EM, Vinson C, Puzas JE, Rosier R, Reynolds PR, O'Keefe RJ (2001) PTHrP modulates chondrocyte differentiation through AP-1 and CREB signaling. J Biol Chem 276:11639–11647
Itsumura N, Inamo Y, Okazaki F, Teranishi F, Narita H, Kambe T, Kodama H (2013) Compound heterozygous mutations in SLC30A2/ZnT2 results in low milk zinc concentrations: a novel mechanism for zinc deficiency in a breast-fed infant. PLoS One 8:e64045
Jeon YH, Heo YS, Kim CM, Hyun YL, Lee TG, Ro S, Cho JM (2005) Phosphodiesterase: overview of protein structures, potential therapeutic applications and recent progress in drug development. Cell Mol Life Sci 62:1198–1220
Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M (2004) Overview of mammalian zinc transporters. Cell Mol Life Sci 61:49–68
Kim JH, Jeon J, Shin M, Won Y, Lee M, Kwak JS, Lee G, Rhee J, Ryu JH, Chun CH et al (2014) Regulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1 axis. Cell 156:730–743
Kitabayashi C, Fukada T, Kanamoto M, Ohashi W, Hojyo S, Atsumi T, Ueda N, Azuma I, Hirota H, Murakami M et al (2010) Zinc suppresses Th17 development via inhibition of STAT3 activation. Int Immunol 22:375–386
Kobayashi T, Chung UI, Schipani E, Starbuck M, Karsenty G, Katagiri T, Goad DL, Lanske B, Kronenberg HM (2002) PTHrP and Indian hedgehog control differentiation of growth plate chondrocytes at multiple steps. Development (Camb) 129:2977–2986
Kronenberg HM (2003) Developmental regulation of the growth plate. Nature (Lond) 423:332–336
Kronenberg HM (2006) PTHrP and skeletal development. Ann N Y Acad Sci 1068:1–13
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
Lemaire K, Ravier MA, Schraenen A, Creemers JW, Van de Plas R, Granvik M, Van Lommel L, Waelkens E, Chimienti F, Rutter GA et al (2009) Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. Proc Natl Acad Sci USA 106:14872–14877
Liu MJ, Bao S, Galvez-Peralta M, Pyle CJ, Rudawsky AC, Pavlovicz RE, Killilea DW, Li C, Nebert DW, Wewers MD et al (2013) ZIP8 regulates host defense through zinc-mediated inhibition of NF-kappaB. Cell Rep 3:386–400
Liuzzi JP, Lichten LA, Rivera S, Blanchard RK, Aydemir TB, Knutson MD, Ganz T, Cousins RJ (2005) Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc Natl Acad Sci USA 102:6843–6848
MacDonald RS (2000) The role of zinc in growth and cell proliferation. J Nutr 130:1500S–1508S
Maret W (2011) Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. Biometals 24:411–418
Maret W (2013) Zinc and the zinc proteome. Met Ions Life Sci 12:479–501
Matsui T, Yamaguchi M (1995) Zinc modulation of insulin-like growth factor's effect in osteoblastic MC3T3-E1 cells. Peptides 16:1063–1068

Mayo KE, Godfrey PA, Suhr ST, Kulik DJ, Rahal JO (1995) Growth hormone-releasing hormone: synthesis and signaling. Recent Prog Horm Res 50:35–73

Miyai T et al (2014) Zinc transporter SLC39A10/ZIP10 facilitates antiapoptotic signaling during early B-cell development. Proc Natl Acad Sci U S A 111:11780–11785

Mizuguchi T, Collod-Beroud G, Akiyama T, Abifadel M, Harada N, Morisaki T, Allard D, Varret M, Claustres M, Morisaki H et al (2004) Heterozygous TGFBR2 mutations in Marfan syndrome. Nat Genet 36:855–860

Nicolson TJ, Bellomo EA, Wijesekara N, Loder MK, Baldwin JM, Gyulkhandanyan AV, Koshkin V, Tarasov AI, Carzaniga R, Kronenberger K et al (2009) Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. Diabetes 58:2070–2083

Nilsson O, Marino R, De Luca F, Phillip M, Baron J (2005) Endocrine regulation of the growth plate. Horm Res 64:157–165

Nishida K, Hasegawa A, Nakae S, Oboki K, Saito H, Yamasaki S, Hirano T (2009) Zinc transporter Znt5/Slc30a5 is required for the mast cell-mediated delayed-type allergic reaction but not the immediate-type reaction. J Exp Med 206:1351–1364

Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC (1998) Growth hormone and bone. Endocr Rev 19:55–79

Oner G, Bhaumick B, Bala RM (1984) Effect of zinc deficiency on serum somatomedin levels and skeletal growth in young rats. Endocrinology 114:1860–1863

Percival MD, Yeh B, Falgueyret JP (1997) Zinc dependent activation of cAMP-specific phosphodiesterase (PDE4A). Biochem Biophys Res Commun 241:175–180

Peters JL, Dufner-Beattie J, Xu W, Geiser J, Lahner B, Salt DE, Andrews GK (2007) Targeting of the mouse Slc39a2 (Zip2) gene reveals highly cell-specific patterns of expression, and unique functions in zinc, iron, and calcium homeostasis. Genesis 45:339–352

Prasad AS (1995) Zinc: an overview. Nutrition 11:93–99

Prasad AS (2008) Zinc in human health: effect of zinc on immune cells. Mol Med 14:353–357

Prasad AS, Oberleas D, Wolf P, Horwitz JP (1969) Effect of growth hormone on nonhypophysectomized zinc-deficient rats and zinc on hypophysectomized rats. J Lab Clin Med 73:486–494

Procter AM, Phillips JA 3rd, Cooper DN (1998) The molecular genetics of growth hormone deficiency. Hum Genet 103:255–272

Quadri M, Federico A, Zhao T, Breedveld GJ, Battisti C, Delnooz C, Severijnen LA, Di Toro Mammarella L, Mignarri A, Monti L et al (2012) Mutations in SLC30A10 cause parkinsonism and dystonia with hypermanganesemia, polycythemia, and chronic liver disease. Am J Hum Genet 90:467–477

Rauch F, Glorieux FH (2004) Osteogenesis imperfecta. Lancet 363:1377–1385

Rink L, Gabriel P (2000) Zinc and the immune system. Proc Nutr Soc 59:541–552

Root AW, Duckett G, Sweetland M, Reiter EO (1979) Effects of zinc deficiency upon pituitary function in sexually mature and immature male rats. J Nutr 109:958–964

Rossi L, Migliaccio S, Corsi A, Marzia M, Bianco P, Teti A, Gambelli L, Cianfarani S, Paoletti F, Branca F (2001) Reduced growth and skeletal changes in zinc-deficient growing rats are due to impaired growth plate activity and inanition. J Nutr 131:1142–1146

Roth HP, Kirchgessner M (1994) Influence of alimentary zinc deficiency on the concentration of growth hormone (GH), insulin-like growth factor I (IGF-I) and insulin in the serum of force-fed rats. Horm Metab Res 26:404–408

Sakharova AA, Horowitz JF, Surya S, Goldenberg N, Harber MP, Symons K, Barkan A (2008) Role of growth hormone in regulating lipolysis, proteolysis, and hepatic glucose production during fasting. J Clin Endocrinol Metab 93:2755–2759

Steinmann B, Royce PM (eds) (2002) Connective tissue and its heritable disorders, 2nd edn. Wiley-Liss, New York

Steinmann B, Royce PM, Superti-Furga A (2002) The Ehlers–Danlos syndrome. In: Steinmann B, Royce PM (eds) Connective tissue and its heritable disorders. Wiley-Liss, New York, pp 431–524

Struthers RS, Vale WW, Arias C, Sawchenko PE, Montminy MR (1991) Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant. Nature (Lond) 350:622–624

Sugarman B (1983) Zinc and infection. Rev Infect Dis 5:137–147

Tamaki M, Fujitani Y, Hara A, Uchida T, Tamura Y, Takeno K, Kawaguchi M, Watanabe T, Ogihara T, Fukunaka A et al (2013) The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. J Clin Invest 123:4513–4524

Tuschl K, Clayton PT, Gospe SM Jr, Gulab S, Ibrahim S, Singhi P, Aulakh R, Ribeiro RT, Barsottini OG, Zaki MS et al (2012) Syndrome of hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia caused by mutations in SLC30A10, a manganese transporter in man. Am J Hum Genet 90:457–466

Underwood LE (1996) Nutritional regulation of IGF-I and IGFBPs. J Pediatr Endocrinol Metab 9 (suppl 3):303–312

von Bulow V, Rink L, Haase H (2005) Zinc-mediated inhibition of cyclic nucleotide phosphodiesterase activity and expression suppresses TNF-alpha and IL-1 beta production in monocytes by elevation of guanosine 3′,5′-cyclic monophosphate. J Immunol 175:4697–4705

Wang X, Zhou B (2010) Dietary zinc absorption: a play of zips and ZnTs in the gut. IUBMB Life 62:176–182

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

Wang X, Wu Y, Zhou B (2009) Dietary zinc absorption is mediated by ZnT1 in *Drosophila melanogaster*. FASEB J 23:2650–2661

Wang B, He L, Dong H, Dalton TP, Nebert DW (2011) Generation of a Slc39a8 hypomorph mouse: markedly decreased ZIP8 Zn(2)(+)/(HCO(3)(-))(2) transporter expression. Biochem Biophys Res Commun 410:289–294

Wapnir RA, Chemical Rubber Company (1990) Protein nutrition and mineral absorption. CRC Press, Boca Raton

Weaver BP, Dufner-Beattie J, Kambe T, Andrews GK (2007) Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse Slc39a4 and Slc39a5 zinc transporters (Zip4 and Zip5). Biol Chem 388:1301–1312

Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW et al (2007) The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci USA 104:17040–17045

Yamaguchi M, Gao YH (1998) Potent effect of zinc acexamate on bone components in the femoral-metaphyseal tissues of elderly female rats. Gen Pharmacol 30:423–427

Yamaguchi M, Hashizume M (1994) Effect of beta-alanyl-L-histidinato zinc on protein components in osteoblastic MC3T3-El cells: increase in osteocalcin, insulin-like growth factor-I and transforming growth factor-beta. Mol Cell Biochem 136:163–169

Yamashita S, Miyagi C, Fukada T, Kagara N, Che YS, Hirano T (2004) Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature (Lond) 429:298–302

Chapter 13
Zinc and Its Role in the Pathogenesis of Type 2 Diabetes

Yoshio Fujitani, Motoyuki Tamaki, Ayako Fukunaka, and Hirotaka Watada

Abstract Although zinc ions have important roles in the biosynthesis and storage of insulin, many previous studies have reported the possible involvement of zinc deficiency in the pathogenesis of diabetes. Zinc may counteract the deleterious effects of oxidative stress, which contributes to increased insulin sensitivity, and may also protect pancreatic β cells from glucolipotoxicity. The recent discovery that loss of SLC30A8/ZnT8 function may increase the susceptibility to type 2 diabetes provides novel insights into the role of zinc in diabetes. Here, we discuss the roles of zinc in glucose homeostasis, particularly in relationship to ZnT8 function, based on our knowledge of mice and humans with ZnT8 loss-of-function mutations.

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13.1 Zinc and Pancreatic β Cells

Zinc was reported as an essential factor for insulin crystal formation in the 1930s (Scott 1934), and zinc content in the pancreas of diabetic patients was shown to be reduced by 50 % (Scott and Fisher 1938). These findings suggested a close relationship between zinc and diabetes. Follow-up studies have shown that insulin exists as a hexamer consisting of six insulin and two zinc molecules (Dodson et al. 1979; Dunn 2005) (Fig. 13.1). Crystallized insulin in insulin secretory granules is observed as "dense-core granules" upon electron microscopic analysis (Hou et al. 2009; Tamaki et al. 2013) (Fig. 13.2; ZnT8-KO mice).

After folding, the proinsulin protein forms dimers via electrostatic interactions. The residues B24–B26 (amino acids 24–26 of the B-chain) are thought to be important for this dimer formation (Zoete et al. 2005). In fact, amino acid substitutions of these residues yielded a series of ultra-rapid-acting insulin analogues that are now widely used in clinical practice (Brange et al. 1990; Kang et al. 1991). The electrostatically coupled proinsulin dimers and zinc bind with B10 histidine, resulting in proinsulin hexamer formation (Havu et al. 1977) (Fig. 13.1). This insulin hexamer formation is accomplished by prohormone convertase-mediated c-peptide dissociation (Steiner et al. 1996). Because the c-peptide portion of proinsulin is located outside the proinsulin hexamer, it has been considered that the formation of the hexamer contributes to c-peptide dissociation (Dunn 2005). Insulin crystals are formed under specific conditions within insulin granules in which both insulin and zinc exist in high concentrations and acid pH is maintained (Emdin et al. 1980; Hutton 1989). Given that the addition of zinc to commercial insulin products improves its stability, hexamer formation and crystallization are thought to similarly contribute to insulin stabilization in vivo. Once insulin crystals are secreted from pancreatic β cells, they are believed to rapidly dissociate into monomers and flow into the bloodstream.

13.2 Zinc Deficiency and Diabetes

The relationship between zinc deficiency and diabetes has been extensively examined in mouse models. Mice with zinc deficiency demonstrated decreased insulin granules in their pancreatic β cells (Boquist and Lernmark 1969), as well as impaired glucose-stimulated insulin secretion (GSIS) (Huber and Gershoff 1973). Because pancreatic β cells actively synthesize a large amount of ATP, they are prone to exposure to and damage by oxidative stress (Donath et al. 2005). Because zinc is an essential component of antioxidative enzymes, including Cu–Zn-SOD

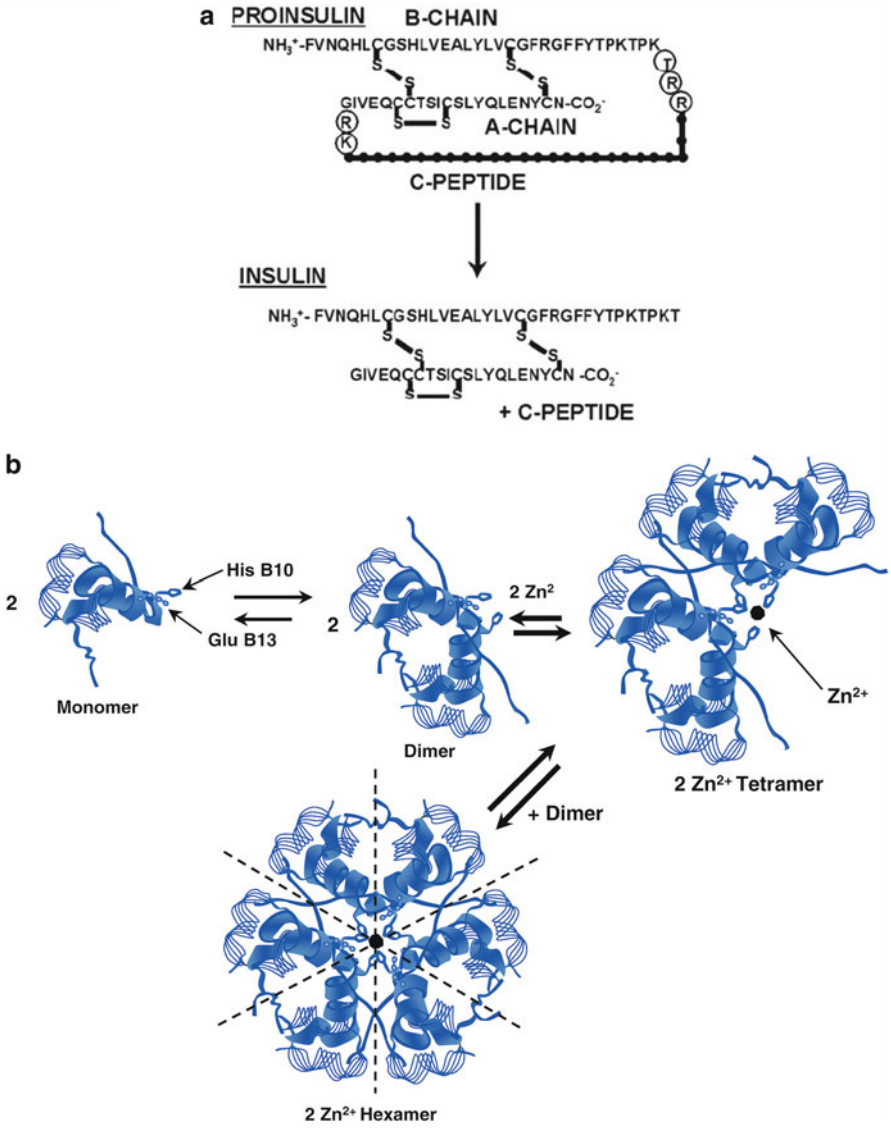

Fig. 13.1 The biosynthesis of insulin. **a** The insulin precursor preproinsulin contains a signal sequence that is proteolytically cleaved to yield proinsulin, in which the C-peptide links the future A- and B-chains of mature insulin. Cleavage of the C-peptide converts proinsulin to insulin. **b** Association of insulin monomers in the presence of zinc. Insulin readily associates into dimers, and then aggregates into hexameric forms in the presence of zinc. His B10 is implicated in the zinc-mediated insulin hexamer formation

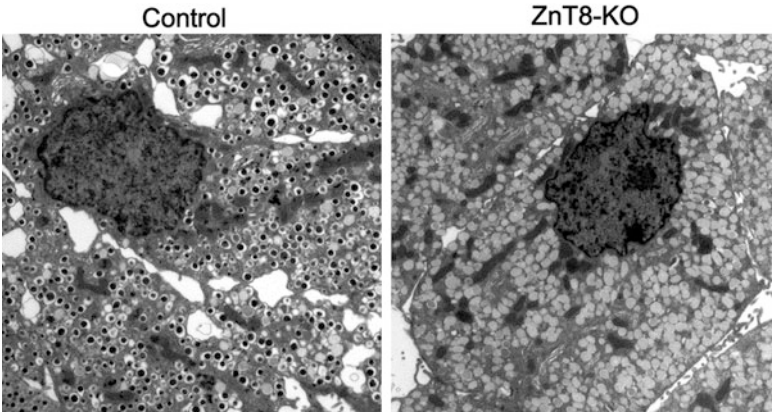

Fig. 13.2 Electron microscopic images of pancreatic β cells from a control and a ZnT8-KO mouse. Numerous dense-core granules are observed in the control β cells. ZnT8-KO β cells are devoid of dense-core granules, suggesting a deficiency in insulin crystal formation

(superoxide dismutase) (Mysore et al. 2005) or catalase (Marklund et al. 1982), zinc deficiency could worsen the condition of pancreatic β cells under oxidative stress. These findings support the idea that zinc deficiency may result in impaired pancreatic β-cell function and survival. The supplementation of zinc also improved insulin sensitivity in obese diabetic *db*/*db* or *ob*/*ob* mice (Begin-Heick et al. 1985; Simon and Taylor 2001). A recent study by Adachi et al. reported that oral administration of zinc (II) complex for 4 weeks to obese diabetic KKA^{y} mice significantly improved their glucose tolerance and insulin sensitivity, which was accompanied by increased plasma adiponectin levels (Adachi et al. 2006). The insulin-sensitizing effect of zinc has been explained by the inhibition of the tyrosine phosphatase activity of PTP1B (protein tyrosine phosphatase-1B) (Haase and Maret 2005). The enzymatic activity of PTP1B is inhibited by zinc ions. The concentration of available cellular zinc is in the nanomolar or picomolar range, and hence inhibition of PTPs by zinc occurs within this low range of available zinc (Haase and Maret 2003). Experiments in cell culture provided evidence for a physiological role of zinc in modulating PTP activity. Incubation of cells with zinc increases tyrosine phosphorylation of the insulin receptor, and its effects are mediated by the inhibition of dephosphorylation rather than enhanced phosphorylation. The concentration of cellular available zinc is not constant but fluctuates under various conditions. Zinc fluctuations are controlled by zinc-buffering systems, and one of these systems involves metallothionein (MT) (Maret 2009). Metallothionein and zinc have been implicated in cellular defense mechanisms against oxidative stress (Eibl et al. 2010). This finding is consistent with the observation that zinc administration to streptozotocin-induced diabetic rats reduced their glucose levels through robust induction of MT in the pancreas and liver (Yang and Cherian 1994). Thus, zinc appears to show antidiabetic activity not only through potentiating β-cell function

but also through insulin-sensitizing effects on the liver and other insulin-sensitive tissues.

In humans, a prospective cohort study employing 82,000 women in the United States revealed that women with low zinc intake have a 17 % increased risk of onset of diabetes compared to women who take adequate amounts of zinc (Sun et al. 2009). In a Brazilian study, 30 mg/day of zinc supplementation to obese women improved insulin sensitivity (Marreiro et al. 2006), and another cross-sectional study in North India reported that lower consumption of dietary zinc and low serum zinc levels are associated with an increased prevalence of coronary artery disease (CAD) and diabetes, as well as several of their associated risk factors including hypertension and hypertriglyceridemia (Singh et al. 1998). Recently, a negative correlation between plasma zinc concentrations and diabetes onset has been reported in China (Shan et al. 2014). Interestingly, this report describes the possible interaction of SLC30A8 [zinc transporter 8 (ZnT8)] dysfunction and decreased plasma zinc concentrations in regulating glucose tolerance and diabetes. According to this report, lower plasma zinc concentrations and decreased ZnT8 function coordinately increase the risk of diabetes. Although these data suggest that zinc supplementation can prevent the deterioration of glucose homeostasis, particularly in people with zinc deficiency, prospective interventional studies are necessary to confirm the efficacy of zinc supplementation in preventing diabetes onset.

13.3 Structure and Function of ZnT8

ZnT8 is a member of the ZnT family, which has a predicted membrane topology of six membrane-spanning domains with both N- and C-terminal ends, four conserved amino acid residues within transmembrane domains (TMDs) II and V, and a cytoplasmic His-rich loop between TMDs IV and V (Kambe 2012). ZnT8 is closely related to ZnT2, ZnT3, and ZnT4, which are members of a subfamily of ZnT proteins that is involved in secretory/synaptic vesicle transport and lysosomal/endosomal zinc storage (Chimienti et al. 2004). The expression of ZnT8 is two to three orders of magnitude higher in the islet than in all other tissues examined (Nicolson et al. 2009). Thus, ZnT8 is responsible for transporting zinc into insulin-containing granules in islet β cells, where insulin is stored as a hexamer bound with two zinc ions before secretion (Fig. 13.1b). ZnT8 is also expressed in other tissues, including islet α cells, pancreatic polypeptide (PP) cells, and a subset of endocrine cells in the thyroid and adrenal glands (Tamaki et al. 2009). The islet-specific expression of ZnT8 was shown to be regulated by the β-cell-enriched transcriptional factor Pdx-1 through an intronic enhancer (Pound et al. 2011).

Several studies have shown that ZnT8 is a bona fide zinc transporter by using ZnT8-overexpressed cells (Lefebvre et al. 2012), ZnT8-knockdown cells (Fu et al. 2009), or ZnT8-KO mice (Nicolson et al. 2009). The mechanism of zinc transport is still incompletely understood. However, experiments using fluorescence-based functional measurements have shown that ZnT transporters

function as Zn^{2+}/H^{+} exchangers (Ohana et al. 2009). The zinc transport activity of ZnT3 in neurons has been reported to be potentiated by chloride channels and vesicular glutamate transporters (Salazar et al. 2005; Salazar et al. 2004a). Similar mechanisms may also operate in ZnT8. Furthermore, similarly to ZnT3 in which synaptic localization is controlled by AP-3 (Salazar et al. 2004b), ZnT8 localization in the insulin granule might be controlled by other molecules, because the sorting signal of ZnT8 to the insulin granule has not been identified.

The expression of ZnT transporters is transcriptionally regulated by various stimuli. In β cells, it has been shown that glucose and cytokines play roles in the regulation of zinc transporter expression. Cytokines [interleukin (IL-1β), tumor necrosis factor (TNF-α)] decrease the expression of zinc transporters, particularly ZnT8 (El Muayed et al. 2010), suggesting that the expression of ZnT8 may be decreased during diabetes that accompanies pancreatitis. Moreover, ZnT8 expression was found to be remarkably downregulated in the early stages of diabetes in diabetic model mice, such as *db*/*db* and *Akita* (Tamaki et al. 2009). These findings demonstrated that the expression of ZnT8 is reduced during the course of hyperglycemia and inflammation in type 2 diabetes. Further analyses are required to determine the factors that might regulate ZnT8 expression in such situations.

Homology modeling studies based on the bacterial zinc transporter and ZnT homologue YiiP have shown that ZnT8 forms homodimers held together by four zinc ions located at the interface of the cytoplasmic domains, with two transmembrane domains swinging out to yield a Y-shaped structure (Nicolson et al. 2009). Recent genome-wide association studies demonstrated that the nonsynonymous single-nucleotide polymorphism rs1326634 in the *SLC30A8* gene, resulting in the replacement of tryptophan-325 with arginine, increases the risk of type 2 diabetes (Fig. 13.3) (Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research et al. 2007; Scott et al. 2007; Sladek et al. 2007). To examine the potential physiological differences between the low-risk and increased-risk variants of ZnT8, Guy Rutter's group showed,, using fluorescent dyes to monitor the vacuolar accumulation of zinc, that the increased-risk R-form of ZnT8 is less active as a zinc transporter (Nicolson et al. 2009).

13.4 The Role of ZnT8 in Glucose Homeostasis

In pancreatic β cells, ZnT8 is located within the plasma membrane of insulin secretory granules and is implicated in zinc transport into insulin secretory granules (Fig. 13.3) (Murgia et al. 2009). How is the transport of zinc into insulin granules related to the increased risk of diabetes onset? To address this question, we examined the physiological role of ZnT8 by generating pancreatic β-cell-specific Slc30a8-deficient mice (ZnT8-KO) (Tamaki et al. 2013).

As expected, immunohistochemical analysis demonstrated that ZnT8 expression is largely absent from ZnT8-KO islets. Furthermore, dense-core granules, a

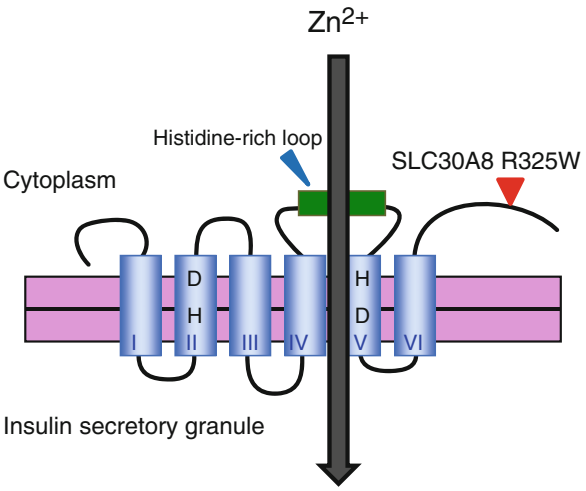

Fig. 13.3 Zinc transporter 8 encoded by SLC30A8 is localized on the membranes of insulin secretory granules. Like other ZnT family members, it contains six transmembrane domains (TMDs) and a histidine-rich loop between TMDs IV and V (thick green line). Four conserved amino acid residues (two histidine (H) and two aspartic acid (D)) are located within TMDs II and V. A nonsynonymous single nucleotide polymorphism affecting amino acids 325 on ZnT8 is implicated in type 2 diabetes from genome-wide association studies

hallmark of crystallized insulin usually seen in normal β cells, were absent in the ZnT8-KO β cells (Fig. 13.2). In agreement with these observations, intra-islet zinc content was severely depleted in ZnT8-KO mice. Because ZnT8-KO mice showed mildly impaired glucose tolerance, we expected insulin secretion from the ZnT8-KO islets to be decreased. Indeed, after glucose challenge, lower peripheral blood insulin levels were detected in ZnT8 mice compared with control mice (Tamaki et al. 2013). Glucose-stimulated insulin secretion (GSIS) from the isolated ZnT8-KO islets was, in contrast to our expectations, almost twice that from the control islets. Enhanced GSIS from islets usually results in increased blood insulin levels. Although enhanced insulin secretion from the ZnT8-KO islets was observed, insulin concentrations in the blood circulation of ZnT8-KO mice were lower than those in control mice, for some unknown reason. To explain this discrepancy, the pancreas perfusion experiment was performed. In this experiment, pancreata were perfused with a glucose solution, so that more physiologically relevant insulin secretion profiles can be assessed compared with experiments using isolated islets. Enhanced insulin secretion was still noted in ZnT8-KO islets upon pancreas perfusion, further supporting that insulin secretion is actually increased in vivo in ZnT8-KO islets. Next, to examine insulin metabolism, pancreas–liver dual perfusion was performed (Tamaki et al. 2013). A comparison of the results obtained by pancreas perfusion versus pancreas–liver perfusion revealed that a large proportion of the secreted insulin was degraded during the first liver passage.

The idea that ZnT8 regulates hepatic insulin clearance is intriguing. It has been reported that insulin clearance is dynamically altered before and after meals (Caumo et al. 2007). In the fasting state, clearance of unnecessary insulin by the liver plays a major role in preventing inappropriate hyperinsulinemia, which may otherwise cause hypoglycemia and obesity (Poy et al. 2002). In the postprandial state, on the other hand, hepatic insulin degradation is inhibited to ensure a sufficient supply of insulin to the peripheral target tissues. Although previous studies proposed that incretin hormones, such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), that are secreted with food intake regulate hepatic insulin clearance (Ahren et al. 2005; Rudovich et al. 2004), a later study argued against such a possibility (Meier et al. 2007). Another report suggested that the insulin pulse mass from pulsatile insulin secretion into the portal vein is involved in suppressing the hepatic insulin clearance rate (Meier et al. 2005), but the mechanism underlying this process remains largely unknown. As it was reported that zinc secretion is absent in ZnT8-KO islets because of the loss of zinc storage within secretory granules (Lemaire et al. 2009), we then speculated the possibility that the zinc co-secreted with insulin from β cells may have some regulatory roles in insulin clearance. A set of in vivo and in vitro experiments revealed that zinc inhibits hepatic insulin uptake by counteracting clathrin-mediated endocytosis of the insulin receptor (Ceresa et al. 1998). Because of the suppressive role of zinc in hepatic insulin clearance, glucose tolerance can be maintained by optimizing hepatic insulin degradation (Fig. 13.4).

The next question is this: Why is insulin secretion from ZnT8-KO islets enhanced? It was reported that zinc secreted from β cells can affect neighboring endocrine cells (Ishihara et al. 2003) (Kim et al. 2000). Previous studies showed that zinc binds to specific histidine residues of the K-ATP channel and thus hyperpolarizes pancreatic β cells by enhancing the outward current of the K-ATP channel (Bancila et al. 2005; Prost et al. 2004). Therefore, we hypothesized that the absence of zinc secretion from β cells causes derepression of insulin hypersecretion from ZnT8-KO islets. As expected, addition of zinc (30 μM) to the perfusate during pancreas perfusion markedly suppressed insulin hypersecretion in ZnT8-KO mice, almost to control levels. These results indicated that the zinc co-secreted with insulin from β cells acts to decrease the insulin secretion of neighboring β cells (Fig. 13.3). These findings are consistent with a previous report by Nicolson et al. in which similar hypersecretion of insulin from ZnT8-deficient islets was observed (Nicolson et al. 2009).

There is increasing evidence that glucagon secretion is regulated by various stimuli, including insulin, glucose (Gylfe and Gilon 2014), amino acids (Cabrera et al. 2008), amylin (Young 2005), GLP-1 (Meier and Nauck 2005), and zinc (Egefjord et al. 2010). Whether zinc can suppress glucagon secretion (Ishihara et al. 2003) (Slucca et al. 2010) or cannot (Ravier and Rutter 2005) is currently an active topic of debate. As two independent studies reported that there was no alteration in glucagon secretion in ZnT8 global KO mice (Nicolson et al. 2009), we measured glucagon secretion from islets isolated from pancreatic β-cell-specific ZnT8-KO mice and controls. The average glucagon secretion from ZnT8-KO islets

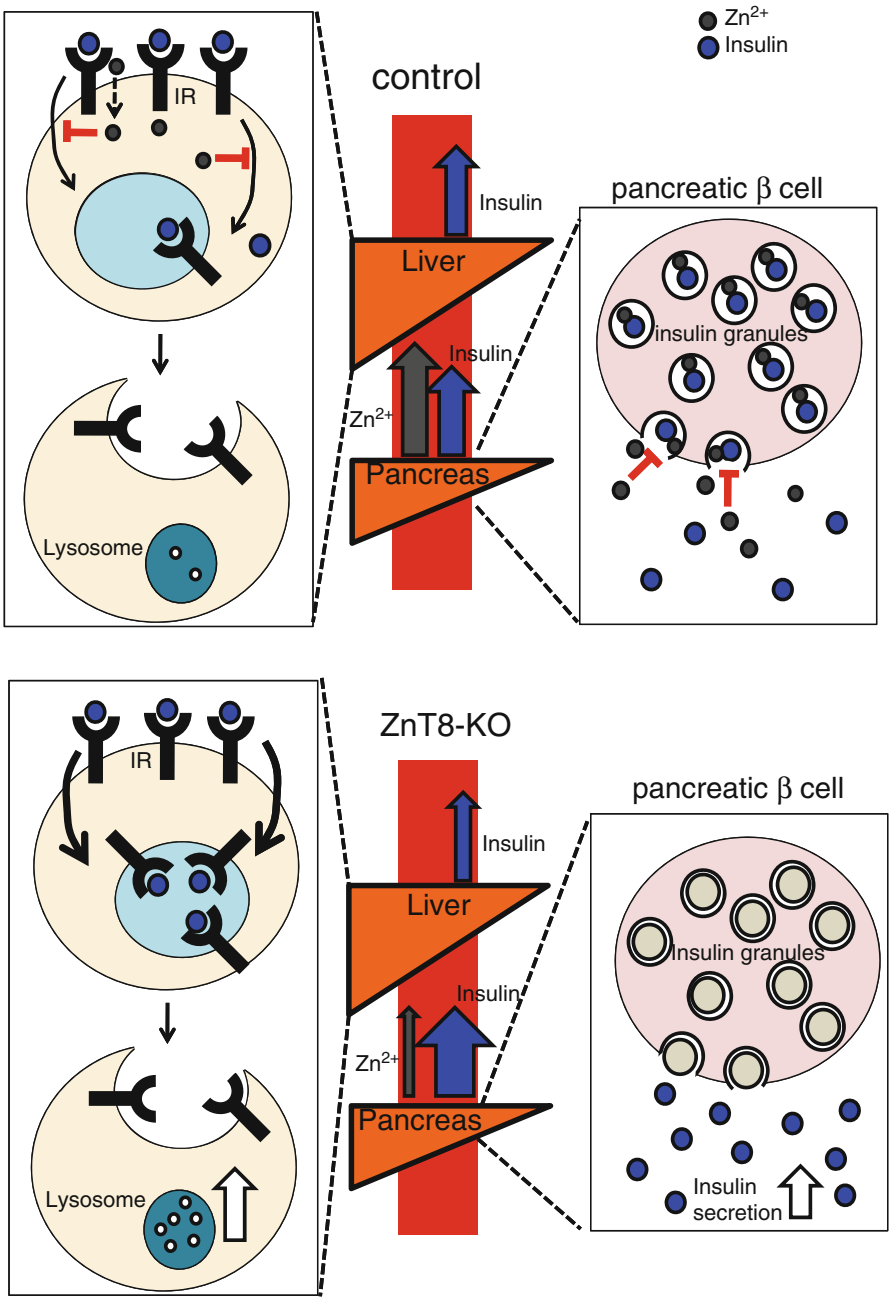

Fig. 13.4 Schematic representation of insulin clearance in control and ZnT8-KO mice. Zinc co-secreted with insulin suppresses insulin secretion from pancreatic β cells (*upper right*) and inhibits hepatic insulin clearance (*upper left*) in *control* mice. Although the reduced zinc secretion allows enhanced insulin secretion from β cells in ZnT8-KO mice (*lower right*), hepatic insulin clearance is not suppressed (*lower left*). Thus, peripheral insulin levels in ZnT8-KO mice are maintained at lower levels than in control mice IR: insulin receptor

under a low glucose condition appeared to be larger than that from control islets, but there was no statistically significant difference. We also measured blood glucagon concentrations to test whether glucose-stimulated insulin plus zinc secretion can suppress glucagon secretion in vivo. However, there were no differences in the levels of glucagon suppression between ZnT8-KO and control mice after glucose challenge. In conclusion, consistent with the reports by Nicolson et al. (2009) and Hardy et al. (2011), glucagon secretion was not changed in ZnT8-KO mice.

13.5 Insulin Metabolism and Hepatic Insulin Clearance

Insulin secreted from the islets of Langerhans flows directly into the portal vein (PV). About half the insulin that enters the liver is cleared; the rest flows into the systemic circulation (Eaton et al. 1983). Thus, the rate of hepatic insulin clearance is an important regulator of peripheral insulin levels. In the postprandial state, hepatic insulin clearance is estimated to be suppressed by 20 % (Caumo et al. 2007). Insulin clearance by the liver involves the following three steps: (i) binding of insulin to the insulin receptor (IR) on the cell surface of hepatocytes, (ii) internalization of insulin via IR-mediated endocytosis, and (iii) degradation of insulin by insulin-degrading enzyme (IDE) within endosomes (Authier et al. 1996). We found that zinc does not affect either steps (i) or (iii) and that step (ii) is the zinc-sensitive process. It was reported that endocytosis of the transferrin receptor in hepatocytes is inhibited by zinc in a clathrin-dependent manner (Liu et al. 2010; McAbee and Jiang 1999). The same mechanism appears to be involved in endocytosis of the insulin receptor (IR). Blockade of IR endocytosis was enhanced by pyrithione, a zinc ionophore, suggesting that internalized zinc is responsible for the inhibitory effects. However, the effect was independent of the zinc transporter ZIP14 (Hojyo et al. 2011).

As already discussed, ZnT8 has a pivotal role in determining the levels of insulin to be delivered to the liver versus other peripheral organs, and thus optimizes the effect of insulin on whole-body glucose metabolism. Insulin and c-peptide are generated from proinsulin at a 1:1 ratio by prohormone convertase-mediated digestion (Fig. 13.1a). Although c-peptide, which is not degraded by the liver, represents insulin secretion per se from pancreatic β cells, insulin is dynamically degraded during liver passage. Accordingly, the c-peptide/insulin ratio can be used as a marker for assessing insulin clearance in vivo (Meier et al. 2007; Poy et al. 2002). In wild-type animals, the released Zn^{2+} inhibited further insulin release whereas loss of ZnT8 resulted in sustained insulin secretion (Tamaki et al. 2013). Intriguingly, despite increased insulin secretion, peripheral insulin levels were lower and the c-peptide/insulin ratios were increased in KO mice (Tamaki et al. 2013). These data are in agreement with the idea that lower insulin levels might result from increased insulin clearance from the bloodstream, rather than

decreased insulin secretion from β cells. Studies of c-peptide/insulin ratios and rates of insulin clearance in humans with the SLC30A8 R325W polymorphism were also consistent with this idea (Tamaki et al. 2013).

13.6 Clinical Significance of Zinc-Mediated Pancreas–Liver Communication

In the Eugene study, human homozygous carriers of the *SLC30A8* risk allele had low peripheral insulin levels in the early phase of intravenous glucose tolerance test (i.v. GTT) (Boesgaard et al. 2008). However, in previous studies that characterized the ZnT8-deficient mice, there was no agreement on the underlying mechanism by which *SLC30A8* regulates the susceptibility to type 2 diabetes (Lemaire et al. 2009; Nicolson et al. 2009; Pound et al. 2009; Wijesekara et al. 2010). Furthermore, there is a lack of consensus on the effect of *Slc30a8* deletion on insulin secretion and circulation in mice, as summarized in the recent mini-review by O'Halloran et al. (2013). Although some studies reported that deletion of SLC30A8/ZnT8 results in impaired glucose tolerance (Nicolson et al. 2009; Wijesekara et al. 2010), others reported that loss of ZnT8 exhibits essentially no change in glucose tolerance (Lemaire et al. 2009; Pound et al. 2009). These differences might have been caused by differences in the study designs or the genetic backgrounds of the mice generated (Pound et al. 2012; Tamaki et al. 2013). We demonstrated that SLC30A8/ZnT8 regulates hepatic insulin clearance, by extensive examination of β-cell-specific ZnT8-KO mice, and importantly, the same mechanism appears to be conserved also in humans (Tamaki et al. 2013). In general clinical practice, hypoinsulinemia is usually interpreted to result from low insulin secretion by pancreatic β cells. However, this cannot be confirmed without measuring insulin secretion from the pancreas, because insulin levels in the systemic circulation are determined by a balance between insulin secretion and insulin clearance. Our study highlighted a zinc-mediated, novel inter-organ communication between the pancreas and liver; together with the pancreas, the liver is actively involved in the regulation of postprandial plasma insulin levels by modulating insulin clearance (Fig. 13.4). Indeed, inhibition of hepatic insulin clearance has been a target for the pharmacological intervention of diabetes. A previous study reported the therapeutic potential of designed small molecule inhibitors of insulin-degrading enzyme (IDE) that regulate the catabolism of insulin (Leissring et al. 2010). Our findings provide a novel insight into the molecular pathology of diabetes: dysregulated hepatic insulin clearance, which could be an attractive future therapeutic target for diabetes.

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Note added in the proof During the author's proofreading of this manuscript, Flannick et al. report 12 rare protein-truncating mutations in *SLC30A8*, which collectively explain a 65% reduction in diabetes risk (Flannick et al. 2014 Nat Genet 46(4):357–363). They report a study including genotyping and sequencing of ~150,000 individuals that concludes that rare loss-of-function mutations in *SCL30A8* are protective against the development of diabetes. This finding challenges the recent studies suggesting the reverse relationship in this disease association.

References

Adachi Y, Yoshida J, Kodera Y, Kiss T, Jakusch T, Enyedy EA, Yoshikawa Y, Sakurai H (2006) Oral administration of a zinc complex improves type 2 diabetes and metabolic syndromes. Biochem Biophys Res Commun 351:165–170

Ahren B, Thomaseth K, Pacini G (2005) Reduced insulin clearance contributes to the increased insulin levels after administration of glucagon-like peptide 1 in mice. Diabetologia 48:2140–2146

Authier F, Posner BI, Bergeron JJ (1996) Insulin-degrading enzyme. Clin Invest Med 19:149–160

Bancila V, Cens T, Monnier D, Chanson F, Faure C, Dunant Y, Bloc A (2005) Two SUR1-specific histidine residues mandatory for zinc-induced activation of the rat KATP channel. J Biol Chem 280:8793–8799

Begin-Heick N, Dalpe-Scott M, Rowe J, Heick HM (1985) Zinc supplementation attenuates insulin secretory activity in pancreatic islets of the ob/ob mouse. Diabetes 34:179–184

Boesgaard TW, Zilinskaite J, Vanttinen M, Laakso M, Jansson PA, Hammarstedt A, Smith U, Stefan N, Fritsche A, Haring H et al (2008) The common SLC30A8 Arg325Trp variant is associated with reduced first-phase insulin release in 846 non-diabetic offspring of type 2 diabetes patients–the EUGENE2 study. Diabetologia 51:816–820

Boquist L, Lernmark A (1969) Effects on the endocrine pancreas in Chinese hamsters fed zinc deficient diets. Acta Pathol Microbiol Scand 76:215–228

Brange J, Owens DR, Kang S, Volund A (1990) Monomeric insulins and their experimental and clinical implications. Diabetes Care 13:923–954

Cabrera O, Jacques-Silva MC, Speier S, Yang SN, Kohler M, Fachado A, Vieira E, Zierath JR, Kibbey R, Berman DM et al (2008) Glutamate is a positive autocrine signal for glucagon release. Cell Metab 7:545–554

Caumo A, Florea I, Luzi L (2007) Effect of a variable hepatic insulin clearance on the postprandial insulin profile: insights from a model simulation study. Acta Diabetol 44:23–29

Ceresa BP, Kao AW, Santeler SR, Pessin JE (1998) Inhibition of clathrin-mediated endocytosis selectively attenuates specific insulin receptor signal transduction pathways. Mol Cell Biol 18:3862–3870

Chimienti F, Devergnas S, Favier A, Seve M (2004) Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. Diabetes 53:2330–2337

Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research, Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, Roix JJ et al (2007) Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science 316:1331–1336

Dodson EJ, Dodson GG, Hodgkin DC, Reynolds CD (1979) Structural relationships in the two-zinc insulin hexamer. Can J Biochem 57:469–479

Donath MY, Ehses JA, Maedler K, Schumann DM, Ellingsgaard H, Eppler E, Reinecke M (2005) Mechanisms of beta-cell death in type 2 diabetes. Diabetes 54(suppl 2):S108–S113

Dunn MF (2005) Zinc–ligand interactions modulate assembly and stability of the insulin hexamer: a review. Biometals 18:295–303

Eaton RP, Allen RC, Schade DS (1983) Hepatic removal of insulin in normal man: dose response to endogenous insulin secretion. J Clin Endocrinol Metab 56:1294–1300
Egefjord L, Petersen AB, Rungby J (2010) Zinc, alpha cells and glucagon secretion. Curr Diabetes Rev 6:52–57
Eibl JK, Abdallah Z, Ross GM (2010) Zinc-metallothionein: a potential mediator of antioxidant defence mechanisms in response to dopamine-induced stress. Can J Physiol Pharmacol 88:305–312
El Muayed M, Billings LK, Raja MR, Zhang X, Park PJ, Newman MV, Kaufman DB, O'Halloran TV, Lowe WL Jr (2010) Acute cytokine-mediated downregulation of the zinc transporter ZnT8 alters pancreatic beta-cell function. J Endocrinol 206:159–169
Emdin SO, Dodson GG, Cutfield JM, Cutfield SM (1980) Role of zinc in insulin biosynthesis. Some possible zinc-insulin interactions in the pancreatic B-cell. Diabetologia 19:174–182
Fu Y, Tian W, Pratt EB, Dirling LB, Shyng SL, Meshul CK, Cohen DM (2009) Downregulation of ZnT8 expression in INS-1 rat pancreatic beta cells reduces insulin content and glucose-inducible insulin secretion. PLoS One 4:e5679
Gylfe E, Gilon P (2014) Glucose regulation of glucagon secretion. Diabetes Res Clin Pract 103:1–10
Haase H, Maret W (2003) Intracellular zinc fluctuations modulate protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Exp Cell Res 291:289–298
Haase H, Maret W (2005) Protein tyrosine phosphatases as targets of the combined insulinomimetic effects of zinc and oxidants. Biometals 18:333–338
Hardy AB, Serino AS, Wijesekara N, Chimienti F, Wheeler MB (2011) Regulation of glucagon secretion by zinc: lessons from the beta cell-specific Znt8 knockout mouse model. Diabetes Obes Metab 13(suppl 1):112–117
Havu N, Lundgren G, Falkmer S (1977) Zinc and manganese contents of micro-dissected pancreatic islets of some rodents. A microchemical study in adult and newborn guinea pigs, rats, Chinese hamsters and spiny mice. Acta Endocrinol (Copenh) 86:570–577
Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin BH, Koseki H, Hirano T (2011) The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. PLoS One 6:e18059
Hou JC, Min L, Pessin JE (2009) Insulin granule biogenesis, trafficking and exocytosis. Vitam Horm 80:473–506
Huber AM, Gershoff SN (1973) Effect of zinc deficiency in rats on insulin release from the pancreas. J Nutr 103:1739–1744
Hutton JC (1989) The insulin secretory granule. Diabetologia 32:271–281
Ishihara H, Maechler P, Gjinovci A, Herrera PL, Wollheim CB (2003) Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. Nat Cell Biol 5:330–335
Kambe T (2012) Molecular architecture and function of ZnT transporters. Curr Top Membr 69:199–220
Kang S, Brange J, Burch A, Volund A, Owens DR (1991) Subcutaneous insulin absorption explained by insulin's physicochemical properties. Evidence from absorption studies of soluble human insulin and insulin analogues in humans. Diabetes Care 14:942–948
Kim BJ, Kim YH, Kim S, Kim JW, Koh JY, Oh SH, Lee MK, Kim KW, Lee MS (2000) Zinc as a paracrine effector in pancreatic islet cell death. Diabetes 49:367–372
Lefebvre B, Vandewalle B, Balavoine AS, Queniat G, Moerman E, Vantyghem MC, Le Bacquer O, Gmyr V, Pawlowski V, Kerr-Conte J et al (2012) Regulation and functional effects of ZNT8 in human pancreatic islets. J Endocrinol 214:225–232
Leissring MA, Malito E, Hedouin S, Reinstatler L, Sahara T, Abdul-Hay SO, Choudhry S, Maharvi GM, Fauq AH, Huzarska M et al (2010) Designed inhibitors of insulin-degrading enzyme regulate the catabolism and activity of insulin. PLoS One 5:e10504
Lemaire K, Ravier MA, Schraenen A, Creemers JW, Van de Plas R, Granvik M, Van Lommel L, Waelkens E, Chimienti F, Rutter GA et al (2009) Insulin crystallization depends on zinc

transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. Proc Natl Acad Sci USA 106:14872–14877
Liu AP, Aguet F, Danuser G, Schmid SL (2010) Local clustering of transferrin receptors promotes clathrin-coated pit initiation. J Cell Biol 191:1381–1393
Maret W (2009) Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals. Biometals 22:149–157
Marklund SL, Westman NG, Lundgren E, Roos G (1982) Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. Cancer Res 42:1955–1961
Marreiro DN, Geloneze B, Tambascia MA, Lerario AC, Halpern A, Cozzolino SM (2006) Effect of zinc supplementation on serum leptin levels and insulin resistance of obese women. Biol Trace Elem Res 112:109–118
McAbee DD, Jiang X (1999) Copper and zinc ions differentially block asialoglycoprotein receptor-mediated endocytosis in isolated rat hepatocytes. J Biol Chem 274:14750–14758
Meier JJ, Nauck MA (2005) Glucagon-like peptide 1 (GLP-1) in biology and pathology. Diabetes Metab Res Rev 21:91–117
Meier JJ, Veldhuis JD, Butler PC (2005) Pulsatile insulin secretion dictates systemic insulin delivery by regulating hepatic insulin extraction in humans. Diabetes 54:1649–1656
Meier JJ, Holst JJ, Schmidt WE, Nauck MA (2007) Reduction of hepatic insulin clearance after oral glucose ingestion is not mediated by glucagon-like peptide 1 or gastric inhibitory polypeptide in humans. Am J Physiol Endocrinol Metab 293:E849–E856
Murgia C, Devirgiliis C, Mancini E, Donadel G, Zalewski P, Perozzi G (2009) Diabetes-linked zinc transporter ZnT8 is a homodimeric protein expressed by distinct rodent endocrine cell types in the pancreas and other glands. Nutr Metab Cardiovasc Dis 19:431–439
Mysore TB, Shinkel TA, Collins J, Salvaris EJ, Fisicaro N, Murray-Segal LJ, Johnson LE, Lepore DA, Walters SN, Stokes R et al (2005) Overexpression of glutathione peroxidase with two isoforms of superoxide dismutase protects mouse islets from oxidative injury and improves islet graft function. Diabetes 54:2109–2116
Nicolson TJ, Bellomo EA, Wijesekara N, Loder MK, Baldwin JM, Gyulkhandanyan AV, Koshkin V, Tarasov AI, Carzaniga R, Kronenberger K et al (2009) Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. Diabetes 58:2070–2083
O'Halloran TV, Kebede M, Philips SJ, Attie AD (2013) Zinc, insulin, and the liver: a menage a trois. J Clin Invest 123:4136–4139
Ohana E, Hoch E, Keasar C, Kambe T, Yifrach O, Hershfinkel M, Sekler I (2009) Identification of the Zn^{2+} binding site and mode of operation of a mammalian Zn^{2+} transporter. J Biol Chem 284:17677–17686
Pound LD, Sarkar SA, Benninger RK, Wang Y, Suwanichkul A, Shadoan MK, Printz RL, Oeser JK, Lee CE, Piston DW et al (2009) Deletion of the mouse Slc30a8 gene encoding zinc transporter-8 results in impaired insulin secretion. Biochem J 421:371–376
Pound LD, Sarkar SA, Cauchi S, Wang Y, Oeser JK, Lee CE, Froguel P, Hutton JC, O'Brien RM (2011) Characterization of the human SLC30A8 promoter and intronic enhancer. J Mol Endocrinol 47:251–259
Pound LD, Sarkar SA, Ustione A, Dadi PK, Shadoan MK, Lee CE, Walters JA, Shiota M, McGuinness OP, Jacobson DA et al (2012) The physiological effects of deleting the mouse SLC30A8 gene encoding zinc transporter-8 are influenced by gender and genetic background. PLoS One 7:e40972
Poy MN, Yang Y, Rezaei K, Fernstrom MA, Lee AD, Kido Y, Erickson SK, Najjar SM (2002) CEACAM1 regulates insulin clearance in liver. Nat Genet 30:270–276
Prost AL, Bloc A, Hussy N, Derand R, Vivaudou M (2004) Zinc is both an intracellular and extracellular regulator of KATP channel function. J Physiol 559:157–167

Ravier MA, Rutter GA (2005) Glucose or insulin, but not zinc ions, inhibit glucagon secretion from mouse pancreatic alpha-cells. Diabetes 54:1789–1797

Rudovich NN, Rochlitz HJ, Pfeiffer AF (2004) Reduced hepatic insulin extraction in response to gastric inhibitory polypeptide compensates for reduced insulin secretion in normal-weight and normal glucose tolerant first-degree relatives of type 2 diabetic patients. Diabetes 53:2359–2365

Salazar G, Love R, Styers ML, Werner E, Peden A, Rodriguez S, Gearing M, Wainer BH, Faundez V (2004a) AP-3-dependent mechanisms control the targeting of a chloride channel (ClC-3) in neuronal and non-neuronal cells. J Biol Chem 279:25430–25439

Salazar G, Love R, Werner E, Doucette MM, Cheng S, Levey A, Faundez V (2004b) The zinc transporter ZnT3 interacts with AP-3 and it is preferentially targeted to a distinct synaptic vesicle subpopulation. Mol Biol Cell 15:575–587

Salazar G, Craige B, Love R, Kalman D, Faundez V (2005) Vglut1 and ZnT3 co-targeting mechanisms regulate vesicular zinc stores in PC12 cells. J Cell Sci 118:1911–1921

Scott DA (1934) Crystalline insulin. Biochem J 28(4):1592–1602

Scott DA, Fisher AM (1938) The insulin and the zinc content of normal and diabetic pancreas. J Clin Invest 17:725–728

Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ, Li Y, Duren WL, Erdos MR, Stringham HM, Chines PS, Jackson AU et al (2007) A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. Science 316:1341–1345

Shan Z, Bao W, Zhang Y, Rong Y, Wang X, Jin Y, Song Y, Yao P, Sun C, Hu FB et al (2014) Interactions between zinc transporter-8 gene (SLC30A8) and plasma zinc concentrations for impaired glucose regulation and type 2 diabetes. Diabetes 63:1796–1803

Simon SF, Taylor CG (2001) Dietary zinc supplementation attenuates hyperglycemia in db/db mice. Exp Biol Med (Maywood) 226:43–51

Singh RB, Niaz MA, Rastogi SS, Bajaj S, Gaoli Z, Shoumin Z (1998) Current zinc intake and risk of diabetes and coronary artery disease and factors associated with insulin resistance in rural and urban populations of North India. J Am Coll Nutr 17:564–570

Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S et al (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature (Lond) 445:881–885

Slucca M, Harmon JS, Oseid EA, Bryan J, Robertson RP (2010) ATP-sensitive K^+ channel mediates the zinc switch-off signal for glucagon response during glucose deprivation. Diabetes 59:128–134

Steiner DF, Rouille Y, Gong Q, Martin S, Carroll R, Chan SJ (1996) The role of prohormone convertases in insulin biosynthesis: evidence for inherited defects in their action in man and experimental animals. Diabetes Metab 22:94–104

Sun Q, van Dam RM, Willett WC, Hu FB (2009) Prospective study of zinc intake and risk of type 2 diabetes in women. Diabetes Care 32:629–634

Tamaki M, Fujitani Y, Uchida T, Hirose T, Kawamori R, Watada H (2009) Downregulation of ZnT8 expression in pancreatic beta-cells of diabetic mice. Islets 1:124–128

Tamaki M, Fujitani Y, Hara A, Uchida T, Tamura Y, Takeno K, Kawaguchi M, Watanabe T, Ogihara T, Fukunaka A et al (2013) The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. J Clin Invest 123:4513–4524

Wijesekara N, Dai FF, Hardy AB, Giglou PR, Bhattacharjee A, Koshkin V, Chimienti F, Gaisano HY, Rutter GA, Wheeler MB (2010) Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion. Diabetologia 53:1656–1668

Yang J, Cherian MG (1994) Protective effects of metallothionein on streptozotocin-induced diabetes in rats. Life Sci 55:43–51

Young A (2005) Inhibition of glucagon secretion. Adv Pharmacol 52:151–171

Zoete V, Meuwly M, Karplus M (2005) Study of the insulin dimerization: binding free energy calculations and per-residue free energy decomposition. Proteins 61:79–93

Chapter 14
Zinc Signaling and Cancer

Thirayost Nimmanon and Kathryn M. Taylor

Abstract The intracellular level of zinc is tightly controlled by ZnT (SLC30A) and ZIP (SLC39A) zinc transport proteins, as well as zinc-binding proteins such as metallothioneins. The ZIP channels are responsible for zinc influx into the cytoplasm, either from the extracellular space or intracellular storage compartments, such as the endoplasmic reticulum and the Golgi, whereas the ZnT transporters transport zinc in the opposite direction. Malfunctions of some zinc transport proteins, resulting in cellular zinc dyshomeostasis and subsequent effects on zinc signaling pathways, have been associated with cancer in a tissue-specific manner. In this chapter we detail what is known about the association between zinc channel and transporter dysregulations and the impact that this has on zinc signaling in different cancers. A particular emphasis is placed on the types of cancer in which the role of zinc dyshomeostasis on carcinogenesis or cancer progression has been most thoroughly investigated, including cancers of the breast, prostate, liver, pancreas, and colorectum. Posttranslational modification by phosphorylation as a novel regulatory mechanism of ZIP channels is also discussed as an important mechanism that may provide a clinical biomarker or target mechanism.

Keywords Cancer • SLC30A • SLC39A • Zinc channel • Zinc transport • ZIP6 • ZIP7

14.1 Introduction

As has already been described in previous chapters, zinc is clearly an essential trace element of extraordinary biological and public health significance (Hambidge and Krebs 2007). It is the only metal that can be found in all six enzyme classes, according to the IUPAC system of enzyme classification (Food and Nutrition Board of Institute of Medicine 2001). Recent bioinformatic analysis has revealed that 2,800 to 3,200 human proteins, or approximately 10 % of the proteome, are predicted to be zinc-binding proteins (Andreini et al. 2006; Passerini et al. 2007),

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and more than 1,000, or 3 %, of all 32,000 identified genes in the human genome encode proteins with zinc-finger domains (Maret 2001). Not surprisingly, zinc is known to play an important role in various biological processes that are essential for both cell survival and cell death (John et al. 2010), including signal transduction, gene expression, and apoptosis (Hambidge and Krebs 2007). Zinc not only protects the body from invaders by promoting the functions of immune cells (John et al. 2010), but it also protects cells from free radical damage by modulating the free radical scavenging system (Oteiza 2012). Through its functional and structural association with enzymes, zinc is also involved in the metabolism of carbohydrates, lipids, proteins, nucleic acids, and other micronutrients, as well as control of cellular proliferation, growth, and differentiation (World Health Organization and Food and Agriculture Organization of the United Nations 2002). Mechanistically, intracellular zinc is suggested to exert its functions in three interrelated biological activities, consisting of catalysis, structural arrangement of proteins, and regulation of cellular events (Swinkels et al. 1994).

Furthermore, zinc has recently been classified as an intracellular second messenger, because it is capable of transducing an extracellular stimulus into intracellular signaling cascades, which was first experimentally demonstrated in mast cells (Yamasaki et al. 2007). In this study, the intracellular zinc was shown to be increased by release from intracellular storage compartments, such as the endoplasmic reticulum (ER), which was termed a zinc wave (Yamasaki et al. 2007). This intracellular store release of zinc in the form of a zinc wave results in activation of multiple intracellular signaling pathways and subsequent stimulation of the growth potential of cells (Taylor et al. 2008). Thereafter, the role of zinc as a second messenger has been further confirmed in other types of immune cells, such as lymphocytes (Yu et al. 2011), as well as breast cancer cells (Taylor et al. 2012a). These data confirm that an extracellular stimulus of cells can produce a store release of zinc inside the cell with dramatic effects on cellular signaling, manifesting a response on the time scale of minutes, independently of the much slower transcriptional and DNA-binding effects of zinc, which require hours or days to attain. This demonstration of zinc as a second messenger has elevated zinc signaling to an intracellular role potentially as important as that of calcium, yet relatively little is known about the mechanism of how zinc is released within cells and its relevance to disease.

14.2 Control of Intracellular Zinc Level

Both zinc deficiency and excess result in detrimental cellular consequences (Plum et al. 2010). For example, zinc deficiency abrogates cell proliferation (MacDonald 2000) and induces programmed cell death or apoptosis (Fraker 2005), whereas zinc excess results in apoptosis and necrosis (Kim et al. 1999). Therefore, it is necessary for a cell to tightly regulate the cellular level of free zinc. This control is contributed by three groups of proteins, consisting of ZnT transporters (formerly called cation

diffusion family, SLC30A), ZIP channels (Zrt- and Irt-like proteins, SLC39A), and cytosolic zinc-binding proteins, particularly metallothioneins (Cousins et al. 2006; Lichten and Cousins 2009). The ZnT transporters reduce cytosolic zinc concentration by promoting zinc efflux from the cytoplasm to the extracellular space or intracellular compartments depending on their cellular location, whereas the ZIP channels increase cytosolic zinc bioavailability by mobilizing zinc in the opposite direction (Fukada and Kambe 2011; Lichten and Cousins 2009). Additionally, the cysteine-rich metallothioneins also play a crucial role in intracellular zinc homeostasis by orchestrating the zinc buffering and muffling reactions and serving as an intracellular zinc supply for redox signaling (Colvin et al. 2010). The buffering reaction occurs under steady-state conditions to regulate a cytosolic free zinc concentration within picomolar levels, whereas the muffling reaction modulates the cytosolic free zinc concentration that transiently changes under a non-steady-state situation (Colvin et al. 2010). According to the muffler model, the increase in intracellular free zinc level, which results from either zinc uptake from the extracellular space or zinc release from the intracellular stores after activation, is tightly regulated by the binding of the zinc ion to the muffler metallothioneins before delivery into the internal storage compartments or by removal from the cells (Colvin et al. 2010). A further detailed description of this mechanism has already been provided in Chap. 2.

14.3 Classification and Predicted Membrane Topology of Zinc Transport Proteins

Most zinc transport proteins that have been associated with cancer belong to the LIV-1 subfamily of the ZIP channels, which constitute the main focus of this chapter. However some information on ZnT transporters and cancer does exist and is also mentioned.

14.3.1 ZIP Channels

The ZIP channels (SLC39A) are zinc influx transport proteins mobilizing zinc into the cytoplasm, either from the extracellular space or intracellular stores, such as the ER or the Golgi, depending on their cellular location. There are 14 human members of this family, ZIP1 to ZIP14 (Cousins et al. 2006; Eide 2004). Phylogenetically, the ZIP channels can be classified into four subfamilies: subfamily I (ZIP9), subfamily II (ZIP1, ZIP2, ZIP3), gufA subfamily (ZIP11), and the most recently characterized LIV-1 subfamily (ZIP4–ZIP8, ZIP10, ZIP12–ZIP14, previously called LZT) (Taylor and Nicholson 2003).

Structurally, the ZIP channels are predicted to have eight transmembrane (TM) domains with extracellular or extracytoplasmic amino- and carboxyl-termini,

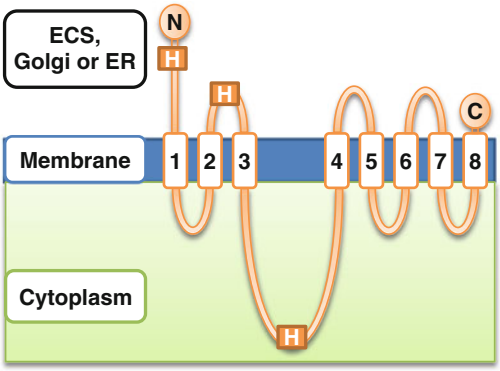

Fig. 14.1 Topological model of Zrt- and Irt-like protein (ZIP) channels. The predicted structure of the ZIP channels proposes they have eight transmembrane (TM) domains, with a long amino-terminus, a long histidine-rich intracytoplasmic loop between TM III and TM IV, and a short carboxyl-terminus. *ECS* extracellular space, *ER* endoplasmic reticulum, *H* histidine

and a long variable histidine-rich intracytoplasmic loop between TM III and IV, which is predicted to be a metal-binding site (Fig. 14.1) (Taylor and Nicholson 2003). In contrast to the other subfamilies of ZIP channels, those belonging to the LIV-1 subfamily have some unique features, including additional histidine-rich regions in the extracellular amino-terminus and the extracellular loop between TM II and III. They also have a unique motif HEXPHEXGD (where H = histidine, E = glutamate, P = proline, D = aspartate, G = glycine and X = any amino acid) in TM V (Taylor and Nicholson 2003) which is the consensus motif for this subfamily of zinc channels and fits the catalytic zinc-binding site of the zinc in PDF metalloproteases (Taylor and Nicholson 2003).

All the ZIP channels are localized to the plasma membrane, being responsible for zinc uptake from the extracellular space, except ZIP7, which has been demonstrated on the intracellular membranes of the ER (Taylor et al. 2004) and the Golgi (Huang et al. 2005), and ZIP13, which is found in the Golgi (Bin et al. 2011). The unique locations of ZIP7 and ZIP13 suggest that they have a role in mobilization of zinc from the intracellular stores into the cytoplasm. As a result of its unique location in the cells, ubiquitous expression throughout the body (Taylor et al. 2004), and the recently discovered posttranslational activation mechanism by phosphorylation by protein kinase CK2 (Taylor et al. 2012a), ZIP7 is proposed to be a gatekeeper for intracellular zinc release (Hogstrand et al. 2009), which may be a prerequisite for zinc-mediated signaling cascades via the well-known zinc inhibitory effect on protein tyrosine phosphatases (PTP) (Haase and Maret 2005). This discovery, that CK2 phosphorylation of ZIP7 is the stimulus to open the ZIP7 gate and cause release of zinc from stores into the cytoplasm (Taylor et al. 2012a), presumably down a concentration gradient, now allows the SLC39A family of zinc transporters to be redesignated as zinc channels rather than transporters. This crucial role of ZIP7 in release of zinc from stores has been confirmed by a study conducted by our group in which ZIP7-mediated zinc release is shown to be

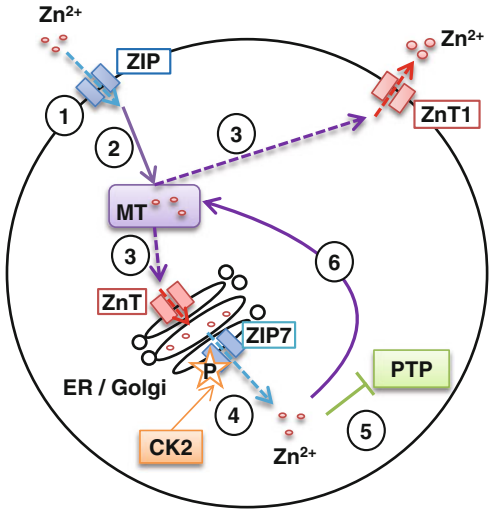

Fig. 14.2 Intracellular zinc homeostasis and regulation of ZIP7 shown in a schematic representation of the muffler model (Colvin et al. 2010). (*1*) Zinc influxes from the extracellular space into a cell via a ZIP channel. (*2*) Cytosolic free zinc is immediately muffled by the cysteine-rich metallothioneins (*MT*). (*3*) The muffled zinc is either shuttled directly to the intracellular stores, such as the endoplasmic reticulum (*ER*) or the *Golgi*, or eliminated from the cells via ZnT1, the only ZnT transporter located on the plasma membrane (Palmiter and Findley 1995). (*4*) Upon activation by an appropriate extracellular stimulus, protein kinase CK2 triggers ZIP7-mediated zinc release from the stores, resulting in a zinc wave (Taylor et al. 2012a). (*5*) The released zinc ions activate intracellular signaling molecules via an inhibitory effect on protein tyrosine phosphatases (*PTP*) (Taylor et al. 2012a). (*6*) The excess cytoplasmic zinc ions are tightly regulated by the muffling reaction. Eventually, the muffled zinc ion is either delivered back to intracellular stores or removed from the cell via ZnT1 (step 3)

triggered by protein kinase CK2 phosphorylation on serine residues S^{275} and S^{276}, resulting in activation of mitogen-activated protein kinases and tyrosine kinases (Taylor et al. 2012a), which are known substrates of the PTPs (Haase and Maret 2005). A simplified schematic of zinc homeostasis according to the muffler model with ZIP7 regulation is illustrated in Fig. 14.2.

Interestingly, the activation of ZIP9, which is located on the Golgi membrane (Matsuura et al. 2009), is also demonstrated to decrease PTP activity, resulting in activation of the same kinases in the chicken DT40 B cell line (Taniguchi et al. 2013). Given that ZIP7 expression is not detectable in chickens (Kambe et al. 2006), this further highlights the important role of the gatekeepers of zinc release from the stores, such as ZIP7 for mammalian cells and ZIP9 for chicken cells, in zinc signaling.

14.3.2 ZnT Transporters

The ZnT transporters (SLC30A), functioning as Zn^{2+}/H^{+} exchangers (Ohana et al. 2009), are responsible for zinc efflux from the cytoplasm. This family of zinc efflux transporters contains ten members, ZnT1 to ZnT10, which can be grouped into subfamilies I, II, and III (Huang and Tepaamorndech 2013). The majority of ZnT transporters have six TM domains, with intracytoplasmic amino- and carboxyl-termini and a long intracytoplasmic loop between TM IV and V, which includes the histidine-rich region, except for ZnT6, which has a serine-rich region instead (Seve et al. 2004). All ZnT transporters are located on intracellular membranes, except ZnT1, which is the only ZnT transporter on the plasma membrane, thereby being essential for zinc efflux from the cell (Palmiter and Findley 1995).

14.4 Zinc and Cancer

Zinc has been widely studied for its potential involvement in human diseases. Because of its antioxidant properties and beneficial influences on the immune system and cell growth, zinc has been believed to protect the body against age-related diseases such as age-related macular degeneration (Age-Related Eye Disease Study Research Group 2001), to accelerate wound healing (Lansdown et al. 2007), and to reduce morbidities of the common cold (Science et al. 2012) and gastroenteritis (Walker and Black 2010).

The beneficial effects of zinc should generally help decrease the risk of cancer development and can be a potential dietary cancer-chemopreventive agent (Dhawan and Chadha 2010; Ho 2004; Prasad 2009). This hypothesis is supported by the epidemiological evidence that serum zinc levels are significantly decreased in patients with most types of cancers, including carcinomas of the head and neck (Buntzel et al. 2007), the breast (Arinola and Charles-Davies 2008), the gastrointestinal tract (Boz et al. 2005), the gallbladder (Gupta et al. 2005), and the female genital tract (Martin-Lagos et al. 1997; Naidu et al. 2007). Zinc levels in hair samples, which are likely to provide less variable results, confirm the decrease in zinc levels for cancers of the lung (Piccinini et al. 1996), breast (Memon et al. 2007), and ovary (Memon et al. 2007).

14.4.1 Cancer-Preventive Role of Zinc

Theoretically, the cancer-preventive effects of zinc are mainly attributed to its crucial role in both prevention of oxidative DNA damage, via its involvement in the free radical scavenging system, and response and repair of damaged DNA

(Ho et al. 2003). The involvement of zinc in DNA repair is supported by downregulation of some DNA repair genes in zinc-depleted cells (Ho et al. 2003). Interestingly, even though p53 expression, one of the most important tumor suppressor and DNA-repair genes, is increased by cellular zinc deficiency, its ability to induce an effective DNA repair is impaired in the absence of zinc, resulting from the loss of its binding capability to DNA sequences (Ho and Ames 2002). Zinc supplementation has also been suggested to help control the growth of cancers by inhibiting angiogenesis, inducing inflammatory cytokines, and promoting apoptosis in cancer cells (Prasad et al. 2009). Furthermore, in vivo studies have demonstrated that zinc supplementation prevents the development of *cis*-platinum- and melphalan-induced lung cancers in mice (Satoh et al. 1993), 1,2-dimethylhydrazine-induced colonic cancer in rats (Dani et al. 2007), and *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal cancer in zinc-deficient rats (Fong et al. 2001).

Regardless of these anti-cancer properties of zinc, it is noteworthy that clinical studies have reported conflicting results. For instance, instead of preventing prostrate carcinogenesis (Kristal et al. 1999), the use of zinc supplementation was instead shown to either increase the risk of prostate cancer (Kolonel et al. 1988; Leitzmann 2003) or have no association at all (Chang et al. 2004). Nevertheless, the cancer-promoting effect of zinc was possibly confounded by cadmium in some zinc supplements (Krone and Harms 2003). Collectively, the data from many studies to date have suggested that zinc has some cancer chemopreventive or even chemotherapeutic properties, at least for some types of cancers, even though the effectiveness and risk of zinc supplementation are yet to be proved.

Notwithstanding, some other studies have also reported contradictory results of zinc levels in patients with cancers of the breast, lung, stomach, and prostate (Navarro Silvera and Rohan 2007). Moreover, zinc levels in individual malignant tissues are not always well correlated with body zinc status: rather, they vary in a cancer type-specific manner. For example, zinc levels are significantly reduced in prostate cancer, but, regardless of the decrease in serum zinc levels, they are elevated in most other types of cancers, including cancers of the breast (Margalioth et al. 1983; Mulay et al. 1971) and the lung (Mulay et al. 1971). Therefore, the alterations in zinc levels in cancerous tissues may not necessarily be affected by systemic zinc status.

14.4.2 Cancer Tissue Zinc Levels with Aberrant Expressions of Zinc Transport Proteins

Many studies support the fact that the changes in tumor tissue zinc levels are actually attributed to tissue-specific zinc dysregulation, resulting mainly from aberrant expressions of various ZIP channels. The uniquely decreased zinc level in prostatic cancer tissue directly corresponds to the decrease in the expression of

zinc channels ZIP1 (Huang et al. 2006), ZIP2, and ZIP3 (Desouki et al. 2007), as well as ZIP4 (Chen 2012). In contrast, the increased tissue zinc levels in various types of cancers are reasonably related to the increase in the expression of particular ZIP channels, such as ZIP6 (Taylor et al. 2003) and ZIP10 (Kagara et al. 2007) in breast cancer cells, ZIP7 in both tamoxifen-resistant and fulvestrant-resistant breast cancer models, and ZIP8 in a fulvestrant-resistant breast cancer model (Taylor et al. 2007).

Although no relevant data of tissue zinc levels are available to our knowledge, ZIP6 is known to increase in adenocarcinomas of the cervix and the lung (Taylor and Nicholson 2003). Parenthetically, even though bronchogenic carcinoma of the lung has been reported to have increased tissue zinc level, the tumor demonstrated squamous phenotypes, suggesting the diagnosis of squamous cell carcinoma, rather than adenocarcinoma (Mulay et al. 1971). Similarly, cervical cancer tissue is found to have no difference in zinc content from corresponding normal tissue, but no histological subtype was indicated (Margalioth et al. 1983). According to the much higher incidence of squamous cell carcinoma before the use of the Papanicolaou smear test as a screening method (Wang et al. 2004), it is more likely to be this histological type rather than adenocarcinoma, for which the increase in zinc channel ZIP6 is known.

Additionally, ZIP4 is shown to be overexpressed in 94 % of pancreatic adenocarcinoma specimens, and the experimentally induced ZIP4 overexpression results in zinc accumulation and cancer progression (Li et al. 2007). The zinc levels and zinc transport protein expression in tumor tissues or models are summarized in Table 14.1.

14.5 Zinc Signaling and Cancer

Currently available data suggest that zinc dysregulation resulting from aberrant expression of zinc transport proteins is associated with particular types of cancers in a cancer-specific manner. We therefore discuss each type of cancer individually in the remainder of this chapter. The roles of zinc transport proteins in carcinogenesis and cancer progression have been investigated most thoroughly for breast cancer and prostate cancer, which are the main focus of this review. However, we also discuss recent discoveries in other types of cancers.

14.5.1 Zinc Signaling in Breast Cancer

As all relevant studies have been performed in invasive ductal carcinoma of no special type, which is the most common type of breast cancer, comprising up to 79 % of breast carcinoma (Lester 2010), breast cancer in this chapter refers to this particular cancer type.

Table 14.1 Zinc levels and zinc transport protein expression in tumor tissues or models

Cancer	Tissue zinc level	Aberrant expression of zinc transport proteins
Prostate adenocarcinoma	Decreased (Desouki et al. 2007)	Decreased
		• *ZIP1* (Huang et al. 2006)
		• *ZIP2* (Desouki et al. 2007)
		• *ZIP3* (Desouki et al. 2007)
		• *ZIP4* (Chen 2012)
Breast cancer (invasive ductal carcinoma)	Increased (Margalioth et al. 1983; Mulay et al. 1971)	Increased
		• *ZIP6* (Taylor et al. 2003)
		• *ZIP10* (Kagara et al. 2007)
Anti-hormone-resistant breast cancer	Increased in tamoxifen-resistant breast cancer model (Taylor et al. 2007)	Increased
		• *ZIP7* (Taylor et al. 2007) (tamoxifen-resistant and fulvestrant-resistant breast cancer model)
		• *ZIP8* (Taylor et al. 2007) (fulvestrant-resistant breast cancer model)
Pancreatic adenocarcinoma	N/A	Increased
		• *ZIP4* (Li et al. 2007)

14.5.1.1 Zinc Status in Breast Cancer

Physiologically, the intact functions of particular zinc transporters are important during lactation, as impairment in zinc secretion into breast milk can result in zinc deficiency in a nursing infant (Kelleher et al. 2011). Pathologically, epidemiological data have long implicated zinc dysregulation in breast cancer, because significant elevation in zinc level has been consistently reported in cancerous tissue when compared with noncancerous breast tissue (Margalioth et al. 1983; Mulay et al. 1971). Zinc content in breast cancer tissue is demonstrated to be twice as high as the adjacent nonneoplastic tissue, and increase in metallothionein expression consistent with an elevation in zinc content is also observed (Jin et al. 1999).

The robustly increased zinc content in breast cancer and the role of zinc dysregulation in breast carcinogenesis have further been reinforced by experimental in vivo studies employing the *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumorigenesis model in rats, which is one of the most widely used animal models for investigating human breast carcinogenesis because of the biological similarities between MNU-induced mammary tumours and human breast cancer (Welsch 1985). These studies demonstrated the accumulation of zinc in the MNU-induced mammary tumors, with an increase in zinc concentration up to 19 fold when compared with nontumorous mammary glands (Lee et al. 2003; Woo and Xu 2002). Interestingly, the increase in zinc content was consistently observed, regardless of various dietary zinc intake levels (Woo and Xu 2002). In humans, the increase in zinc content in benign breast tissue has also been confirmed to be associated with a modestly increased risk of subsequent breast cancer (Cui

et al. 2007). Collectively, these data further support the role of zinc in breast cancer carcinogenesis and confirm the local, rather than systemic, zinc dysregulations, most likely resulting from malfunctions of zinc transport proteins and irrespective of dietary zinc consumption.

14.5.1.2 Zinc Transport Proteins in Breast Cancer

The elevation in zinc content in breast cancer tissue has been attributed to aberrantly increased expressions of ZIP6 (Taylor et al. 2003) and ZIP10 (Kagara et al. 2007). ZIP6 has been linked to positive immunohistochemical staining for estrogen receptor (Manning et al. 1993) and breast cancers with lymph node involvement (Manning et al. 1994). It has been established to be an estrogen-regulated gene (Manning et al. 1994) and as such has become a reliable biomarker of estrogen receptor-positive luminal type A breast cancer (Perou et al. 2000; Tozlu et al. 2006). Using polymerase chain reaction (PCR) analysis, our group has confirmed the relationship between ZIP6 expression and estrogen-positive breast cancer both in cell lines and in clinical material (Taylor et al. 2007). Recently, using our unique ZIP6 antibodies, ZIP6 protein has been experimentally proved to be involved in epithelial-mesenchymal transition (EMT) and anoikis resistance, indicating ZIP6 as a cause of cell motility, migration, and metastasis (Hogstrand et al. 2013).

Furthermore, another ZIP channel, ZIP10, has also been connected to the increased invasiveness of cancer cells (Kagara et al. 2007). ZIP7, which increases in both tamoxifen-resistant and fulvestrant-resistant breast cancer models, has been implicated as a contributory factor in the development of anti-hormone resistance (Taylor et al. 2008), and additionally associated with poor clinical outcome in human breast cancer, according to the Oncomine database (Taylor et al. 2007). Recently, in association with protein kinase CK2, ZIP7 has also been shown to promote cell proliferation and migration (Taylor et al. 2012a). Therefore, there appear to be increasing data available to suggest that in the context of breast cancer, zinc and a number of ZIP channels are considered to be cancer promoting. The association between ZIP channels and breast cancer biology is summarized in Table 14.2. The mechanisms whereby these zinc channels contribute to particular malignant behaviors are discussed in detail next.

14.5.1.3 Zinc Channel ZIP6 in Breast Cancer

Epithelial-mesenchymal transition (EMT) is crucial for embryonic development, as well as various pathological processes, including tissue repair and tumor metastasis (Thiery et al. 2009). As the name implies, it is the process whereby the cells lose their epithelial markers, such as adhesion molecule E-cadherin, and adopt a mesenchymal phenotype, promoting cell detachment and migration. Among the metastatic oncogenes, the transcription factor Snail plays an important role in EMT after

Table 14.2 Association between ZIP channels and breast cancer biology

ZIP channel	Association with breast cancer biology
ZIP6	• Reliable marker of estrogen-positive disease (Perou et al. 2000; Taylor et al. 2007; Tozlu et al. 2006)
	• Lymph node involvement (Manning et al. 1994; Taylor et al. 2007)
	• Cell motility, migration, and metastasis (Hogstrand et al. 2013)
	• Part of a five-gene molecular signature of outcome (Andres et al. 2013)
ZIP10	• Migration and metastasis (Kagara et al. 2007)
ZIP7	• Development of anti-hormone resistance (Taylor et al. 2008)
	• Proliferation and migration (Taylor et al. 2012a)

its nuclear localization by repressing the transcription of E-cadherin (Peinado et al. 2007). Snail has been demonstrated to be negatively regulated by glycogen synthase kinase-3-beta (GSK-3β)-mediated phosphorylation, which promotes its nuclear export, ubiquitination, and consequential degradation (Zhou et al. 2004).

Importantly, with its molecular link to signal transducer and activator of transcription 3 (STAT3), ZIP6 is known to be essential for EMT in zebrafish gastrulation, by promoting the nuclear localization of Snail, resulting in the repression of E-cadherin and increased cell migration (Yamashita et al. 2004). Given that the roles of both STAT3 (Pakala et al. 2013) and Snail (Zhang et al. 2013) in metastasis have been well documented in breast cancer, with an additional strongly positive correlation between the expression of ZIP6 and STAT3 (Taylor et al. 2007), the potential mechanistic link between the role of ZIP6 in gastrulation and breast cancer metastasis has come to light.

Recently, the mechanism of action of zinc channel ZIP6 in breast cancer migration has been unraveled by a study in which ZIP6 was demonstrated to be transcriptionally transactivated by STAT3, resulting in its expression as a pro-protein (Hogstrand et al. 2013). The ZIP6 pro-protein resides on the endoplasmic reticulum membrane until the appropriate stimulus triggers cleavage at the N-terminus between residues 155 and 237, which causes it to relocate to the plasma membrane where it can influx zinc into the cell. This posttranslational processing of ZIP6 was discovered using a panel of anti-peptide antibodies with different epitopes (Hogstrand et al. 2013) and observing that the ZIP6 antibody epitope near the far N-terminus and upstream of residue 155 never recognized plasma membrane-located ZIP6, but only ZIP6 located in the endoplasmic reticulum. These data now explain some apparently conflicting results in the literature suggesting that ZIP6 was a good indicator of favorable breast cancer outcome (Kasper et al. 2005). The antibody epitope used in this latter study was upstream of the N-terminal cleavage site of ZIP6 and would only be able to recognize endoplasmic reticulum-located ZIP6. This study therefore agrees with new data, provided the endoplasmic reticulum-located ZIP6 is considered to be the inactive pro-protein form of ZIP6.

After ZIP6 had been activated by N-terminal cleavage and relocated to the plasma membrane (Hogstrand et al. 2013), it was able to mediate zinc influx into

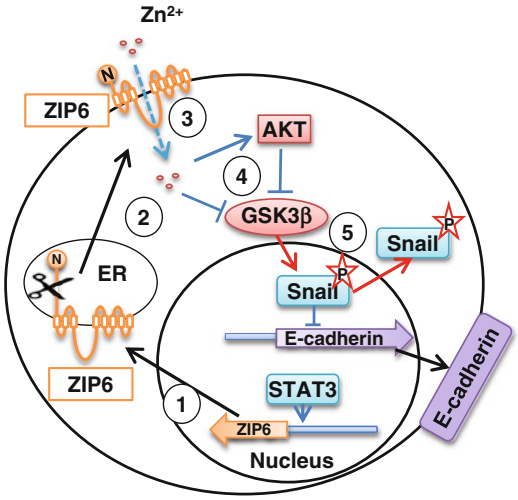

Fig. 14.3 Schematic illustration of ZIP6 involvement in epithelial-mesenchymal transition (EMT) demonstrates the ZIP6 signaling pathway associated with EMT, as proposed by a recent study (Hogstrand et al. 2013). (*1*) After transcriptional activation by STAT3, ZIP6 protein is produced as a pro-protein and located on the endoplasmic reticulum. (*2*) Upon activation by N-terminal cleavage, the ZIP6 channel is translocated to the plasma membrane. (*3*) The plasma membrane-located ZIP6 mediates zinc influx into the cytoplasm. (*4*) Glycogen synthase kinase-3-beta (*GSK-3β*) is inhibited by phosphorylation, mediated either directly by zinc or indirectly by zinc-activated *AKT*. (*5*) Normally, GSK-3β negatively regulates Snail by phosphorylation, resulting in nuclear export, ubiquitination, and degradation of Snail. Inhibition of GSK-3β therefore causes nuclear retention of Snail, leading to repression of the adhesion molecule E-cadherin, and consequential cell migration and metastasis

the cytoplasm, resulting in inhibitory phosphorylation of GSK-3β, either directly by zinc (Ilouz et al. 2002) or indirectly by zinc-activated AKT (Lee et al. 2009), with subsequent nuclear retention of Snail, transcriptional repression of E-cadherin, and increased cell detachment (Hogstrand et al. 2013). Interestingly, being resistant to anoikis, the detached cells retained their capability to proliferate even after detachment (Hogstrand et al. 2013), thereby having a high potential to migrate and metastasize. A simplified schematic illustration of this mechanism is depicted in Fig. 14.3.

14.5.1.4 Zinc Channel ZIP10 in Breast Cancer

ZIP10 is known to be the closest paralogue to ZIP6 (Taylor and Nicholson 2003). ZIP6, an estrogen-regulated gene, and ZIP10 have a positive correlation with estrogen receptor in breast cancer tissue, even though the latter does not have such potential clinical relevance because of its low expression (Taylor et al. 2007). Similar to ZIP6, ZIP10 is also demonstrated to be involved in migration and metastasis of breast cancer (Kagara et al. 2007). Furthermore, among all the

members of the LIV-1 subfamily, ZIP10 is the only zinc channel other than ZIP6 that contains a potential PEST cleavage site (Taylor and Nicholson 2003), and it has been shown to be proteolytically modified at its ectodomain (Ehsani et al. 2012). However, it is yet to be determined whether it is posttranslationally processed in the same manner as ZIP6 and whether it synergistically participates with ZIP6 in the process of migration and metastasis in breast cancer.

14.5.1.5 Zinc Channel ZIP7 in Breast Cancer

In routine clinical practice, patients with both early and late estrogen receptor-positive breast cancers, pathologically diagnosed by tissue biopsies, are treated with anti-hormonal drugs, such as tamoxifen (Osborne 1998). Unfortunately, as many as 40 % of patients who initially respond to the adjuvant tamoxifen therapy eventually acquire drug resistance, resulting in relapse and death from the disease (Ring and Dowsett 2004). To investigate the mechanisms of acquired resistance, our group has developed a tamoxifen-resistant breast cancer cell line (TamR), which was derived from estrogen receptor-positive tamoxifen-responsive MCF-7 breast cancer cells (Knowlden et al. 2003). Interestingly, not only are the TamR cells able to continue growing in the presence of the drug, but they also exhibit an even more aggressive phenotype, such as increased cell motility and invasiveness (Hiscox et al. 2004). The aggressive phenotype was attained by hyperactivation of alternative pathways, including those mediated by epidermal growth factor receptor (EGFR) (Knowlden et al. 2003), insulin-like growth factor-1 receptor (IGF-1R) (Jones et al. 2004). and non-receptor tyrosine kinase Src (Hiscox et al. 2006), all of which are included among the known substrates of PTP1B (Bourdeau et al. 2005).

Given that ZIP7 is the only ZIP channel to be overexpressed in TamR cells, which have been demonstrated to have twice as much zinc as the responsive MCF-7 cells (Taylor et al. 2007), the potential role of ZIP7 in the development of anti-hormone resistance and the associated aggressive behaviour cannot be overstated. We have investigated this in TamR cells and proved that ZIP7-mediated zinc release from the stores was responsible for activation of tyrosine kinase receptor signaling pathways as well as Src-dependent pathways (Taylor et al. 2008), which was likely mediated by the zinc inhibitory effect on PTP1B (Haase and Maret 2005). The stimulation of these signaling pathways resulted in activation of their downstream effectors, including mitogen-activated protein kinase (MAPK) such as extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and AKT, with consequential increases in cell growth and motility (Taylor et al. 2008). It is noteworthy that Src not only activates the tyrosine kinase receptor signaling pathways itself, but it also contributes to cell proliferation, cell survival, invasiveness, and angiogenesis (Summy and Gallick 2006), all of which result in increased aggressiveness of the malignancy. Moreover, we demonstrated that concentrations of zinc as low as 20 μM could overcome the effects of Src inhibitor on cancer invasiveness, conveying the efficiency of the intracellular zinc ions to activate this oncogenic tyrosine kinase (Taylor et al. 2008). Altogether, these data introduce the

concept that blocking ZIP7-mediated zinc release could be a new and worthwhile strategy for the treatment of anti-endocrine-resistant breast cancer.

Regardless of the well-established involvement of ZIP7 in cancer, little is known of its molecular regulatory mechanisms. Remarkably, posttranslational regulation by phosphorylation of ZIP7 has been demonstrated in a recent study, in which CK2 phosphorylated serine residues S^{275} and S^{276}, which reside within the long histidine-rich intracytoplasmic loop between TM III and TM IV. This phosphorylation triggered the ZIP7-mediated release of zinc from internal stores (Taylor et al. 2012a), and the released zinc in turn activated downstream signaling pathways, including phosphorylation of ERK1/2 and AKT, and promoted cell migration. This information now provides new insight into how the ZIP channels are regulated. Furthermore, the protein kinase that phosphorylates ZIP7, CK2, is itself involved in cell growth and proliferation, as well as oncogenesis and pathobiological features of neoplasms, with numerous possible substrates that may be responsible for these effects (Tawfic et al. 2001). Therefore, these findings also suggest a possibility that CK2 may cause a number of these cancer-promoting effects by mediating the phosphorylation of zinc channel ZIP7 (Taylor et al. 2012b). A simplified schematic demonstrating ZIP7-mediated zinc release from the stores with activations of downstream effectors is shown in Fig. 14.4.

According to the phosphorylation prediction databases, there are at least four residues in the cytoplasmic loop between TM III and IV of zinc channel ZIP7 that have been experimentally confirmed by mass spectrometry (Daub et al. 2008; Kim et al. 2005; Zahedi et al. 2008) to be phosphorylated in mammalian cells (Fig. 14.5). Serine residues S^{275} and S^{276} (Kim et al. 2005) are the only two sites that have to date been confirmed by site-specific methods to be phosphorylated by CK2 (Taylor et al. 2012a). Interestingly, the kinases predicted according to the consensus sequences for the other two sites, S^{293} and T^{394} (Olsen et al. 2010), have some cancer-promoting properties. The kinases MAPKAPK2 and MAPKAPK3, predicted for S^{293}, are involved in cell migration (Menon et al. 2009), while the kinases PIM1, PIM2, and PIM3, predicted for T^{394}, promote cell proliferation (Chen et al. 2005) and survival (Amaravadi and Thompson 2005). It is yet to be investigated whether these two sites also orchestrate the regulation of ZIP7, whether they are phosphorylated by the particular predicted kinases, and whether ZIP7-mediated zinc release is responsible for the cancer-promoting properties of these predicted kinases. Given that ZIP7 is activated by phosphorylation, it is also to be questioned if other ZIP transporters can be activated in the same manner.

14.5.2 Zinc Signaling in Prostate Cancer

A role for zinc and zinc signaling has also been demonstrated for prostate cancer. However, in contrast to that observed for breast cancer, the level of zinc appears to decrease in prostate cancer compared to normal prostate.

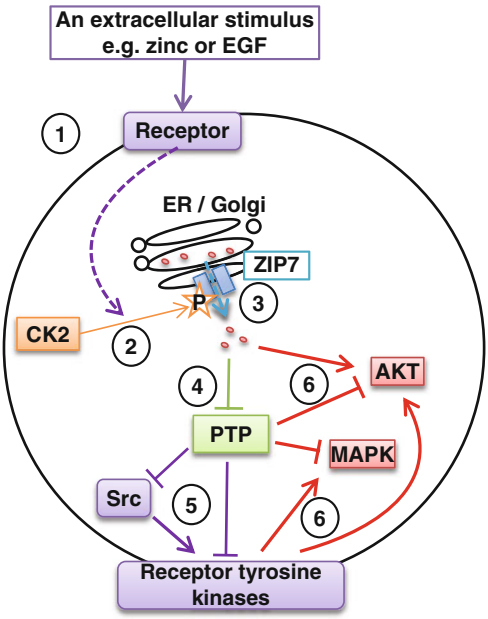

Fig. 14.4 ZIP7-mediated zinc release from stores and the downstream cascades. (*1*) ZIP7-mediated zinc release from stores can be activated by an extracellular stimulus, such as zinc or epidermal growth factor (*EGF*) (Taylor et al. 2012a). (*2*) This activation results in association between protein kinase CK2 and ZIP7 and consequential phosphorylation on S^{275} and S^{276} of ZIP7 (Taylor et al. 2012a). The connection between the extracellular stimulus and ZIP7 activation is as yet unknown. (*3*) The phosphorylation of ZIP7 activates zinc release from the stores on which ZIP7 resides, such as the endoplasmic reticulum (*ER*) or the *Golgi*, thereby producing a zinc wave (Taylor et al. 2008, 2012a). (*4*) The released zinc in turn inhibits *PTP*s (Haase and Maret 2005). (*5*) The inhibition of PTPs, such as PTP1B, results in sustained activation of tyrosine kinases, including EGFR, IGF-1R, and nonreceptor tyrosine kinase *Src* (Taylor et al. 2008). (*6*) Eventually, this zinc release results in activation of various kinases downstream of these tyrosine kinases such as *MAPK* and *AKT*, either via Src-dependent receptor tyrosine kinase activation (Taylor et al. 2008) or via the zinc inhibitory effect on PTPs (Haase and Maret 2005). Additionally, AKT is directly activated by zinc (Lee et al. 2009)

14.5.2.1 Zinc Status in Prostate Cancer

Along with the choroid of the eye, the prostate gland has the highest concentration of zinc in the body (World Health Organization and Food and Agriculture Organization of the United Nations 2002). Zinc is known to be involved in male fertility, as it is required for sperm release and motility (Yoshida et al. 2008). Physiologically, high zinc levels in the prostate gland spare citrate from the citric acid cycle by inhibiting the enzyme m-aconitase, thereby helping supply high amounts of citrate into prostatic fluid (Costello et al. 2005). In pathological conditions, prostate tissue zinc levels increase in nonneoplastic prostate disease, whereas they dramatically decrease in prostate cancer (Zaichick et al. 1997). These epidemiological data introduce zinc decrease as a cancer-promoting condition and zinc supplementation

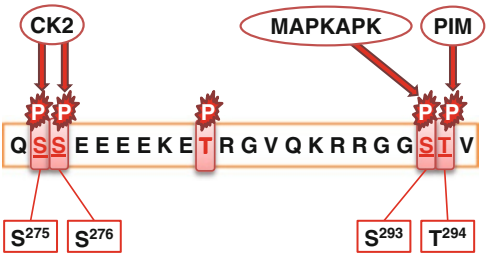

Fig. 14.5 Phosphorylation sites for ZIP7 with kinase prediction. The amino acid sequence of ZIP7 between residues 274 and 295 is located on the intracytoplasmic loop between TM III and TM IV. We show the predicted phosphorylation sites that have been experimentally confirmed in mammalian cells for ZIP7 (Daub et al. 2008; Kim et al. 2005; Zahedi et al. 2008), and the predicted kinases with the highest scores for these sites using PhosphoNET (Kinexus Bioinformatics Corp.), PhosphoSitePlus (Hornbeck et al. 2012), and Phosida (Gnad et al. 2007)

as a promising cancer-preventive strategy, even though clinical studies have failed to show consistent results, as mentioned earlier in Sect. 14.4.1.

14.5.2.2 Zinc and Prostate Carcinogenesis

Mechanistically, the role of zinc in prostate cancer prevention or suppression is at least twofold: it prevents prostate epithelial cells from oxidative DNA damage, and it induces apoptosis. A simplified schematic is shown in Fig. 14.6.

Zinc-deficient prostate epithelial cells have been reported to have increased DNA damage (Yan et al. 2008), which suggests that high zinc content is essential for prevention of oxidative DNA damage in the prostate gland. This beneficial effect of zinc can be linked to its physiological action of m-aconitase inhibition (Costello et al. 1997). Loss of any zinc inhibitory effect on the enzyme encourages citrate to enter into the citric acid cycle and the subsequent oxidative phosphorylation. Even though this ATP-producing process is vital for life, it also results in the production of reactive oxygen species, a major cause of DNA damage. Furthermore, the decrease in zinc with its antioxidant properties (Oteiza 2012) results in uncontrolled generation of free radicals. It therefore can be concluded that decrease in the zinc content of prostate glands may eventually cause excessive production of free radicals and consequential DNA damage.

Moreover, high zinc concentration also produces pro-apoptotic conditions in the prostate gland (Feng et al. 2002), supported by the finding that zinc directly induces bax-mediated mitochondrial cytochrome *c* release, which in turn activates the caspase cascade, ultimately leading to apoptotic cell death (Feng et al. 2008). Therefore, the cellular zinc deficiency observed in the prostate promotes cell survival by inhibiting apoptosis in prostate epithelial cells.

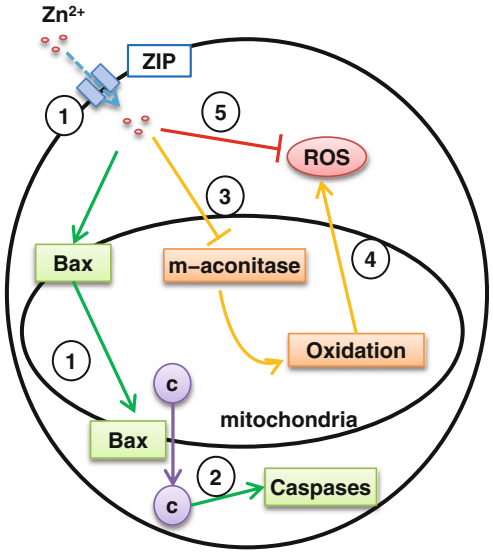

Fig. 14.6 Role of zinc in prostate cancer prevention or suppression. Schematic illustrates the mechanism whereby zinc suppresses prostate cancer. (*1*) In the normal prostate, ZIP channels, including ZIP1, ZIP2, ZIP3, and ZIP4, assure high zinc levels. Zinc directly causes *Bax*-mediated cytochrome *c* release from the mitochondria (Feng et al. 2008). (*2*) The released cytochrome *c* activates a caspase cascade, resulting in apoptotic cell death. (*3*) To spare citrate for secretion into prostatic fluid, zinc inhibits *m-aconitase* (Costello et al. 1997), preventing citrate from entering into the citric acid cycle and oxidation. (*4*) As normal oxidative phosphorylation produces free radicals, the inhibitory effect of zinc on m-aconitase results in a decrease in free radical production. (*5*) Moreover, zinc itself has anti-oxidant properties (Oteiza 2012), thereby preventing oxidative DNA damage

14.5.2.3 Zinc Signaling in Prostate Cancer

The reduced zinc level in prostate cancer is associated with decreased expression of ZIP channels, including ZIP1 (Huang et al. 2006), ZIP2, and ZIP3 (Desouki et al. 2007), as well as ZIP4 (Chen 2012). Among these zinc channels, ZIP1 is demonstrated to be highly expressed in the peripheral zone, the most common site of cancer, and its gene expression is massively downregulated at an early stage of the cancer (Franklin et al. 2005), explaining why it is known as a tumor suppressor gene in prostate cancer (Costello and Franklin 2006; Franklin and Costello 2007).

Surprisingly, zinc transporters ZnT1 (Hasumi et al. 2003) and ZnT4 (Beck et al. 2004) are found to decrease in prostate cancer tissue, but their involvement in prostate carcinogenesis is rather obscure and is yet to be unraveled.

14.5.3 Zinc Signaling in Other Cancers

In the past few years a number of studies have implicated aberrant zinc transporter expression in other cancers. For example, ZIP4 has been implicated in the acquisition of zinc by hepatocellular carcinomas (Weaver et al. 2010). In these cancers, ZIP4 was present in increased amounts and as such able to repress apoptosis, enhance growth rate, and enhance the invasive capacity of the cancers. Moreover, ZIP6 mRNA was increased in liver cancer cells compared to normal cells and demonstrated an inverse relationship with E-cadherin (Shen et al. 2013), agreeing with the role for ZIP6 in EMT and migration of breast cancer cells (Hogstrand et al. 2013). A decrease in another ZIP channel, ZIP14, in contrast to ZIP1, ZIP2, and ZIP3, has also been associated with hepatocellular cancer and thought to be involved with the depletion of zinc observed in these cells (Franklin et al. 2012).

Although the exact role for zinc in pancreatic cancer has not yet been elucidated, there is certainly new evidence emerging that zinc may have a role. Levels of ZIP transporters appear considerably decreased in the disease (Costello and Franklin 2013; Yang et al. 2013), especially levels of ZIP3, which has also been implicated in the early development of pancreatic adenocarcinoma (Costello et al. 2011, 2012). Additionally, ZIP4 has been demonstrated to regulate pancreatic cell growth and does not show the decrease in cancerous tissue observed with other family members (Yang et al. 2013). Furthermore, the pathway of action of ZIP4 was elucidated to involve increases of IL-6, activating STAT3 and leading to increased cell proliferation by increases of cyclinD1 (Zhang et al. 2010). As the zinc-associated events appear to take place in the early phase of oncogenesis, this would suggest a worthy area for future investigation.

Recently, three independent groups have implicated ZIP14 as having a role in colorectal cancer, encompassing ZIP14 splice variants in the disease (Thorsen et al. 2011) and their use as a potential biomarker (Sveen et al. 2012) or therapeutic target (Miura et al. 2012). Furthermore, ZIP10, a molecule with a recognized role in breast cancer (Kagara et al. 2007), is one of seven genes upregulated in activated colon tumor cells.

It seems increasingly obvious that aberrant expression of a variety of zinc transporters, depending on the tissue in question, may be involved in providing more zinc to tumor cells than is required for normal growth and thus encouraging excessive or cancerous growth. Further examination of these molecules is now required, as well as investigation of their phosphorylation status, to understand their influence on zinc signaling pathways in tumors.

14.6 Use of Zinc Signaling as a Target or Cancer Biomarker

This is an exciting time in zinc biology with respect to targeting cancer growth, as the mechanisms of action of zinc transporters and channels within cells are just beginning to emerge with the hope of delivering new potential biological targets that could impact greatly on clinical disease. Furthermore, the proven involvement of certain zinc channels and transporters could also provide new biomarkers for this type of treatment. Moreover, the realization that zinc channels require phosphorylation to release zinc opens the door for biomarkers using antibodies that only recognize the phosphorylated, and therefore active, forms of these molecules.

14.6.1 Zinc Signaling as a Cancer Target

As more information is discovered defining the mechanism of action of zinc transporters in intracellular zinc release, more opportunities are made available for producing agents that may be useful new inhibitors of the signaling pathways known to drive the aggressive growth of tumors. Two particular examples are discussed in the following sections.

14.6.1.1 Targeting ZIP7 Using Protein Kinase CK2 Inhibitors

The discovery that zinc channel ZIP7 requires phosphorylation by protein kinase CK2 to open the channel gate and release zinc from intracellular stores (Taylor et al. 2012a) has provided a means for targeting this mechanism of zinc release by using CK2 inhibitors. Interestingly, Cylene Pharmaceuticals has developed a CK2 inhibitor that is currently under evaluation in clinical cancer trials (Siddiqui-Jain et al. 2010). This inhibitor, called CX-4945, has not only shown promise in combination with EGFR inhibitors in killing cancer cells (Bliesath et al. 2012), but also and more recently has been demonstrated as a potential new treatment for glioblastoma (Zheng et al. 2013) and leukemia (Martins et al. 2013).

Protein kinase CK2 is a ubiquitously expressed threonine/serine kinase composed of two catalytic α-subunits and two regulatory β-subunits (Niefind et al. 2009) that has a multitude of cellular targets. Protein kinase CK2 has a proven role in cell survival and proliferation (St.-Denis and Litchfield 2009) and shuttles between the cytosol and nuclei of cells to support apoptosis or mitosis, respectively. Furthermore, CK2 levels are known to be increased in many different cancer types, suggesting deregulation and a potential target. Importantly, the ZIP7-mediated zinc release from the ER and the subsequent activation of multiple downstream pathways that enhance cell proliferation and migration rely on phosphorylation of zinc channel ZIP7 by protein kinase CK2 (Taylor et al. 2012b).

ZIP7 abundance is increased in tumors, being one of the top 10 % of genes overexpressed in many poor prognostic cancer states, and shows a statistically significant positive correlation of mRNA expression with known indicators of poor outcome in breast cancer, such as the proliferation marker Ki67 (Taylor et al. 2007). This finding, together with current thinking that CK2 is essential for the neoplastic phenotype and can also act as an oncogene when overexpressed (Duncan and Litchfield 2008), suggests that these two molecules may have a common role in cell survival. The fact that CK2 inhibitors decrease the viability of various cancer cells, including prostate cancer cells and tamoxifen-resistant MCF-7 breast cancer cells (Yde et al. 2007), adds further weight to this view, supporting the possibility that CK2 and ZIP7 may function together to mediate intracellular zinc release.

Targeting ZIP7-mediated release of zinc in breast cancers leads to loss of growth, invasion, and signaling (Taylor et al. 2008). The identification here of a CK2-mediated phosphorylation switch for activating ZIP7 to release zinc opens the door to the use of CK2 inhibitors, which are well tolerated by cancer patients (Solares et al. 2009), to treat breast cancer. Furthermore, because intracellular zinc signals inhibit protein tyrosine phosphatases (Haase and Maret 2005), targeting zinc release could prevent activation of multiple tyrosine kinases and their associated signaling pathways, thereby benefiting cancer patients (Taylor et al. 2008, 2011).

ZIP7 has a recognized role in driving the growth of tamoxifen-resistant breast cancers (Taylor et al. 2008). Therefore, targeting zinc release, in conjunction with anti-hormones such as tamoxifen, the usual treatment for these types of cancer, is likely to provide a successful method of inhibiting multiple signaling pathways because of the ability of intracellular zinc to inhibit multiple phosphatases (Haase and Maret 2005). This method should prevent the cancer from harnessing alternative pathways and ensure a continual response to current anti-hormone treatment.

14.6.1.2 Targeting ZIP6

ZIP6 is an estrogen-regulated gene and as such used as a marker of luminal A breast cancer (Perou et al. 2000). We have also shown that ZIP6, after N-terminal cleavage, is enriched on the plasma membrane of migratory cancer cells (Hogstrand et al. 2013) as part of an EMT mechanism. Evidence that ZIP6 associates with ER-positive and triple-negative breast cancers has been used to generate a new therapeutic for breast cancer (Sussman et al. 2013). SGN-LIV1A is an antibody drug-conjugate targeting ZIP6 in metastatic breast cancer that consists of a humanized ZIP6 antibody conjugated to the microtubule-disrupting agent, monomethyl auristatin E, via a protease-cleavable linker. Clinical trials using this agent are currently in the initiation phase by Seattle Genetics, with the hope that the ZIP6 antibody would attach to cancer cells and the toxin would destroy them.

14.6.2 Zinc Signaling as a Cancer Biomarker

ZIP7 associates with STAT3 and cancer spread in anti-hormone-resistant breast cancer (Taylor et al. 2007). The level of ZIP7 may be indicative of the potential for zinc release, although the level of the phosphorylated form may be a better indicator of the activated ZIP7 than the total. There is existing evidence from Affymetrix analysis of a variety of anti-hormone-resistant breast cancer cell models (Taylor et al. 2007) that ZIP7 expression is not only increased but that the zinc level in these cells is also increased. Furthermore, it has also been suggested that this increased zinc, as a direct result of increased ZIP7-mediated zinc release into the cytoplasm (Taylor et al. 2008), is responsible for the activation of multiple signaling pathways that promote aggressive growth and migration of the cancers. All these data collectively suggest that levels of ZIP7 or phosphorylated ZIP7 may, together or individually, be useful markers of cancers that are likely to become resistant to anti-hormones. Our group are currently testing an antibody that detects only the phosphorylated form of ZIP7 as such a biomarker in breast cancer disease states.

ZIP6 also has a significant association with STAT3 in breast cancer samples (Taylor et al. 2007) and has been demonstrated recently to have a causative role in cell detachment (Hogstrand et al. 2013). This mechanism requires N-terminal cleavage of ZIP6 to cause relocation of ZIP6 from the endoplasmic reticulum to the plasma membrane where it can influx zinc into cells. These data suggest that ZIP6 could be present on the plasma membrane of cancer cells and also those cancer cells that have detached from the original tumor. This mechanism could be harnessed to deliver a novel biomarker for metastatic cells, provided the ZIP6 antibody was directed to the correct extracellular location of ZIP6.

14.7 Conclusions and Perspectives

From the foregoing discussions, there appears to be an emerging role for zinc in driving the excessive growth of cancer cells. Most cancer tissues examined to date, with the exception of prostate cancer, have elevated intracellular zinc, which could easily cause tyrosine kinase-driven growth by the known ability of zinc to inhibit tyrosine phosphatases. The realization that zinc channel ZIP7 requires phosphorylation to transport zinc (Hogstrand et al. 2013) now raises the question of whether other zinc channels also require phosphorylation. If this is true, then much work is needed to examine which kinases are involved in activating which zinc channels. Further discoveries in this area should shed light on this and open the door for new and novel small kinase inhibitors that can be used to inhibit the effects of excessive zinc in cancer cells.

The knowledge of the three-dimensional structure of the bacterial ZnT family member, YiiP (Lu and Fu 2007), has provided much insight into the workings of these transporters. There is now a need for the complementary structure of the ZIP

family to be discovered, which would enable the design of specific inhibitory small molecules to target the phosphorylation of individual zinc channels. The hope would be that these small molecules would be more specific than broad kinase inhibitors and prevent the often unwanted side effects observed with these types of drugs in the clinic.

References

Age-Related Eye Disease Study Research Group (2001) A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS Report No. 8. Arch Ophthalmol 119(10):1417–1436. doi:10.1001/archopht.119.10.1417

Amaravadi R, Thompson CB (2005) The survival kinases Akt and Pim as potential pharmacological targets. J Clin Invest 115:2618–2624

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196–201

Andres SA, Brock GN, Wittliff JL (2013) Interrogating differences in expression of targeted gene sets to predict breast cancer outcome. BMC Cancer 13:326

Arinola OG, Charles-Davies MA (2008) Micronutrient levels in the plasma of Nigerian females with breast cancer. Afr J Biotechnol 7:1620–1623

Beck FW, Prasad AS, Butler CE, Sakr WA, Kucuk O, Sarkar FH (2004) Differential expression of hZnT-4 in human prostate tissues. Prostate 58:374–381

Bin BH, Fukada T, Hosaka T, Yamasaki S, Ohashi W, Hojyo S, Miyai T, Nishida K, Yokoyama S, Hirano T (2011) Biochemical characterization of human ZIP13 protein: a homo-dimerized zinc transporter involved in the spondylocheiro dysplastic Ehlers–Danlos syndrome. J Biol Chem 286:40255–40265

Bliesath J, Huser N, Omori M, Bunag D, Proffitt C, Streiner N, Ho C, Siddiqui-Jain A, O'Brien SE, Lim JK et al (2012) Combined inhibition of EGFR and CK2 augments the attenuation of PI3K-Akt-mTOR signaling and the killing of cancer cells. Cancer Lett 322:113–118

Bourdeau A, Dube N, Tremblay ML (2005) Cytoplasmic protein tyrosine phosphatases, regulation and function: the roles of PTP1B and TC-PTP. Curr Opin Cell Biol 17:203–209

Boz A, Evliyaoglu O, Yildirim M, Erkan N, Karaca B (2005) The value of serum zinc, copper, ceruloplasmin levels in patients with gastrointestinal tract cancers. Turk J Gastroenterol 16:81–84

Buntzel J, Bruns F, Glatzel M, Garayev A, Mucke R, Kisters K, Schafer U, Schonekaes K, Micke O (2007) Zinc concentrations in serum during head and neck cancer progression. Anticancer Res 27:1941–1943

Chang ET, Hedelin M, Adami HO, Gronberg H, Balter KA (2004) Re: Zinc supplement use and risk of prostate cancer. J Natl Cancer Inst 96:1108, author reply 1108–1109

Chen QG (2012) The role of zinc transporter ZIP4 in prostate carcinoma. Urol Oncol 30:906–911

Chen WW, Chan DC, Donald C, Lilly MB, Kraft AS (2005) Pim family kinases enhance tumor growth of prostate cancer cells. Mol Cancer Res 3:443–451

Colvin RA, Holmes WR, Fontaine CP, Maret W (2010) Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. Metallomics 2:306–317

Costello LC, Franklin RB (2006) The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots. Mol Cancer 5:17

Costello LC, Franklin RB (2013). A review of the current status and concept of the emerging implications of zinc and zinc transporters in the development of pancreatic cancer. Pancreat Disord Ther Suppl 4:002

Costello LC, Liu Y, Franklin RB, Kennedy MC (1997) Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. J Biol Chem 272:28875–28881

Costello LC, Franklin RB, Feng P, Tan M, Bagasra O (2005) Zinc and prostate cancer: a critical scientific, medical, and public interest issue (United States). Cancer Causes Control 16:901–915

Costello LC, Levy BA, Desouki MM, Zou J, Bagasra O, Johnson LA, Hanna N, Franklin RB (2011) Decreased zinc and downregulation of ZIP3 zinc uptake transporter in the development of pancreatic adenocarcinoma. Cancer Biol Ther 12:297–303

Costello LC, Zou J, Desouki MM, Franklin RB (2012) Evidence for changes in RREB-1, ZIP3, and zinc in the early development of pancreatic adenocarcinoma. J Gastrointest Cancer 43:570–578

Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. J Biol Chem 281:24085–24089

Cui Y, Vogt S, Olson N, Glass AG, Rohan TE (2007) Levels of zinc, selenium, calcium, and iron in benign breast tissue and risk of subsequent breast cancer. Cancer Epidemiol Biomarkers Prev 16:1682–1685

Dani V, Goel A, Vaiphei K, Dhawan DK (2007) Chemopreventive potential of zinc in experimentally induced colon carcinogenesis. Toxicol Lett 171:10–18

Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, Korner R, Greff Z, Keri G, Stemmann O, Mann M (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol Cell 31:438–448

Desouki MM, Geradts J, Milon B, Franklin RB, Costello LC (2007) hZip2 and hZip3 zinc transporters are down regulated in human prostate adenocarcinomatous glands. Mol Cancer 6:37

Dhawan DK, Chadha VD (2010) Zinc: a promising agent in dietary chemoprevention of cancer. Indian J Med Res 132:676–682

Duncan JS, Litchfield DW (2008) Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. Biochim Biophys Acta 1784:33–47

Ehsani S, Salehzadeh A, Huo H, Reginold W, Pocanschi CL, Ren H, Wang H, So K, Sato C, Mehrabian M et al (2012) LIV-1 ZIP ectodomain shedding in prion-infected mice resembles cellular response to transition metal starvation. J Mol Biol 422:556–574

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

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

Feng P, Li T, Guan Z, Franklin RB, Costello LC (2008) The involvement of Bax in zinc-induced mitochondrial apoptogenesis in malignant prostate cells. Mol Cancer 7:25

Food and Nutrition Board of Institute of Medicine (2001) Zinc. In: Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. National Academy Press, Washington, D.C., pp 442–501

Fong LY, Nguyen VT, Farber JL (2001) Esophageal cancer prevention in zinc-deficient rats: rapid induction of apoptosis by replenishing zinc. J Natl Cancer Inst 93:1525–1533

Fraker PJ (2005) Roles for cell death in zinc deficiency. J Nutr 135:359–362

Franklin RB, Costello LC (2007) Zinc as an anti-tumor agent in prostate cancer and in other cancers. Arch Biochem Biophys 463:211–217

Franklin RB, Feng P, Milon B, Desouki MM, Singh KK, Kajdacsy-Balla A, Bagasra O, Costello LC (2005) hZIP1 zinc uptake transporter down regulation and zinc depletion in prostate cancer. Mol Cancer 4:32

Franklin RB, Levy BA, Zou J, Hanna N, Desouki MM, Bagasra O, Johnson LA, Costello LC (2012) ZIP14 zinc transporter downregulation and zinc depletion in the development and progression of hepatocellular cancer. J Gastrointest Cancer 43:249–257

Fukada T, Kambe T (2011) Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics 3:662–674
Gnad F, Ren S, Cox J, Olsen JV, Macek B, Oroshi M, Mann M (2007) PHOSIDA (phosphorylation site database): management, structural and evolutionary investigation, and prediction of phosphosites. Genome Biol 8:R250
Gupta SK, Singh SP, Shukla VK (2005) Copper, zinc, and Cu/Zn ratio in carcinoma of the gallbladder. J Surg Oncol 91:204–208
Haase H, Maret W (2005) Protein tyrosine phosphatases as targets of the combined insulinomimetic effects of zinc and oxidants. Biometals 18:333–338
Hambidge KM, Krebs NF (2007) Zinc deficiency: a special challenge. J Nutr 137:1101–1105
Hasumi M, Suzuki K, Matsui H, Koike H, Ito K, Yamanaka H (2003) Regulation of metallothionein and zinc transporter expression in human prostate cancer cells and tissues. Cancer Lett 200:187–195
Hiscox S, Morgan L, Barrow D, Dutkowskil C, Wakeling A, Nicholson RI (2004) Tamoxifen resistance in breast cancer cells is accompanied by an enhanced motile and invasive phenotype: inhibition by gefitinib ('Iressa', ZD1839). Clin Exp Metastasis 21:201–212
Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RI (2006) Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. Breast Cancer Res Treat 97:263–274
Ho E (2004) Zinc deficiency, DNA damage and cancer risk. J Nutr Biochem 15:572–578
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
Ho E, Courtemanche C, Ames BN (2003) Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. J Nutr 133:2543–2548
Hogstrand C, Kille P, Nicholson RI, Taylor KM (2009) Zinc transporters and cancer: a potential role for ZIP7 as a hub for tyrosine kinase activation. Trends Mol Med 15:101–111
Hogstrand C, Kille P, Ackland ML, Hiscox S, Taylor KM (2013) A mechanism for epithelial-mesenchymal transition and anoikis resistance in breast cancer triggered by zinc channel ZIP6 and STAT3 (signal transducer and activator of transcription 3). Biochem J 455:229–237
Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, Sullivan M (2012) PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res 40:D261–D270
Huang L, Tepaamorndech S (2013) The SLC30 family of zinc transporters: a review of current understanding of their biological and pathophysiological roles. Mol Aspects Med 34:548–560
Huang L, Kirschke CP, Zhang Y, Yu YY (2005) The ZIP7 gene (Slc39a7) encodes a zinc transporter involved in zinc homeostasis of the Golgi apparatus. J Biol Chem 280:15456–15463
Huang L, Kirschke CP, Zhang Y (2006) Decreased intracellular zinc in human tumorigenic prostate epithelial cells: a possible role in prostate cancer progression. Cancer Cell Int 6:10
Ilouz R, Kaidanovich O, Gurwitz D, Eldar-Finkelman H (2002) Inhibition of glycogen synthase kinase-3beta by bivalent zinc ions: insight into the insulin-mimetic action of zinc. Biochem Biophys Res Commun 295:102–106
Jin R, Bay B, Tan P, Tan BK (1999) Metallothionein expression and zinc levels in invasive ductal breast carcinoma. Oncol Rep 6:871–875
John E, Laskow TC, Buchser WJ, Pitt BR, Basse PH, Butterfield LH, Kalinski P, Lotze MT (2010) Zinc in innate and adaptive tumor immunity. J Transl Med 8:118
Jones HE, Goddard L, Gee JM, Hiscox S, Rubini M, Barrow D, Knowlden JM, Williams S, Wakeling AE, Nicholson RI (2004) Insulin-like growth factor-I receptor signalling and acquired resistance to gefitinib (ZD1839; Iressa) in human breast and prostate cancer cells. Endocr Relat Cancer 11:793–814

Kagara N, Tanaka N, Noguchi S, Hirano T (2007) Zinc and its transporter ZIP10 are involved in invasive behavior of breast cancer cells. Cancer Sci 98:692–697

Kambe T, Suzuki T, Nagao M, Yamaguchi-Iwai Y (2006) Sequence similarity and functional relationship among eukaryotic ZIP and CDF transporters. Genomics Proteomics Bioinformatics 4:1–9

Kasper G, Weiser AA, Rump A, Sparbier K, Dahl E, Hartmann A, Wild P, Schwidetzky U, Castanos-Velez E, Lehmann K (2005) Expression levels of the putative zinc transporter LIV-1 are associated with a better outcome of breast cancer patients. Int J Cancer 117:961–973

Kelleher SL, McCormick NH, Velasquez V, Lopez V (2011) Zinc in specialized secretory tissues: roles in the pancreas, prostate, and mammary gland. Adv Nutr 2:101–111

Kim YH, Kim EY, Gwag BJ, Sohn S, Koh JY (1999) Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals. Neuroscience 89:175–182

Kim JE, Tannenbaum SR, White FM (2005) Global phosphoproteome of HT-29 human colon adenocarcinoma cells. J Proteome Res 4:1339–1346

Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE, Nicholson RI (2003) Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology 144:1032–1044

Kolonel LN, Yoshizawa CN, Hankin JH (1988) Diet and prostatic cancer: a case-control study in Hawaii. Am J Epidemiol 127:999–1012

Kristal AR, Stanford JL, Cohen JH, Wicklund K, Patterson RE (1999) Vitamin and mineral supplement use is associated with reduced risk of prostate cancer. Cancer Epidemiol Biomarkers Prev 8:887–892

Krone CA, Harms LC (2003) Re: Zinc supplement use and risk of prostate cancer. J Natl Cancer Inst 95:1556–1557

Lansdown AB, Mirastschijski U, Stubbs N, Scanlon E, Agren MS (2007) Zinc in wound healing: theoretical, experimental, and clinical aspects. Wound Repair Regen 15:2–16

Lee R, Woo W, Wu B, Kummer A, Duminy H, Xu Z (2003) Zinc accumulation in *N*-methyl-*N*-nitrosourea-induced rat mammary tumors is accompanied by an altered expression of ZnT-1 and metallothionein. Exp Biol Med 228:689–696

Lee S, Chanoit G, McIntosh R, Zvara DA, Xu Z (2009) Molecular mechanism underlying Akt activation in zinc-induced cardioprotection. Am J Physiol 297:H569–H575

Leitzmann MF (2003) Zinc supplement use and risk of prostate cancer. J Natl Cancer Inst 95:1004–1007

Lester SC (2010) The breast. In: Kumar V, Abbas AK, Fausto N, Aster JC (eds) Robbins and Cotran pathologic basis of disease, 8th edn. Saunders Elsevier, Philadelphia

Li M, Zhang Y, Liu Z, Bharadwaj U, Wang H, Wang X, Zhang S, Liuzzi JP, Chang SM, Cousins RJ et al (2007) Aberrant expression of zinc transporter ZIP4 (SLC39A4) significantly contributes to human pancreatic cancer pathogenesis and progression. Proc Natl Acad Sci USA 104:18636–18641

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr 29:153–176

Lu M, Fu D (2007) Structure of the zinc transporter YiiP. Science 317:1746–1748

MacDonald RS (2000) The role of zinc in growth and cell proliferation. J Nutr 130:1500S–1508S

Manning DL, McClelland RA, Gee JM, Chan CM, Green CD, Blamey RW, Nicholson RI (1993) The role of four oestrogen-responsive genes, pLIV1, pS2, pSYD3 and pSYD8, in predicting responsiveness to endocrine therapy in primary breast cancer. Eur J Cancer 29A:1462–1468

Manning DL, Robertson JF, Ellis IO, Elston CW, McClelland RA, Gee JM, Jones RJ, Green CD, Cannon P, Blamey RW et al (1994) Oestrogen-regulated genes in breast cancer: association of pLIV1 with lymph node involvement. Eur J Cancer 30A:675–678

Maret W (2001) Zinc biochemistry, physiology, and homeostasis: recent insights and current trends. Biometals 14:187–190

Margalioth EJ, Schenker JG, Chevion M (1983) Copper and zinc levels in normal and malignant tissues. Cancer (Phila) 52:868–872

Martin-Lagos F, Navarro-Alarcon M, Terres-Martos C, López-G de la Serrana H, Lopez-Martinez MC (1997) Serum copper and zinc concentrations in serum from patients with cancer and cardiovascular disease. Sci Total Environ 204:27–35

Martins LR, Lucio P, Melao A, Antunes I, Cardoso BA, Stansfield R, Bertilaccio MT, Ghia P, Drygin D, Silva MG et al (2013) Activity of the clinical-stage CK2-specific inhibitor CX-4945 against chronic lymphocytic leukemia. Leukemia 28:179–182

Matsuura W, Yamazaki T, Yamaguchi-Iwai Y, Masuda S, Nagao M, Andrews GK, Kambe T (2009) SLC39A9 (ZIP9) regulates zinc homeostasis in the secretory pathway: characterization of the ZIP subfamily I protein in vertebrate cells. Biosci Biotechnol Biochem 73:1142–1148

Memon AU, Kazi TG, Afridi HI, Jamali MK, Arain MB, Jalbani N, Syed N (2007) Evaluation of zinc status in whole blood and scalp hair of female cancer patients. Clin Chim Acta 379:66–70

Menon MB, Ronkina N, Schwermann J, Kotlyarov A, Gaestel M (2009) Fluorescence-based quantitative scratch wound healing assay demonstrating the role of MAPKAPK-2/3 in fibroblast migration. Cell Motil Cytoskeleton 66:1041–1047

Miura K, Fujibuchi W, Unno M (2012) Splice isoforms as therapeutic targets for colorectal cancer. Carcinogenesis (Oxf) 33:2311–2319

Mulay IL, Roy R, Knox BE, Suhr NH, Delaney WE (1971) Trace-metal analysis of cancerous and noncancerous human tissues. J Natl Cancer Inst 47:1–13

Naidu MS, Suryakar AN, Swami SC, Katkam RV, Kumbar KM (2007) Oxidative stress and antioxidant status in cervical cancer patients. Indian J Clin Biochem 22:140–144

Navarro Silvera SA, Rohan TE (2007) Trace elements and cancer risk: a review of the epidemiologic evidence. Cancer Causes Control 18:7–27

Niefind K, Raaf J, Issinger OG (2009) Protein kinase CK2 in health and disease: protein kinase CK2: from structures to insights. Cell Mol Life Sci 66:1800–1816

Ohana E, Hoch E, Keasar C, Kambe T, Yifrach O, Hershfinkel M, Sekler I (2009) Identification of the Zn^{2+} binding site and mode of operation of a mammalian Zn^{2+} transporter. J Biol Chem 284:17677–17686

Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, Jensen LJ, Gnad F, Cox J, Jensen TS, Nigg EA et al (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. Sci Signal 3:ra3

Osborne CK (1998) Tamoxifen in the treatment of breast cancer. N Engl J Med 339:1609–1618

Oteiza PI (2012) Zinc and the modulation of redox homeostasis. Free Radic Biol Med 53:1748–1759

Pakala SB, Rayala SK, Wang RA, Ohshiro K, Mudvari P, Reddy SD, Zheng Y, Pires R, Casimiro S, Pillai MR et al (2013) MTA1 promotes STAT3 transcription and pulmonary metastasis in breast cancer. Cancer Res 73:3761–3770

Palmiter RD, Findley SD (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J 14:639–649

Passerini A, Andreini C, Menchetti S, Rosato A, Frasconi P (2007) Predicting zinc binding at the proteome level. BMC Bioinformatics 8:39

Peinado H, Olmeda D, Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 7:415–428

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA et al (2000) Molecular portraits of human breast tumours. Nature (Lond) 406:747–752

Piccinini L, Borella P, Bargellini A, Medici CI, Zoboli A (1996) A case-control study on selenium, zinc, and copper in plasma and hair of subjects affected by breast and lung cancer. Biol Trace Elem Res 51:23–30

Plum LM, Rink L, Haase H (2010) The essential toxin: impact of zinc on human health. Int J Environ Res Public Health 7:1342–1365

Prasad AS (2009) Impact of the discovery of human zinc deficiency on health. J Am Coll Nutr 28:257–265

Prasad AS, Beck FW, Snell DC, Kucuk O (2009) Zinc in cancer prevention. Nutr Cancer 61:879–887

Ring A, Dowsett M (2004) Mechanisms of tamoxifen resistance. Endocr Relat Cancer 11:643–658

Satoh M, Kondo Y, Mita M, Nakagawa I, Naganuma A, Imura N (1993) Prevention of carcinogenicity of anticancer drugs by metallothionein induction. Cancer Res 53:4767–4768

Science M, Johnstone J, Roth DE, Guyatt G, Loeb M (2012) Zinc for the treatment of the common cold: a systematic review and meta-analysis of randomized controlled trials. Can Med Assoc J 184:E551–E561

Seve M, Chimienti F, Devergnas S, Favier A (2004) In silico identification and expression of SLC30 family genes: an expressed sequence tag data mining strategy for the characterization of zinc transporters' tissue expression. BMC Genomics 5:32

Shen R, Xie F, Shen H, Liu Q, Zheng T, Kou X, Wang D, Yang J (2013) Negative correlation of LIV-1 and E-cadherin expression in hepatocellular carcinoma cells. PLoS One 8:e56542

Siddiqui-Jain A, Drygin D, Streiner N, Chua P, Pierre F, O'Brien SE, Bliesath J, Omori M, Huser N, Ho C et al (2010) CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits prosurvival and angiogenic signaling and exhibits antitumor efficacy. Cancer Res 70:10288–10298

Solares AM, Santana A, Baladron I, Valenzuela C, Gonzalez CA, Diaz A, Castillo D, Ramos T, Gomez R, Alonso DF et al (2009) Safety and preliminary efficacy data of a novel casein kinase 2 (CK2) peptide inhibitor administered intralesionally at four dose levels in patients with cervical malignancies. BMC Cancer 9:146

St.-Denis NA, Litchfield DW (2009) Protein kinase CK2 in health and disease: from birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. Cell Mol Life Sci 66:1817–1829

Summy JM, Gallick GE (2006) Treatment for advanced tumors: SRC reclaims center stage. Clin Cancer Res 12:1398–1401

Sussman D, Smith LM, Anderson ME, Duniho S, Hunter JH, Kostner H, Miyamoto JB, Nesterova A, Westendorf L, Van Epps HA et al (2013) SGN-LIV1A: a development stage antibody drug-conjugate targeting LIV-1 for the treatment of metastatic breast cancer. Cancer Res 73(abstr 3962)

Sveen A, Bakken AC, Agesen TH, Lind GE, Nesbakken A, Nordgard O, Brackmann S, Rognum TO, Lothe RA, Skotheim RI (2012) The exon-level biomarker SLC39A14 has organ-confined cancer-specificity in colorectal cancer. Int J Cancer 131:1479–1485

Swinkels JW, Kornegay ET, Verstegen MW (1994) Biology of zinc and biological value of dietary organic zinc complexes and chelates. Nutr Res Rev 7:129–149

Taniguchi M, Fukunaka A, Hagihara M, Watanabe K, Kamino S, Kambe T, Enomoto S, Hiromura M (2013) Essential role of the zinc transporter ZIP9/SLC39A9 in regulating the activations of Akt and Erk in B-cell receptor signaling pathway in DT40 cells. PLoS One 8:e58022

Tawfic S, Yu S, Wang H, Faust R, Davis A, Ahmed K (2001) Protein kinase CK2 signal in neoplasia. Histol Histopathol 16:573–582

Taylor KM, Nicholson RI (2003) The LZT proteins: the LIV-1 subfamily of zinc transporters. Biochim Biophys Acta 1611:16–30

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, Morgan HE, Johnson A, Nicholson RI (2004) Structure–function analysis of HKE4, a member of the new LIV-1 subfamily of zinc transporters. Biochem J 377:131–139

Taylor KM, Morgan HE, Smart K, Zahari NM, Pumford S, Ellis IO, Robertson JF, Nicholson RI (2007) The emerging role of the LIV-1 subfamily of zinc transporters in breast cancer. Mol Med 13:396–406

Taylor KM, Vichova P, Jordan N, Hiscox S, Hendley R, Nicholson RI (2008) ZIP7-mediated intracellular zinc transport contributes to aberrant growth factor signaling in antihormone-resistant breast cancer Cells. Endocrinology 149:4912–4920

Taylor KM, Gee JMW, Kille P (2011) Zinc and cancer. In: Rink L (ed) Zinc in human health. IOS Press, Amsterdam, pp 283–304

Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P (2012a) Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal 5:ra11

Taylor KM, Kille P, Hogstrand C (2012b) Protein kinase CK2 opens the gate for zinc signaling. Cell Cycle 11

Thiery JP, Acloque H, Huang RY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139:871–890

Thorsen K, Mansilla F, Schepeler T, Oster B, Rasmussen MH, Dyrskjot L, Karni R, Akerman M, Krainer AR, Laurberg S et al (2011) Alternative splicing of SLC39A14 in colorectal cancer is regulated by the Wnt pathway. Mol Cell Proteomics 10(M110):002998

Tozlu S, Girault I, Vacher S, Vendrell J, Andrieu C, Spyratos F, Cohen P, Lidereau R, Bieche I (2006) Identification of novel genes that co-cluster with estrogen receptor alpha in breast tumor biopsy specimens, using a large-scale real-time reverse transcription-PCR approach. Endocr Relat Cancer 13:1109–1120

Walker CL, Black RE (2010) Zinc for the treatment of diarrhoea: effect on diarrhoea morbidity, mortality and incidence of future episodes. Int J Epidemiol 39(Suppl 1):i63–i69

Wang SS, Sherman ME, Hildesheim A, Lacey JV Jr, Devesa S (2004) Cervical adenocarcinoma and squamous cell carcinoma incidence trends among white women and black women in the United States for 1976–2000. Cancer (Phila) 100:1035–1044

Weaver BP, Zhang Y, Hiscox S, Guo GL, Apte U, Taylor KM, Sheline CT, Wang L, Andrews GK (2010) Zip4 (Slc39a4) expression is activated in hepatocellular carcinomas and functions to repress apoptosis, enhance cell cycle and increase migration. PLoS One 5

Welsch CW (1985) Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res 45:3415–3443

Woo W, Xu Z (2002) Body zinc distribution profile during *N*-methyl-*N*-nitrosourea-induced mammary tumorigenesis in rats at various levels of dietary zinc intake. Biol Trace Elem Res 87:157–169

World Health Organization and Food and Agriculture Organization of The United Nations (2002) Zinc. In: FAO/WHO expert consultation on human vitamin and mineral requirements. Rome, pp 257–270

Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, Kurosaki T, Yamashita S, Tokunaga M, Nishida K et al (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645

Yamashita S, Miyagi C, Fukada T, Kagara N, Che YS, Hirano T (2004) Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature (Lond) 429:298–302

Yan M, Song Y, Wong CP, Hardin K, Ho E (2008) Zinc deficiency alters DNA damage response genes in normal human prostate epithelial cells. J Nutr 138:667–673

Yang J, Zhang Y, Cui X, Yao W, Yu X, Cen P, Hodges SE, Fisher WE, Brunicardi FC, Chen C et al (2013) Gene profile identifies zinc transporters differentially expressed in normal human organs and human pancreatic cancer. Curr Mol Med 13:401–409

Yde CW, Frogne T, Lykkesfeldt AE, Fichtner I, Issinger OG, Stenvang J (2007) Induction of cell death in antiestrogen resistant human breast cancer cells by the protein kinase CK2 inhibitor DMAT. Cancer Lett 256:229–237

Yoshida K, Kawano N, Yoshiike M, Yoshida M, Iwamoto T, Morisawa M (2008) Physiological roles of semenogelin I and zinc in sperm motility and semen coagulation on ejaculation in humans. Mol Hum Reprod 14:151–156

Yu M, Lee WW, Tomar D, Pryshchep S, Czesnikiewicz-Guzik M, Lamar DL, Li G, Singh K, Tian L, Weyand CM et al (2011) Regulation of T cell receptor signaling by activation-induced zinc influx. J Exp Med 208:775–785

Zahedi RP, Lewandrowski U, Wiesner J, Wortelkamp S, Moebius J, Schutz C, Walter U, Gambaryan S, Sickmann A (2008) Phosphoproteome of resting human platelets. J Proteome Res 7:526–534

Zaichick V, Sviridova TV, Zaichick SV (1997) Zinc in the human prostate gland: normal, hyperplastic and cancerous. Int Urol Nephrol 29:565–574

Zhang Y, Bharadwaj U, Logsdon CD, Chen C, Yao Q, Li M (2010) ZIP4 regulates pancreatic cancer cell growth by activating IL-6/STAT3 pathway through zinc finger transcription factor CREB. Clin Cancer Res 16:1423–1430

Zhang A, Wang Q, Han Z, Hu W, Xi L, Gao Q, Wang S, Zhou J, Xu G, Meng L et al (2013) Reduced expression of Snail decreases breast cancer cell motility by downregulating the expression and inhibiting the activity of RhoA GTPase. Oncol Lett 6:339–346

Zheng Y, McFarland BC, Drygin D, Yu H, Bellis SL, Kim H, Bredel M, Benveniste EN (2013) Targeting protein kinase CK2 suppresses prosurvival signaling pathways and growth of glioblastoma. Clin Cancer Res 19(23):6484–6494. doi:10.1158/1078-0432.CCR-13-0265

Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC (2004) Dual regulation of snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol 6:931–940

Chapter 15
Zinc as a Key Meiotic Cell-Cycle Regulator in the Mammalian Oocyte

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Abstract Mammalian oogenesis is a discontinuous process that begins during fetal development, arrests at birth, and then resumes on a cyclical basis from puberty to menopause to produce fully mature oocytes that are competent for fertilization. Females are born with a fixed number of oocytes arrested at the prophase I stage of meiosis. This arrest is maintained until oocytes are selected to resume growth by gonadotropins, which are released from the pituitary upon entering puberty. At the time of ovulation, a fully grown oocyte completes maturation and arrests again at metaphase of meiosis II until fertilization occurs. Sperm binding triggers egg activation and release from metaphase II arrest. This entire process is tightly controlled, as it has a significant impact on egg quality and the developmental potential of the resulting embryo. The underlying mechanisms regulating oocyte meiotic entry, arrest, and exit—which can occur over a span of decades—have always been of great interest in the reproductive biology field, and many groundbreaking discoveries have revealed the existence of a dynamic network of hormones, receptors, kinases, and second messengers that control this process. Recent work has expanded this regulatory network to include the transition metal zinc, which adds a new level of complexity and fine-tuning to the regulation of the oocyte meiotic cell cycle. Dynamic accrual, sequestration, and exocytosis of zinc through controlled pathways are crucial for appropriate timing of the meiotic cell cycle as oocytes progress through maturation and activation. This work is important to our general understanding of oocytes and will have implications for reproductive interventions in the future.

Keywords Egg • Fertilization • Meiosis • Oocyte • Zinc spark • Zinc

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15.1 Introduction

Zinc is the second most abundant transition metal, after iron, in living organisms, and its roles in enzymatic catalysis, in protein structure, and in signal transduction have been well characterized (Berg and Shi 1996; Vallee and Falchuk 1993). Zinc may also have second messenger roles in a number of physiological and pathological processes such as glucose-responsive insulin secretion, diabetes, and immune cell function (Mocchegiani et al. 2008; O'Halloran et al. 2013; Yamasaki et al. 2007). Excessive levels of zinc are toxic to cells whereas zinc deficiency is associated with a number of abnormal developmental conditions, including neuronal and immune system defects (Fukada et al. 2011). A number of proteins have known functions in regulating the total cellular zinc concentration and subcellular distribution, including zinc-responsive transcription factors, a wide array of zinc transporters, and zinc-sequestering proteins including the metallothioneins (MT) (Blindauer and Leszczyszyn 2010; Finney and O'Halloran 2003; Fukada and Kambe 2011; Guerra and Giedroc 2012; Kambe et al. 2004; Outten and O'Halloran 2001; Vallee 1995; Waldron et al. 2009). Recent studies reveal that changes in zinc content and subcellular distribution are essential for the maturing oocyte to move through reductive division, known as meiosis, and to later successfully navigate fertilization and early embryogenesis. This chapter reviews the emerging role of zinc in female oocyte biology, where zinc plays a necessary role in completing meiosis and providing the key signal to convert from the meiotic cell cycle in the egg to the mitotic cell cycle of the embryo.

15.2 Oocyte Growth, Maturation, Activation, and Fertilization

To put several of the emerging roles of zinc fluxes in meiotic progression into a biological context, we begin with a brief review of mammalian oogenesis. In mammals, oogenesis begins during embryogenesis and continues until just before birth, when germ cells stop dividing and enter meiosis. Females are born with a fixed number of oocytes, which are enclosed by a single layer of somatic cells called granulosa cells to form follicles, the functional unit of the ovary. The smallest follicles, primordial follicles, form around the time of birth, and each follicle contains a prophase I-arrested (PI) immature oocyte. Primordial follicles remain dormant until they are activated and recruited into the growing follicle pool with each reproductive cycle (McGee and Hsueh 2000). A single primordial follicle and its meiotically arrested oocyte may remain dormant for years, even decades, from the time of birth to the time of activation. Following activation, the follicle grows as the granulosa cell layer proliferates and expands. Growth completes upon formation of the antral follicle, which is characterized by the appearance of the fluid-filled cavity (Rodgers and Irving-Rodgers 2010). As the follicle is growing, so

does its enclosed oocyte, expanding from a diameter of 10 μm in a primordial follicle to 70–80 μm at its final stage in mice (Griffin et al. 2006). The coordinated growth of the follicle and its oocyte is critical for production of a fully mature, meiotically competent oocyte that can be successfully fertilized following ovulation and develop into a viable embryo (Erickson and Sorensen 1974).

During early stages of follicle growth, the oocyte remains arrested in prophase I, displaying an enlarged nuclear envelope called the germinal vesicle (GV). In the antral follicle stage, the PI-arrested oocyte is fully grown and becomes transcriptionally quiescent. At this point, a surge in luteinizing hormone (LH) from the pituitary initiates meiotic resumption in the oocyte, inducing germinal vesicle breakdown (GVBD), concurrent with the condensation of the chromosomes, and promoting the expansion of the surrounding cumulus granulosa cells. After GVBD, the oocyte progresses through meiosis I and undergoes asymmetrical cell division by extruding the first polar body. It then enters meiosis II without interphase and arrests again at metaphase II (MII), at which point the oocyte is now called an egg. In mice, the MII egg ovulates approximately 12 h after the LH surge. This entire process (from PI to MII) is called oocyte maturation, and results in a mature egg that is ready for fertilization.

Upon fertilization, the MII egg completes meiosis II. Binding of sperm to the egg triggers a series of reactions referred to as egg activation, which includes calcium oscillations, exocytosis of cortical granules that block polyspermy, and release of the second polar body to reduce the maternal genome by half (Horner and Wolfner 2008; Wakai et al. 2011). The events of MII egg activation can be induced by artificial means in the absence of sperm in a process called parthenogenesis. Soon after fertilization, paternal and maternal pronuclei form separately and then eventually fuse together before initiating embryonic cell division. Transcription ceases before the oocyte resumes meiosis until zygotic genome activation occurs around the two-cell stage in mice; thus, the entire oocyte maturation and the initial embryonic division are under the influence of the maternally stored transcripts (Moore and Lintern-Moore 1974, 1978; Schultz 2002). Although much is known about oocyte maturation, the meiotic cell cycle, and the egg-to-embryo transition, nothing was known about zinc biology in this process, which is reviewed next.

15.3 Massive Zinc Fluxes Are Integral to Oocyte Development and Activation

The role of calcium in egg activation had been recognized for some time, but little was known about the role of other ions or inorganic metals in oocyte biology. Our first clue that zinc was an integral part to this whole process came from our studies using advanced elemental detection technologies, namely synchrotron-based X-ray fluorescence microscopy (XFM) (de Jonge and Vogt 2010). Using XFM, we discovered quantitative dynamic changes in the inorganic signature of the oocyte

during maturation, activation, and fertilization. More specifically, the elemental makeup at three key stages was determined: the PI oocyte, the MII egg, and the two-cell embryo (Kim et al. 2010). Indeed, XFM data showed that zinc is the most abundant transition metal in the mouse oocyte and that its quota is an order of magnitude higher than those of iron or copper. During maturation, the oocyte rapidly acquires 20 billion zinc atoms, an increase of about 50 % in total zinc content that occurs with minimal change in total cell volume. Following fertilization, approximately 12 billion zinc atoms are lost by the time the newly fertilized embryo reaches the two-cell stage (~20 % decrease) (Kim et al. 2010). These large fluxes in zinc content suggested a larger role for zinc during oocyte maturation and egg activation and motivated a number of further studies regarding the possible cellular functions of zinc during these transitions. What is known regarding the role of zinc in the regulation of oocyte maturation, activation, and fertilization is discussed in detail next.

15.3.1 Roles for Zinc in Maintenance of Prophase I Arrest and Meiotic Resumption

The follicle environment inhibits oocyte maturation until the LH surge, as the oocyte undergoes spontaneous maturation once released from the antral follicle in vitro. Prophase I arrest of the follicle-enclosed oocyte is primarily enforced in part by high cAMP levels within the oocyte. This level is maintained by adenylyl cyclase activity, and release from PI arrest is initiated by oocyte-specific cAMP phosphodiesterase-3A (PDE3A) activity, which leads to cAMP turnover (Masciarelli et al. 2004). PI arrest of isolated oocytes can be maintained in vitro by addition of the cAMP analogue dibutyryl cAMP (dbcAMP), as well as phosphodiesterase inhibitors such as milrinone and 3-isobutyl-1-methylxanthine (IBMX). As the oocyte develops in a complex dynamic follicular environment, it is not surprising that more than one signaling molecule participates in maintaining meiotic arrest.

Recently, we showed that zinc depletion through treatment with a chelator could release oocytes from the meiotic PI arrest. Tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) is a heavy metal chelator with high affinity for zinc ($K_d = 2.6 \times 10^{-16}$ M), in addition to other metals including iron ($K_d = 2.5 \times 10^{-15}$ M) and copper ($K_d = 3 \times 10^{-20}$ M) (Sigdel et al. 2006). Disruption of intracellular zinc homeostasis in in vitro cultured oocytes using low, nontoxic levels of TPEN (10 μM) induces meiotic resumption even in the presence of the meiotic inhibitor milrinone (Kong et al. 2012; Tian and Diaz 2012). TPEN acts as a specific zinc chelator, as supplementation of the culture medium with zinc reversed the TPEN-induced meiotic resumption, with oocytes remaining at prophase I. These results can be recapitulated using an in vivo model. Fully grown oocytes from mice fed a zinc-deficient diet for 10 days underwent premature

GVBD, before the LH surge (Tian and Diaz 2012). Thus, both small-molecule zinc chelation in vitro and systemic zinc deficiency in vivo are capable of inducing premature GVBD. We noted that PI oocytes treated transiently with TPEN resumed meiosis normally and proceeded to the MII arrest stage if they were transferred, after completion of GVBD, to TPEN-free medium.

The rapid decrease in cAMP after the LH surge permits meiotic resumption (Norris et al. 2009). A complex bidirectional talk exists between oocytes and its surrounding somatic compartments (Sugiura et al. 2008; Wigglesworth et al. 2013; Zhang et al. 2010). The signaling cascades that mediate meiotic arrest and resumption have been reviewed in great detail elsewhere (Conti et al. 2012; Downs 2010). In brief, the release from prophase I arrest in the oocyte is accompanied by an increase in the activity of maturation-promoting factor (MPF), the activity of which is indirectly inhibited by a high level of cAMP that activates protein kinase A (PKA) during meiotic arrest (Duncan et al. 2006; Han et al. 2005; Oh et al. 2010). Similar to its role in mitotic cell-cycle regulation, MPF is composed of cyclin B1 and cyclin-dependent kinase 1 (CDK1), which drives cell-cycle progression in oocytes. In fact, oocyte growth and gain of meiotic competency are positively correlated with accumulation of MPF (Mitra and Schultz 1996). The kinetics of GVBD induced by TPEN in the presence of milrinone was prolonged, occurring 9 h later in the pulsed TPEN-treated oocytes compared to spontaneously maturing oocytes in vitro (Kong et al. 2012; Tian and Diaz 2012). Accordingly, MPF activity in TPEN-treated oocytes was similarly delayed, which was consistent with the slow GVBD kinetics (Tian and Diaz 2012). The relationship between MPF activity and oocyte zinc levels is not limited to the time of GVBD, as is discussed in the following section.

Surprisingly, in contrast to the decline of cAMP levels before GVBD as one would expect, cAMP levels initially increased in TPEN-treated oocytes and then dropped back down to the baseline around the time of GVBD (Tian and Diaz 2012). This finding is consistent with work by Salustri et al., who reported a transient rise in cAMP level in mouse cumulus-enclosed oocytes cultured in vitro after treatment with a maturation-stimulating dose of follicle-stimulating hormone (FSH) (Salustri et al. 1985). Indeed, short-term incubation of mouse oocytes with cAMP analogues induced meiotic resumption (Chen et al. 2009), likely because of the activation of AMP-activated protein kinase (AMPK), which has been shown to mediate hormone-induced maturation in mouse oocytes (Chen and Downs 2008). Exactly how zinc deficiency leads to cAMP elevation remains elusive. We note that PDE4A hydrolyase activity is enhanced by zinc and that a high concentration of zinc is inhibitory to purified PDE3A protein (Percival et al. 1997; Zhang and Colman 2000). Although we are not sure whether this is the case for oocyte-specific PDE3A in vivo, it still remains possible that TPEN treatment may inactivate some fraction of the PDE3A pool. Altogether, it is likely that TPEN induces meiotic resumption by stimulating more than one pathway.

Further analysis of the mechanisms underlying TPEN-induced GVBD by Kong et al. (2012) suggested that meiotic resumption in zinc-insufficient oocytes is mediated through premature activation of the Mos-mitogen-activated protein

kinase (Mos-MAPK) pathway. The meiosis-inducing action of LH is conveyed to the oocyte through the participation of somatic compartments (Park et al. 2004), as the oocyte does not express LH receptors (Lawrence et al. 1980). Granulosa cell MAPK activation is required for LH signaling in the follicle (Fan et al. 2009; Norris et al. 2008; Su et al. 2003), as mice lacking MAPK3/1 signaling specifically in granulosa cells are infertile and show no signs of GVBD or ovulation (Fan et al. 2009) Although activation of MAPK3/1 in somatic cells is indispensible for oocyte meiotic resumption (Fan et al. 2009; Su et al. 2003), activation of this pathway within the oocyte itself is not required for maturation, which is supported by two lines of evidence. First, both MAPK protein and phosphorylation levels increase in oocytes only after GVBD (Kubiak et al. 1993; Verlhac et al. 1996). Second, *mos*-null oocytes are able to undergo maturation with similar kinetics as wild-type control oocytes (Hashimoto et al. 1994). It therefore appears that mammalian oocytes have evolved to limit Mos-MAPK pathway signaling to later meiotic events. Interestingly, ectopic expression of Mos in bovine oocytes accelerates GVBD (Fissore et al. 1996), and microinjection of *mos*, or MAPK kinase RNA induces GVBD in mouse oocytes maintained in prophase I arrest by IBMX (Choi et al. 1996), suggesting that premature activation of this pathway can trigger meiotic cell-cycle resumption. Indeed, both Mos and phospho-MAPK3/1 protein levels were elevated before MPF activation and GVBD in TPEN-treated oocytes (Kong et al. 2012). Inhibition of protein synthesis and knockdown of Mos-MAPK pathway components abolished the effect of TPEN on delaying meiotic induction, suggesting that premature activation of the Mos-MAPK pathway drives the maturation defect in TPEN-treated oocytes, and that increased zinc availability suppresses the Mos-MAPK pathway to maintain meiotic arrest (Kong et al. 2012).

15.3.2 Zinc Fluxes Regulating Meiotic Progression and Metaphase II Arrest

Following release from PI arrest, we find that the next stage of meiotic progression is dependent on fluctuations in zinc availability in a completely different manner: the choreography of chromosomal movement and cytoskeletal dynamics as the oocyte proceeds from GVBD and into metaphase II (MII) arrest shows a striking requirement for elevated zinc availability (Bernhardt et al. 2011; Kim et al. 2010).

Shortly after GVBD, spindle microtubules nucleated from centriole-lacking microtubule-organizing centers (MTOCs) start polymerizing around the condensed chromosomes. The initial meiotic spindle forms slightly off center in the region of the ooplasm that corresponds to the location of the original GV, and then migrates to the cortex before extrusion of the first polar body, along with half of its chromosomal complement (Li and Albertini 2013). Metaphase II arrest is established following completion of this process. During this same maturation period, the total zinc concentration in oocytes increases by about 50 %, with zinc

levels peaking at the MII stage (Kim et al. 2010). This dramatic change is remarkable and has not been reported in any other cell type investigated thus far (Krężel and Maret 2006). As already discussed, a transient TPEN-induced zinc deficiency at the PI oocyte stage induces meiotic resumption and produces a mature MII egg. However, chronic zinc depletion by TPEN impairs meiotic progression. We found that when oocytes were exposed to 10 μM TPEN during the entire maturation period, a significant number of TPEN-treated oocytes generated unusually large polar bodies (PBs). Further investigation into the spindle morphology of these cells revealed that instead of progressing completely to the metaphase II arrest stage, these cells stalled at a telophase I-like stage and retained a persistent midbody at the site of cytokinesis (Bernhardt et al. 2011; Kim et al. 2010). Subsequent studies reveal that exposure of oocytes to TPEN only after the formation of the MI spindle was sufficient to induce maturation defects (Bernhardt et al. 2012), indicating that premature meiotic arrest is largely caused by zinc insufficiency after the MI stage. In addition to spindle defects, TPEN-treated oocytes exhibited defects in cortical reorganization as an incomplete clearance of cortical granules close to the meiotic spindle region was observed (Bernhardt et al. 2011).

The phenotypes of TPEN-treated oocytes, that is, defects in spindle formation, spindle migration, and cortical reorganization, suggested disruption of the Mos-MAPK pathway. Spindle migration is dependent on an actin filament network, which ensures asymmetrical division so that the majority of the oocyte content is preserved (Azoury et al. 2008). In oocytes, the Mos-MAPK pathway and additional proteins, such as formin-2 and Cdc42, mediate spindle formation and positioning. For instance, *mos*-null oocytes often undergo symmetrical oocyte division and generate a large polar body (Choi et al. 1996; Deng et al. 2005; Verlhac et al. 1996), similar to TPEN-treated oocytes. They also exhibit defects in cortical reorganization, a process that normally includes thickening of actin filaments and loss of microvilli in the region that has close contact with the translocated meiotic spindle (Choi et al. 1996; Longo and Chen 1985; Nicosia et al. 1977). However, *mos*-null oocytes and wild-type oocytes treated with TPEN during the entire maturation period arrest at different developmental stages. Mos-null oocytes were able to complete maturation, whereas zinc-insufficient oocytes stalled at telophase I (Bernhardt et al. 2011; Choi et al. 1996). Interrogation of the potential link between zinc insufficiency and disruption of the Mos-MAPK pathway indicated that TPEN treatment did not affect the Mos-MAPK pathway during the MI to MII transition (Bernhardt et al. 2011). Briefly, zinc-insufficient oocytes had phospho-MAPK3/1 levels comparable to those of controls, despite the decreases in Mos protein levels and MAP2K1/2 phosphorylation in TPEN-treated oocytes; however, this decrease happened later than telophase I arrest, and microinjection of *mos* RNA did not rescue the zinc-insufficient phenotypes (Bernhardt et al. 2011).

Having found that the Mos-MAPK pathway is unlikely to play a role in premature meiotic arrest, Bernhardt et al. explored a potential role for zinc in the regulation of MPF activity (Bernhardt et al. 2011, 2012). To put these observations into context, it is important to consider the cell-cycle regulators controlling oocyte

meiotic progression. MPF levels accumulate until metaphase I (MI), remain high before each meiotic cell division, and then quickly decline afterward (Hashimoto and Kishimoto 1988; Ledan et al. 2001). Similar to mitotic cell division, the onset of anaphase is initiated by Cdc20-containing anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that triggers degradation of substrates including cyclin B1 and securin. Proteasomal degradation of securin liberates separase, which cleaves cohesin and allows the segregation of homologous chromosomes (Terret et al. 2003). APC/C activity is tightly controlled, as premature activation before correct chromosome alignment would cause aneuploidy that compromises later development. Oocytes enter meiosis II without undergoing the S phase and arrest again at MII with fully aligned chromosomes. MPF levels rise quickly after anaphase II and peak during MII arrest; this increase in MPF is caused by active translation as fully grown oocytes are transcriptionally quiescent (Moore and Lintern-Moore 1974, 1978). Meanwhile, APC/C activity is inhibited by the cytostatic factor (CSF), which is essential for maintaining high MPF levels during MII arrest (Madgwick and Jones 2007).

Bernhardt et al. have provided evidence supporting the model that elevated zinc in the GV–MII transition leads to zinc loading of the oocyte-specific APC/C inhibitor, early mitotic inhibitor 2 (Emi2), and that the zinc–Emi2 complex constitutes active CSF (Madgwick and Jones 2007; Shoji et al. 2006). Emi2 contains a zinc-binding region (ZBR) in the C-terminus that is indispensable for blocking the ability of Cdc20 to activate APC/C (Schmidt et al. 2005; Tung et al. 2005). This region resembles an in-between ring (IBR) zinc-finger structural motif and contains eight putative zinc-binding residues, which are composed of seven cysteine residues and one histidine residue (Suzuki et al. 2010a, b). Most importantly, Emi2 not only participates in cytostatic factor (CSF) maintenance, it also establishes CSF activity. Several lines of evidence support the role of Emi2 as a CSF component. First, Emi2 protein levels rise immediately after MI (Liu et al. 2006; Ohe et al. 2007). This timing allows reaccumulation of cyclin B1 and presumably allows the oocyte to proceed through MI, because premature expression of Emi2 induces MI arrest (Madgwick et al. 2006; Suzuki et al. 2010a). Second, the MII spindle fails to assemble in Emi2 morpholino knockdown oocytes after PB extrusion and chromosomes quickly become decondensed; this phenotype can be rescued by nondegradable cyclin B1 (Madgwick et al. 2006), indicating a role of Emi2 in establishment of CSF. Third, Emi2 is rapidly degraded before cyclin B1 destruction upon fertilization, which is consistent with the relief of inhibition on APC/C, thereby allowing the resumption of the meiotic cell cycle (Madgwick et al. 2006). Emi2 was identified in *Xenopus* oocytes as a downstream substrate of polo-like kinase 1 (Plk1) that is important for mediating calcium-dependent activation of APC/C during fertilization (Schmidt et al. 2005; Shoji et al. 2006). Taken together, these data suggest an indispensible and conserved role for Emi2 in maintaining MII arrest in vertebrate oocytes.

Given the importance of the ZBR of Emi2 and the zinc quota change during oocyte maturation, we hypothesize that zinc levels within the oocytes regulate the proper inhibitory functioning of Emi2. We observed that MPF failed to accumulate

after the first meiotic division in TPEN-treated oocytes. Restoration of MPF activity by either expressing nondegradable cyclin B1 or incubating oocytes with protease inhibitor significantly increased the percentage of zinc-insufficient oocytes that progressed to MII (Bernhardt et al. 2011, 2012). This finding implied that inappropriate degradation of cyclin B1, most likely by premature activation of APC/C, accounts for the zinc-insufficient oocyte phenotype. Indeed, oocytes with morpholino knockdown of Emi2, the zinc-binding domain containing APC/C inhibitor, phenocopied zinc-insufficient oocytes, with the formation of large PB and an inability to establish the MII spindle (Shoji et al. 2006). The proper functioning of Emi2 is dependent on zinc availability, as TPEN perturbed the ability of Emi2 to induce metaphase arrest and expression of Emi2 mutated in the zinc-binding region did not completely rescue Emi2-knockdown oocytes (Bernhardt et al. 2012). Based on the data and zinc accumulation profile, we speculate that zinc binding to Emi2 constitutes the active CSF complex, making the zinc flux which leads to loading of Emi2 a critical step in regulating meiotic progression and establishing MII arrest.

15.3.3 Fertilization and Egg Activation

Upon fusion of the sperm with the ooplasmic membrane, the mammalian oocyte goes through a process known as activation. A hallmark of activation is the exit of the oocyte from MII arrest and resumption of meiosis II upon downregulation of CSF activity. Calcium signaling is a remarkably conserved pathway in fertilization in the animal kingdom (Stricker 1999). During fertilization, intracellular calcium is elevated by repetitive calcium rises, referred to as calcium oscillations (Cuthbertson et al. 1981; Ducibella and Fissore 2008). A novel member of the PLC family, PLCζ, is a sperm factor capable of inducing calcium oscillations and triggering egg activation (Saunders et al. 2002). Egg activation can also be achieved artificially without sperm fertilization by chemical treatment (parthenogenesis) such as calcium ionophores (Nakagawa et al. 2001), strontium chloride (Ma et al. 2005; O'Neill et al. 1991), ethanol (Cuthbertson 1983; Kaufman 1982), and phorbol ester (Cuthbertson and Cobbold 1985; Sun et al. 1999). All methods lead to egg activation by increasing intracellular calcium levels.

Zinc chelation can also result in parthenogenetic egg activation, as treatment of MII eggs with 10 μM TPEN results in exit from MII and formation of a single pronucleus (Kim et al. 2010). This TPEN-induced activation is accompanied by reduction in both MPF and MAPK activity, which normally occur when oocytes exit MII arrest (Suzuki et al. 2010b). Furthermore, TPEN treatment successfully induced MII exit in eggs injected with heat-inactivated sperm heads, resulting in live birth of mice following embryo transfer. This result suggested the depletion of zinc at the time of fertilization could also act as an egg-activating method, as heat-inactivated sperm heads alone fail to activate. Strikingly, calcium oscillations did not occur after TPEN treatment in these experiments, arguing against the absolute

requirement for calcium oscillations during egg activation (Suzuki et al. 2010b). The action of TPEN has been attributed in part to its ability to modulate the activity of the ZBR of Emi2 as overexpression of Emi2 in mouse oocytes reduces the ability of TPEN to promote egg activation (Suzuki et al. 2010a). Mutation of putative zinc-binding residues abrogated this effect. These experiments further supported the importance of zinc as a gatekeeping switch for eggs exiting MII by regulating Emi2 and thus as a key component of egg activation.

In a complementary set of experiments, the effect of increasing intracellular zinc levels on egg activation was investigated. The ability of TPEN to activate eggs, and XFM results that indicate a decrease in zinc levels is a characteristic of the egg to embryo transition, suggested that inducing higher than normal zinc levels in MII eggs should prevent activation. Indeed, artificially increasing the zinc level in eggs using the ionophore zinc pyrithione (ZnPT) abolished strontium-induced activation (Bernhardt et al. 2012). Furthermore, ZnPT administration 90 min after strontium-induced egg activation produced eggs with a metaphase-like spindle and suppressed pronucleus formation (Kim et al. 2011). These experiments further emphasize how the oocyte exploits precise cellular zinc levels to control its meiotic cell cycle.

15.3.4 Zinc Sparks: A Novel Facet of the Egg–Embryo Transition

Zinc fluxes not only control resumption and completion of oocyte maturation, but also play a striking role upon fertilization. In contrast to the previous stages where zinc uptake and trafficking plays a central role, zinc efflux is essential in the first several hours of the egg-to-embryo transition. Soon after fertilization, zinc is rapidly expelled from the egg through a series of exocytosis events called zinc sparks (Kim et al. 2011). These concentrated, periodic releases of zinc from the female gamete were detected using live cell fluorescence microscopy and the extracellular zinc dye FluoZin-3. Individual mouse eggs display one to five sparks following $SrCl_2$ activation, with the majority of eggs displaying between two and three sparks. Zinc sparks were also observed upon in vitro fertilization. The cell biology of the coordinated zinc spark events remains uncertain but it is clear that they originate from regions of the egg cortex centered at the microvilli region, that is, distal to the meiotic spindle (Kim et al. 2011). The asymmetrical localization of both total and labile zinc has been established using X-ray fluorescence microscopy (XFM) and in fluorescence microscopy experiments using zinc-selective fluorophores. Z-stack confocal imaging reveals a pattern similar to that observed for the cortical granules that are released at fertilization (Kim et al. 2011). As the cortical granules contain enzymes that are important for zona pellucida hardening and the prevention of polyspermy (Barros and Yanagimachi 1971; Burkart et al. 2012), it is possible that zinc sparks have additional roles during egg activation that have yet to be explored.

Further observation suggested a relationship between intracellular calcium signaling and the release of the zinc sparks. Each zinc spark is preceded by an intracellular rise in calcium, shown using calcium-sensitive and zinc-sensitive dyes simultaneously. The sequestration of intracellular calcium by a calcium chelator, 1,2-bis (*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), inhibited the zinc sparks. In addition, the number of zinc sparks in mouse oocytes positively correlates with the pattern of calcium oscillations triggered by different parthenogenetic activation agents. Strontium chloride induces multiple calcium oscillations in eggs that mimic the effect of sperm contact, whereas ionomycin only triggers a single calcium transient in this organism (Alberio et al. 2001; Bos-Mikich et al. 1995; Kline and Kline 1992; Whittingham and Siracusa 1978). Consistent with these effects, no more than one spark was observed when eggs from either mice (unpublished results) or nonhuman primates (Kim et al. 2011) were activated with ionomycin.

Even after egg activation and fertilization, zinc continues to influence subsequent embryonic development. Maternal zinc deficiency is associated with poor pregnancy outcomes and developmental abnormalities (King 2000). Female rats fed a zinc-deficient diet exhibited reduced litter size and slower growth rate of pups (Hurley and Swenerton 1966). Pregnant patients with acrodermatitis enteropathica, a genetic disorder that affects zinc absorption, are at higher risk of severe abnormalities in fetal development (Hambidge et al. 1975). The negative effect of zinc deficiency traced back to its impact on oocyte quality, as oocytes from zinc-deficient animals underwent premature meiotic resumption and did not ovulate, and also showed abnormal epigenetic programming and aberrant gene expression in oocytes (Tian and Diaz 2013). Of course we cannot negate the systemic effect of zinc deficiency, which might have changed the follicle microenvironment in a way that impairs the ability of follicles to maintain oocyte meiotic arrest, suggesting a negative effect on normal ovarian function as well. Although cultured oocytes treated transiently with TPEN can be fertilized in vitro and proceed through to live birth, the blastocyst rate is significantly reduced (Suzuki et al. 2010b). Similarly, a reduction in fertilization rate and blastocyst formation was observed in the eggs that were collected from the animals on a zinc-deficient diet, and few expended blastocysts were recovered from the reproductive tract of zinc-deficient animals (Tian and Diaz 2013). Abnormal imprinting of embryos from these zinc-deficient animals was also observed. Embryonic development initiated after transient TPEN activation as just described involved a brief exposure to TPEN that was followed by somatic nuclear transfer. However, compared to traditional strontium activation methods, both the activation rate and embryonic development were lower in the TPEN-treated group (Suzuki et al. 2010b). Whether this is caused by general embryo toxicity or lack of calcium oscillations remains to be investigated. Taken together, these results indicate that zinc availability to the developing oocyte is extremely important to generation of both high-quality gametes and subsequent normal, viable embryos.

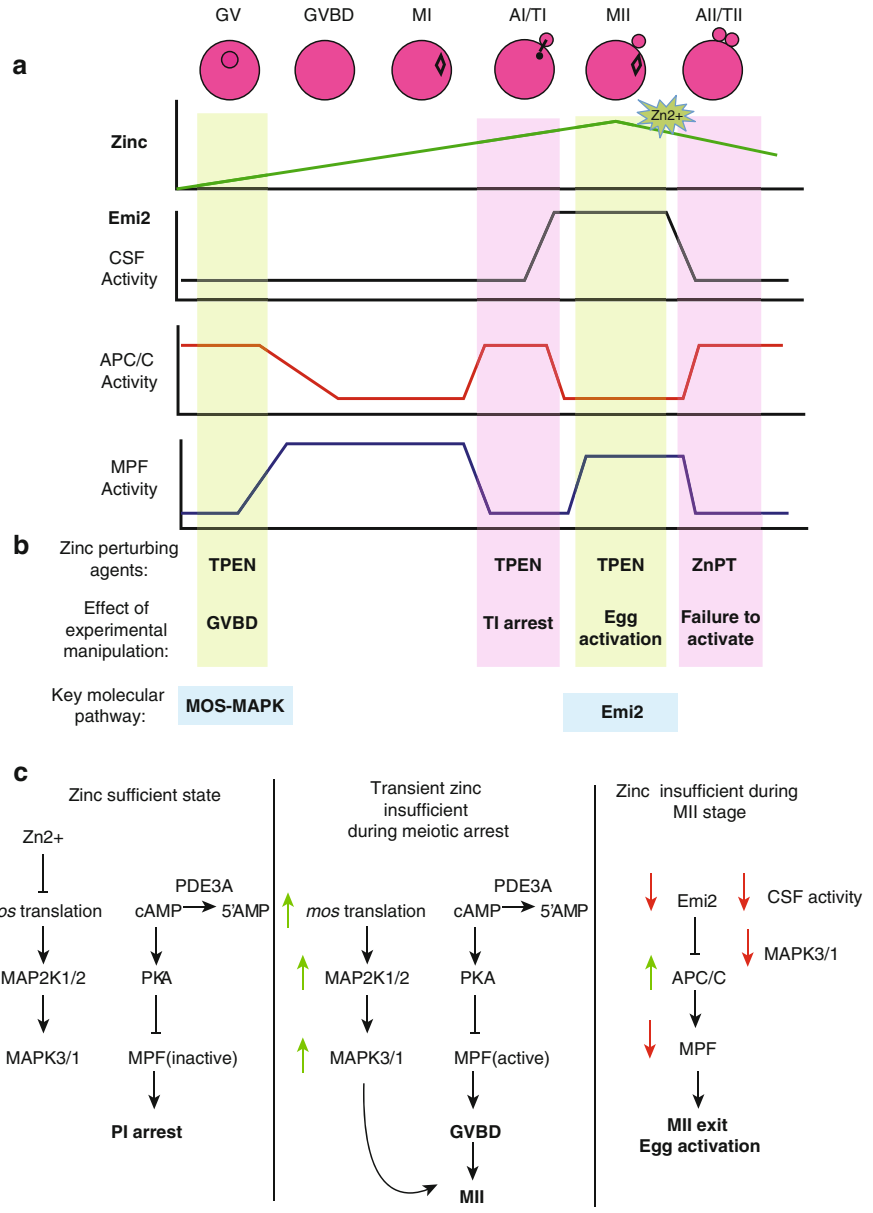

Fig. 15.1 Summary of zinc dynamics throughout meiotic maturation. **a** Total cellular zinc increases by 50 % during meiotic maturation from the prophase I-arrested oocyte to the metaphase II (MII)-arrested egg, and then drops upon fertilization in events described as zinc sparks. The fluctuations of cytostatic factor (CSF), anaphase-promoting complex/cyclosome (APC/C), and maturation-promoting factor (MPF) activity during oocyte maturation and egg activation are also depicted. **b** Perturbation of zinc availability using the heavy metal chelator tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) or the zinc ionophore zinc pyrithione (ZnPT) at critical windows of meiotic maturation and fertilization interferes with specific zinc-dependent pathways,

15.4 Concluding Remarks and Future Directions

Zinc has a central role in many physiological processes. Acute or chronic zinc deficiency causes endoplasmic reticulum stress that could contribute to neuron toxicity (Homma et al. 2013) and is linked to thymic atrophy and lack of cytokine production (Fraker et al. 1986). Zinc is also critical for immune system function (Fraker and King 2004). In this review we have summarized studies of mammalian oocytes in which zinc fluxes play a series of specific and unrelated roles in regulating sequential steps in the meiotic cell cycle. Zinc availability is tightly regulated in the developing oocyte, and its disposition is critical for meiotic cell-cycle progression. First, zinc is required for maintaining prophase I arrest before the LH surge. Second, rapid accumulation of zinc over a short period of time is absolutely necessary for establishing and maintaining metaphase II arrest. Consequently, lowering intracellular zinc content via a series of zinc sparks initiates the second meiotic cell-cycle resumption (summarized in Fig. 15.1). Thus, zinc acts as both a permissive and maintenance factor throughout oocyte maturation and egg activation. These discoveries have opened up a new window onto the role of inorganic elements in egg physiology, adding another layer of complexity to oocyte maturation and egg activation.

The rapid zinc accrual during oocyte maturation is particularly intriguing, and the mechanism by which it occurs is an active area of research. It will be important to identify the specific zinc transporters involved and their temporal and spatial relationships to localization of zinc during oocyte maturation and early embryonic development. Two conserved zinc transporter family proteins mediate cellular zinc transport: ZIP family proteins (Zrt-and Irt-like proteins, e.g., SLC39) and ZnT family proteins (SLC30) (Kambe et al. 2004). ZIP family zinc transporters regulate zinc uptake from the extracellular environment or zinc storage, whereas ZnT family

Fig. 15.1 (continued) resulting in premature arrest or progression through the maturation pathway. Decreasing zinc availability using TPEN at the two crucial arrest points, namely, prophase I and metaphase II, results in premature meiotic progression. Oocytes that undergo germinal vesicle breakdown (GVBD) because of zinc insufficiency have the capacity to develop to MII eggs on return to zinc-replete medium and form blastocysts upon egg activation. This effect of zinc insufficiency in causing meiotic resumption from prophase I arrest is mediated via the MOS-MAPK pathway. As described, experimental manipulation of zinc levels before or after MII arrest, when zinc is actively being acquired or released, respectively, results in meiotic arrest. **c** Model showing proposed action of a zinc-mediated pathway during the first meiotic arrest at prophase I. In a zinc-sufficient state, the MOS-MAPK pathway is maintained in a quiescent state, MPF activity remains low and the oocyte remains arrested at the prophase I-arrested (PI) stage. Transient zinc insufficiency as induced by chelation relieves the inhibition on the MOS-MAPK pathway, allowing meiotic resumption, GVBD, and progression toward MII. Chronic zinc insufficiency or zinc insufficiency during MI–MII transition causes telophase I arrest. Zinc insufficiency during the MII stage induces egg activation by decreasing CSF activity and alleviates APC/C inhibition, which ultimately leads to MII exit. [Figure adapted from Kong et al. (2012) and Bernhardt et al. (2012)]

proteins are responsible for mediating zinc efflux (Fukada and Kambe 2011). Compartmentalization of labile zinc has functional significance; for example, zinc sequestration in macrophages enhances intracellular pathogen clearance (Subramanian Vignesh et al. 2013). Furthermore, zinc compartmentalization by ZnT8 along with insulin in the secretory compartments of pancreatic beta cells is essential to normal systemic glucose utilization (Chimienti et al. 2006). Metallothionein proteins also regulate labile intracellular zinc levels (Blindauer and Leszczyszyn 2010; Vallee 1995). These are but a few examples wherein cell-specific zinc trafficking machinery has a key role in highly differentiated cells. Many zinc transporters are differentially expressed in mouse oocytes and cumulus cells, suggesting the regulation of zinc uptake and sequestration in oocytes by its somatic compartment (Lisle et al. 2013).

The zinc spark phenomenon is striking and raises a myriad of questions. Is the zinc spark a conserved mechanism? What is the function of the zinc spark? If it is as well conserved as calcium oscillations, why do zinc sparks adopt a redundant pathway? The zinc spark is not unique to the mouse egg; it seems to be a conserved biological event in mammals and has been observed in nonhuman primates (Kim et al. 2011). Future studies will clarify whether the zinc spark is conserved in other mammalian species and when it appeared during animal evolution. Current data support the idea that accumulation of zinc during maturation is a component of the gatekeeping mechanisms maintaining MII arrest, as premature parthenogenetic activation is undesired. This role of zinc is supported by the fact that the zinc accumulation profile overlaps with Emi2 expression during oocyte maturation. To resume the meiotic cell cycle upon fertilization, cyclin B1 needs to be degraded by APC/C, which can be accomplished by quick exocytosis of zinc that subsequently reduces the function of Emi2. Therefore, zinc sparks could have a dual effect of maintaining MII arrest in the absence of activation stimuli and facilitating robust meiotic cell-cycle resumption when cued.

Ultimately, it is important to examine the translational value of these findings in a clinical setting. Infertility affects one of every ten couples in the United States, and it is well recognized that decreasing egg quality is associated with increasing maternal age and metabolic dysfunction (Qiao et al. 2013). The insights that we gain from studies of zinc biology in the regulation of oocyte maturation and egg activation may inform improvements in assisted reproductive technologies (ART). In most cases, women who are treated with human chorionic gonadotropin (hCG) to induce in vivo oocyte maturation and ovulation experience mood swings and other adverse effects, and the procedure is associated with a high incidence of multiple births; these outcomes can be circumvented by controlled in vitro oocyte maturation (IVM) (Heijnen et al. 2004). Monitoring and controlling zinc dynamics may ultimately have an impact on IVM outcomes and yield a higher proportion of high-quality fertilizable eggs in several ways. For instance, pharmacological alteration of zinc levels may make it possible to rescue eggs that fail to activate after intracytoplasmic sperm injection (ICSI) (Lu et al. 2006), which is a method of treating male patients with a PLC mutation (Eldar-Geva et al. 2003; Taylor et al. 2010). The discovery of a new hallmark of egg activation—that is, the zinc

spark—holds promise as a noninvasive method to predict embryo developmental potential, giving embryologists a way to select the best quality embryos to implant, thereby reducing the risk of multiple births and other complications.

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References

Alberio R, Zakhartchenko V, Motlik J, Wolf E (2001) Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer. Int J Dev Biol 45:797–809

Azoury J, Lee KW, Georget V, Rassinier P, Leader B, Verlhac M-H (2008) Spindle positioning in mouse oocytes relies on a dynamic meshwork of actin filaments. Curr Biol 18:1514–1519

Barros C, Yanagimachi R (1971) Induction of zona reaction in golden hamster eggs by cortical granule material. Nature (Lond) 233:268–269

Berg JM, Shi Y (1996) The galvanization of biology: a growing appreciation for the roles of zinc. Science 271:1081–1085

Bernhardt ML, Kim AM, O'Halloran TV, Woodruff TK (2011) Zinc requirement during meiosis I–meiosis II transition in mouse oocytes is independent of the MOS-MAPK pathway. Biol Reprod 84:526–536

Bernhardt ML, Kong BY, Kim AM, O'Halloran TV, Woodruff TK (2012) A zinc-dependent mechanism regulates meiotic progression in mammalian oocytes. Biol Reprod 86:114

Blindauer CA, Leszczyszyn OI (2010) Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more. Nat Prod Rep 27:720–741

Bos-Mikich A, Swann K, Whittingham DG (1995) Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. Mol Reprod Dev 41:84–90

Burkart AD, Xiong B, Baibakov B, Jiménez-Movilla M, Dean J (2012) Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. J Cell Biol 197:37–44

Chen J, Downs SM (2008) AMP-activated protein kinase is involved in hormone-induced mouse oocyte meiotic maturation in vitro. Dev Biol 313:47–57

Chen J, Chi MM, Moley KH, Downs SM (2009) cAMP pulsing of denuded mouse oocytes increases meiotic resumption via activation of AMP-activated protein kinase. Reproduction 138:759–770

Chimienti F, Devergnas S, Pattou F, Schuit F, Garcia-Cuenca R, Vandewalle B, Kerr-Conte J, Van Lommel L, Grunwald D, Favier A et al (2006) In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. J Cell Sci 119:4199–4206

Choi T, Fukasawa K, Zhou R, Tessarollo L, Borror K, Resau J, Vande Woude GF (1996) The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. Proc Natl Acad Sci USA 93:7032–7035

Conti M, Hsieh M, Zamah AM, Oh JS (2012) Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. Mol Cell Endocrinol 356:65–73

Cuthbertson KSR (1983) Parthenogenetic activation of mouse oocytes in vitro with ethanol and benzyl alcohol. J Exp Zool 226:311–314

Cuthbertson K, Cobbold P (1985) Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. Nature (Lond) 316:541

Cuthbertson KS, Whittingham DG, Cobbold PH (1981) Free Ca^{2+} increases in exponential phases during mouse oocyte activation. Nature (Lond) 294:754–757

De Jonge MD, Vogt S (2010) Hard X-ray fluorescence tomography; an emerging tool for structural visualization. Curr Opin Struct Biol 20:606–614

Deng M, Williams CJ, Schultz RM (2005) Role of MAP kinase and myosin light chain kinase in chromosome-induced development of mouse egg polarity. Dev Biol 278:358–366

Downs SM (2010) Regulation of the G_2/M transition in rodent oocytes. Mol Reprod Dev 77:566–585

Ducibella T, Fissore R (2008) The roles of Ca^{2+}, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. Dev Biol 315:257–279

Duncan FE, Moss SB, Williams CJ (2006) Knockdown of the cAMP-dependent protein kinase (PKA) type 1-alpha regulatory subunit in mouse oocytes disrupts meiotic arrest and results in meiotic spindle defects. Dev Dyn 235:2961–2968

Eldar-Geva T, Brooks B, Margalioth EJ, Zylber-Haran E, Gal M, Silber SJ (2003) Successful pregnancy and delivery after calcium ionophore oocyte activation in a normozoospermic patient with previous repeated failed fertilization after intracytoplasmic sperm injection. Fertil Steril 79:1656–1658

Erickson GF, Sorensen RA (1974) In vitro maturation of mouse oocytes isolated from late, middle, and pre-antral graafian follicles. J Exp Zool 190:123–127

Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, Richards JS (2009) MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. Science 324:938–941

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

Fissore RA, He CL, Vande Woude GF (1996) Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. Biol Reprod 55:1261–1270

Fraker PJ, King LE (2004) Reprogramming of the immune system during zinc deficiency. Annu Rev Nutr 24:277–298

Fraker PJ, Gershwin ME, Good RA, Prasad A (1986) Interrelationships between zinc and immune function. Fed Proc 45:1474–1479

Fukada T, Kambe T (2011) Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics 3:662–674

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signaling in health and diseases: zinc signaling. J Biol Inorg Chem 16:1123–1134

Griffin J, Emery BR, Huang I, Peterson CM, Carrell DT (2006) Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). J Exp Clin Assist Reprod 3:2

Guerra AJ, Giedroc DP (2012) Metal site occupancy and allosteric switching in bacterial metal sensor proteins. Arch Biochem Biophys 519:210–222

Hambidge K, Neldner K, Walravens P (1975) Zinc, acrodermatitis enteropathica, and congenital malformations. Lancet 1:577–578

Han SJ, Chen R, Paronetto MP, Conti M (2005) Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse. Curr Biol 15:1670–1676

Hashimoto N, Kishimoto T (1988) Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. Dev Biol 126:242–252

Hashimoto N, Watanabe N, Furuta Y, Tamemoto H, Sagata N, Yokoyama M, Okazaki K, Nagayoshi M, Takeda N, Ikawa Y et al (1994) Parthenogenetic activation of c-mos mice. Nature (Lond) 370:68–71

Heijnen EMEW, Macklon NS, Fauser BCJM (2004) What is the most relevant standard of success in assisted reproduction? The next step to improving outcomes of IVF: consider the whole treatment. Hum Reprod 19:1936–1938

Homma K, Fujisawa T, Tsuburaya N, Yamaguchi N, Kadowaki H, Takeda K, Nishitoh H, Matsuzawa A, Naguro I, Ichijo H (2013) SOD1 as a molecular switch for initiating the homeostatic ER stress response under zinc deficiency. Mol Cell 52:75–86

Horner VL, Wolfner MF (2008) Transitioning from egg to embryo: triggers and mechanisms of egg activation. Dev Dyn 237:527–544

Hurley LS, Swenerton H (1966) Congenital malformations resulting from zinc deficiency in rats. Exp Biol Med 123:692–696

Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M (2004) Overview of mammalian zinc transporters. Cell Mol Life Sci C 61:49–68

Kaufman MH (1982) The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment. J Embryol Exp Morphol 71:139–154

Kim AM, Vogt S, O'Halloran TV, Woodruff TK (2010) Zinc availability regulates exit from meiosis in maturing mammalian oocytes. Nat Chem Biol 6:674–681

Kim AM, Bernhardt ML, Kong BY, Ahn RW, Vogt S, Woodruff TK, O'Halloran TV (2011) Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs. ACS Chem Biol 6:716–723

King JC (2000) Determinants of maternal zinc status during pregnancy. Am J Clin Nutr 71:1334s–1343s

Kline D, Kline JT (1992) Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. Dev Biol 149:80–89

Kong BY, Bernhardt ML, Kim AM, O'Halloran TV, Woodruff TK (2012) Zinc maintains prophase I arrest in mouse oocytes through regulation of the MOS-MAPK pathway. Biol Reprod 87(11):1–12

Krężel A, Maret W (2006) Zinc-buffering capacity of a eukaryotic cell at physiological pZn. J Biol Inorg Chem 11:1049–1062

Kubiak JZ, Weber M, de Pennart H, Winston NJ, Maro B (1993) The metaphase II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin B in the presence of CSF. EMBO J 12:3773–3778

Lawrence TS, Dekel N, Beers WH (1980) Binding of human chorionic gonadotropin by rat cumuli oophori and granulosa cells: a comparative study. Endocrinology 106:1114–1118

Ledan E, Polanski Z, Terret ME, Maro B (2001) Meiotic maturation of the mouse oocyte requires an equilibrium between cyclin B synthesis and degradation. Dev Biol 232:400–413

Li R, Albertini DF (2013) The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. Nat Rev Mol Cell Biol 14:141–152

Lisle RS, Anthony K, Randall MA, Diaz FJ (2013) Oocyte–cumulus cell interactions regulate free intracellular zinc in mouse oocytes. Reproduction 145:381–390

Liu J, Grimison B, Lewellyn AL, Maller JL (2006) The anaphase-promoting complex/cyclosome inhibitor Emi2 is essential for meiotic but not mitotic cell cycles. J Biol Chem 281:34736–34741

Longo FJ, Chen D-Y (1985) Development of cortical polarity in mouse eggs: involvement of the meiotic apparatus. Dev Biol 107:382–394

Lu Q, Zhao Y, Gao X, Li Y, Ma S, Mullen S, Critser JK, Chen Z-J (2006) Combination of calcium ionophore A23187 with puromycin salvages human unfertilized oocytes after ICSI. Eur J Obstet Gynecol Reprod Biol 126:72–76

Ma SF, Liu XY, Miao DQ, Han ZB, Zhang X, Miao YL, Yanagimachi R, Tan JH (2005) Parthenogenetic activation of mouse oocytes by strontium chloride: a search for the best conditions. Theriogenology 64:1142–1157

Madgwick S, Jones KT (2007) How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals cytostatic factor. Cell Div 2:4

Madgwick S, Hansen DV, Levasseur M, Jackson PK, Jones KT (2006) Mouse Emi2 is required to enter meiosis II by reestablishing cyclin B1 during interkinesis. J Cell Biol 174:791–801

Masciarelli S, Horner K, Liu C, Park SH, Hinckley M, Hockman S, Nedachi T, Jin C, Conti M, Manganiello V (2004) Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. J Clin Invest 114:196–205

McGee EA, Hsueh AJ (2000) Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200–214

Mitra J, Schultz RM (1996) Regulation of the acquisition of meiotic competence in the mouse: changes in the subcellular localization of cdc2, cyclin B1, cdc25C and wee1, and in the concentration of these proteins and their transcripts. J Cell Sci 109:2407–2415

Mocchegiani E, Giacconi R, Malavolta M (2008) Zinc signalling and subcellular distribution: emerging targets in type 2 diabetes. Trends Mol Med 14:419–428
Moore GPM, Lintern-Moore SUE (1974) A correlation between growth and RNA synthesis in the mouse oocyte. J Reprod Fertil 39:163–166
Moore GPM, Lintern-Moore SUE (1978) Transcription of the mouse oocyte genome. Biol Reprod 18:865–870
Nakagawa K, Yamano S, Nakasaka H, Hinokio K, Yoshizawa M, Aono T (2001) A combination of calcium ionophore and puromycin effectively produces human parthenogenones with one haploid pronucleus. Zygote 9:83–88
Nicosia SV, Wolf DP, Inoue M (1977) Cortical granule distribution and cell surface characteristics in mouse eggs. Dev Biol 57:56–74
Norris RP, Freudzon M, Mehlmann LM, Cowan AE, Simon AM, Paul DL, Lampe PD, Jaffe LA (2008) Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development (Camb) 135:3229–3238
Norris RP, Ratzan WJ, Freudzon M, Mehlmann LM, Krall J, Movsesian MA, Wang H, Ke H, Nikolaev VO, Jaffe LA (2009) Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. Development (Camb) 136:1869–1878
O'Halloran TV, Kebede M, Philips SJ, Attie AD (2013) Zinc, insulin, and the liver: a ménage à trois. J Clin Invest 123:4136–4139
O'Neill G, Rolfe L, Kaufman M (1991) Developmental potential and chromosome constitution of strontium-induced mouse parthenogenones. Mol Reprod Dev 30:214–219
Oh JS, Han SJ, Conti M (2010) Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption. J Cell Biol 188:199–207
Ohe M, Inoue D, Kanemori Y, Sagata N (2007) Erp1/Emi2 is essential for the meiosis I to meiosis II transition in *Xenopus* oocytes. Dev Biol 303:157–164
Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488–2492
Park JY, Su YQ, Ariga M, Law E, Jin SLC, Conti M (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303:682–684
Percival MD, Yeh B, Falgueyret J-P (1997) Zinc dependent activation of cAMP-specific phosphodiesterase (PDE4A). Biochem Biophys Res Commun 241:175–180
Qiao J, Wang ZB, Feng HL, Miao YL, Wang Q, Yu Y, Wei Y-C, Yan J, Wang W-H, Shen W et al (2013) The root of reduced fertility in aged women and possible therapeutic options: current status and future perspects. Mol Aspects Med. doi:10.1016/j.mam.2013.06.001
Rodgers RJ, Irving-Rodgers HF (2010) Formation of the ovarian follicular antrum and follicular fluid. Biol Reprod 82:1021–1029
Salustri A, Petrungaro S, De Felici M, Conti M, Siracusa G (1985) Effect of follicle-stimulating hormone on cyclic adenosine monophosphate level and on meiotic maturation in mouse cumulus cell-enclosed oocytes cultured in vitro. Biol Reprod 33:797–802
Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA (2002) PLCζ: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. Development (Camb) 129:3533–3544
Schmidt A, Duncan PI, Rauh NR, Sauer G, Fry AM, Nigg EA, Mayer TU (2005) *Xenopus* polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity. Genes Dev 19:502–513
Schultz RM (2002) The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum Reprod Update 8:323–331
Shoji S, Yoshida N, Amanai M, Ohgishi M, Fukui T, Fujimoto S, Nakano Y, Kajikawa E, Perry ACF (2006) Mammalian Emi2 mediates cytostatic arrest and transduces the signal for meiotic exit via Cdc20. EMBO J 25:834–845
Sigdel TK, Easton JA, Crowder MW (2006) Transcriptional response of *Escherichia coli* to TPEN. J Bacteriol 188:6709–6713

Stricker SA (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. Dev Biol 211:157–176

Su Y-Q, Denegre JM, Wigglesworth K, Pendola FL, O'Brien MJ, Eppig JJ (2003) Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte–cumulus cell complex. Dev Biol 263:126–138

Subramanian Vignesh K, Landero Figueroa JA, Porollo A, Caruso JA, Deepe GS (2013) Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival. Immunity 39:697–710

Sugiura K, Su Y-Q, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ (2008) Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. Development (Camb) 135:786

Sun Q-Y, Rubinstein S, Breitbart H (1999) MAP kinase activity is downregulated by phorbol ester during mouse oocyte maturation and egg activation in vitro. Mol Reprod Dev 52:310–318

Suzuki T, Suzuki E, Yoshida N, Kubo A, Li H, Okuda E, Amanai M, Perry ACF (2010a) Mouse Emi2 as a distinctive regulatory hub in second meiotic metaphase. Development (Camb) 137:3281–3291

Suzuki T, Yoshida N, Suzuki E, Okuda E, Perry ACF (2010b) Full-term mouse development by abolishing Zn^{2+}-dependent metaphase II arrest without Ca^{2+} release. Development (Camb) 137:2659–2669

Taylor SL, Yoon SY, Morshedi MS, Lacey DR, Jellerette T, Fissore RA, Oehninger S (2010) Complete globozoospermia associated with PLCζ deficiency treated with calcium ionophore and ICSI results in pregnancy. Reprod Biomed Online 20:559–564

Terret ME, Wassmann K, Waizenegger I, Maro B, Peters J-M, Verlhac M-H (2003) The meiosis I-to-meiosis II transition in mouse oocytes requires separase activity. Curr Biol 13:1797–1802

Tian X, Diaz FJ (2012) Zinc depletion causes multiple defects in ovarian function during the periovulatory period in mice. Endocrinology 153:873–886

Tian X, Diaz FJ (2013) Acute dietary zinc deficiency before conception compromises oocyte epigenetic programming and disrupts embryonic development. Dev Biol 376:51–61

Tung JJ, Hansen DV, Ban KH, Loktev AV, Summers MK, Adler JR, Jackson PK (2005) A role for the anaphase-promoting complex inhibitor Emi2/XErp1, a homolog of early mitotic inhibitor 1, in cytostatic factor arrest of *Xenopus* eggs. Proc Natl Acad Sci USA 102:4318–4323

Vallee BL (1995) The function of metallothionein. Neurochem Int 27:23–33

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

Verlhac MH, Kubiak JZ, Weber M, Géraud G, Colledge WH, Evans MJ, Maro B (1996) Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. Development (Camb) 122:815–822

Wakai T, Vanderheyden V, Fissore RA (2011) Ca^{2+} signaling during mammalian fertilization: requirements, players, and adaptations. Cold Spring Harbor Perspect Biol. doi:10.1101/cshperspect.a006767

Waldron KJ, Rutherford JC, Ford D, Robinson NJ (2009) Metalloproteins and metal sensing. Nature (Lond) 460:823–830

Whittingham D, Siracusa G (1978) The involvement of calcium in the activation of mammalian oocytes. Exp Cell Res 113:311–317

Wigglesworth K, Lee K-B, O'Brien MJ, Peng J, Matzuk MM, Eppig JJ (2013) Bidirectional communication between oocytes and ovarian follicular somatic cells is required for meiotic arrest of mammalian oocytes. Proc Natl Acad Sci USA 110:E3723–E3729

Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, Kurosaki T, Yamashita S, Tokunaga M, Nishida K et al (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645

Zhang W, Colman RW (2000) Conserved amino acids in metal-binding motifs of PDE3A are involved in substrate and inhibitor binding. Blood 95:3380–3386

Zhang M, Su Y-Q, Sugiura K, Xia G, Eppig JJ (2010) Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. Science 330:366–369

Future Perspectives

Toshiyuki Fukada

During the past decade, our understanding of the role of zinc as an intracellular and extracellular "zinc signal" has increased dramatically. Advances in molecular, biochemical, genetic, and computational methodologies have undoubtedly contributed to the recent insights into the biological roles of zinc as well as the novel mechanisms by which it functions. At the same time, zinc signaling is still an emerging field, in need of continued sustained efforts to fully understand the impact of zinc on biological processes. Here, I discuss the significant issues that should be addressed in the next decade to facilitate our understanding of this enigmatic molecule.

More than 50 years have passed since zinc was recognized as an essential trace element required for human health. Although we now appreciate the various roles zinc plays in maintaining human health, the molecular basis of the requirement for zinc remains a question. The necessity of zinc for life has been analyzed and accounted for in different ways, invoking a variety of mechanisms. One reasonable answer to this question is that approximately 10 % of human genes encode zinc-binding motifs that may be necessary for protein function or regulation. Thus, the requirement for zinc may, in actuality, be readily comprehensible. Nevertheless, more experimental data are needed to fully validate this observation at the molecular level. At present, a growing body of evidence indicates that zinc acts as both an intracellular and extracellular signaling factor, contributing to our understanding not only of the chemical and biological features of zinc, but also of the zinc-dependent physiological events in human health and disease, which are well described in this book.

The 15 chapters in this volume outline the cutting-edge insights on zinc, ranging from a basic introduction (Chap. 1) to clinical points of view, and identify

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remaining mechanistic questions in the area of zinc signaling. For example, to understand further the mechanisms by which zinc homeostasis is regulated and impacts cell signaling, the various functions of cytosolic zinc-binding proteins involved in zinc "buffering and muffling" in specific cellular locations is needed. The interaction between zinc signaling and other essential or nonessential metal ions is another intriguing subject (Chap. 2). Zinc transporters are the main gatekeepers controlling zinc signaling. Thus, investigation into their activation and inactivation, modification, and three-dimensional structures will provide key insights into the regulation of zinc signaling and may shed light on the molecular basis of their biological specificity (Chap. 3). Oxidative stress activates multiple signaling pathways and induces the elevation of intracellular zinc levels. The complexity of zinc biology will be further clarified by exploring its interaction with other intracellular and extracellular signaling pathways, such as stress responses toward reactive oxygen species (Chap. 4). The development of techniques for detecting zinc signaling in vitro and in vivo will certainly improve our mechanistic understanding of zinc signaling and its interaction with other signaling pathways (Chap. 7). The zinc receptor GPR39, a zinc sensor that converts the extracellular zinc signal to intracellular signaling, is an excellent example of this phenomenon. Because GPR39 is a G protein-coupled receptor (GPCR), the development of GPR39-specific agonists may modulate zinc-signaling processes linked to disease (Chap. 6).

Recent genetic and clinical investigations have advanced our knowledge about zinc signaling and its relationship to various diseases. One of the leading areas is the field of neuroscience. Synaptic zinc homeostasis is involved in both cognitive function and dysfunction including memory loss, depression, and Alzheimer's disease; however, the regulation of zinc homeostasis in the brain is currently unclear. Further investigation into this area may contribute to improved outcomes for patients with zinc-related neurodegenerative diseases (Chaps. 8 and 9). Allergy and immunology, as well as diabetes are also emerging as areas in which zinc signaling is important; zinc has been thought to be required for normal immunity and inflammatory responses since zinc deficiency was discovered. The regulation of zinc signaling in lymphocytes and monocytes (Chaps. 10 and 11), the "zinc wave" in mast cells (Chap. 5), and the "zinc stream" from pancreatic beta cells (Chap. 13) appear to play roles in immunological and inflammatory diseases and diabetes mellitus. It is well established that abnormal zinc homeostasis causes growth retardation and dwarfism; therefore, the proper regulation of zinc homeostasis is indispensable for mammalian growth. Zinc signaling machineries orchestrate other pathways involved in bone homeostasis and elongation (Chap. 12) and in early development (Chap. 15). Therefore, it will be important to define the molecular mechanisms by which zinc signaling regulates the cellular events before and after oocyte fertilization, during growth, in aged individuals, and in a variety of diseases. In particular, zinc signaling appears to be involved in cancer malignancy; thus, further studies dissecting the regulatory mechanisms of zinc transporters may lead to the development of new anti-cancer therapies (Chap. 14).

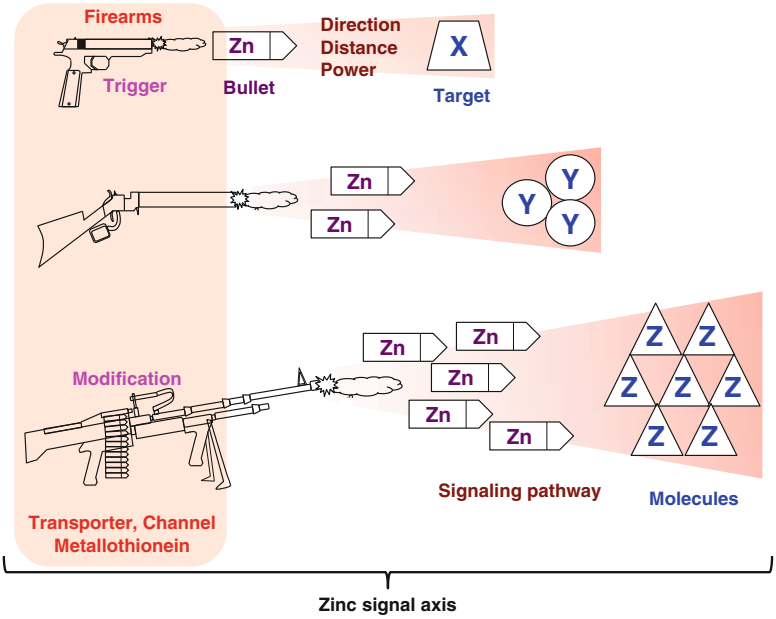

Fig. 1 Each zinc signal from an individual zinc transporter, channel, or metallothionein selectively controls the target molecules and zinc-mediated effects on cellular functions. This situation may be analogous to the relationship between a bullet and a firearm. Even with use of the same bullet and gunpowder (zinc), the use of different firearms (zinc gatekeepers such as transporters, channels, and metallothioneins) will result in hitting different targets (signaling molecules). Each firearm has different triggers and safety devices (modifications or unique motifs), which may influence its efficiency. The objects around the targets (such as chaperons), location of targets and firearms in the field (intracellular distribution), and firearm numbers (expression level of zinc transporters in differential cells and tissues) may affect the results. These complexes form "zinc signal axes" to achieve specific cellular responses and physiological outcomes

The chapters written for this book represent a diverse range of approaches but together raise a simple and common query: How does zinc signaling possess the necessary specificity to correctly regulate the relevant target molecules and biological processes? Although this question undoubtedly requires additional exploration, I would propose that "a bullet and firearm" analogy might provide some mechanistic insight into the specificity of zinc signaling (Fig. 1). Even if the same bullets and gunpowder (zinc in this case) are used, different targets will be hit by using different firearms (i.e., zinc gatekeepers including transporters, channels, and metallothioneins) because the characteristics of an individual firearm affect the distance, direction, speed, and relative timing of the bullets they fire. A machine gun might be likened to a fully open zinc transporter in a constitutively active state, whereas a single-loader gun with safety devices might be more similar to a zinc transporter regulated by negative feedback systems. Similar to firearms, the transporters respond to different triggers (e.g., modification by phosphorylation or oxidation), which also exert profound downstream effects. The aiming of a firearm

may encounter interference by objects around the targets (e.g., intermediate proteins such as chaperones). Even when using the same firearm, the targets hit will depend on their location (i.e., intracellular distribution, differential cell and tissue expression). As of today, many aspects of zinc signaling remain unknown. As we enter the next phase of work in this area, we remain convinced that further studies on zinc signaling will manifest the answers to these key questions in molecular terms, which will shed light on the role of zinc signaling in both normal and disease conditions.

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