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Luis A. del Río · Michael Schrader
Editors

Proteomics of Peroxisomes

Identifying Novel Functions and Regulatory Networks

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Editors
Luis A. del Río
Department of Biochemistry,
Cell and Molecular Biology of Plants
Estación Experimental del Zaidín, CSIC
Granada, Spain

Michael Schrader
Department of Biosciences
University of Exeter
Exeter, UK

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Dedicated to Prof. H. Dariush Fahimi, who has made very important contributions to the understanding of the biology of mammalian peroxisomes, on the occasion of his 85th birthday.

Preface

Peroxisomes are a class of ubiquitous and essential single membrane-bound cell organelles, devoid of DNA, with an essentially oxidative type of metabolism. When these organelles were first isolated from mammalian tissues and characterized by Christian de Duve in the 1960s, it was thought that their main function was the removal, by peroxisomal catalase, of toxic hydrogen peroxide generated in the peroxisomal respiratory pathway through different oxidases (De Duve and Baudhuin 1966). However, in recent years it has become progressively clear that peroxisomes are involved in several important cellular functions in almost all eukaryotic cells (Erdmann 2016; Reumann et al. 2016; del Río 2013; del Río et al. 2006; del Río and López-Huertas 2016; Wanders and Waterham 2006; Wanders 2013; Islinger et al. 2012; Baker and Graham 2002).

The main functions of peroxisomes known so far were elucidated on the basis of peroxisome purification and analysis by classical cell biology and biochemical methods (del Río and López-Huertas 2016; del Río 2013; Wanders and Waterham 2006; Baker and Graham 2002; Palma et al. 2009). However, in recent years a proteomic "burst" has taken place in peroxisome biology. Proteome analysis has confirmed the presence of many proteins previously described in peroxisomes by classical methods, but has also revealed many new peroxisomal proteins, and thus increased our knowledge of peroxisome functions and their metabolic and regulatory networks (Erdmann 2016; Reumann 2011; Reumann et al. 2016; Palma et al. 2009; Saleem et al. 2006; Schuldiner and Zalckvar 2015; Camões et al. 2015; Gronemeyer et al. 2013; Kaur and Hu 2011; Eubel et al. 2008; Islinger et al. 2007; Kikuchi et al. 2004; Ho et al. 2002; Costello et al. 2017).

Proteome studies of peroxisomes have been conducted mainly in organelles from human, animal, plant, and fungal origin. The development of sensitive proteomics and mass spectrometry (MS) technologies now allows the identification of low-abundance and transient peroxisomal proteins and constantly increases our knowledge of the metabolic and regulatory networks of these important cellular organelles (Saleem et al. 2006; Schuldiner and Zalckvar 2015; Islinger et al. 2007). Additionally, the combination of experimental proteomics with bioinformatics

approaches now allows to identify the complete proteome of peroxisomes (Reumann 2011; Gronemeyer et al. 2013).

This volume is organized in four parts which present comprehensively the actual state of the art in proteomics of peroxisomes from multiple origins. In the first part, an updated view of mass spectrometry-based proteomics using peroxisomes from mammalian, fungal, and plant origin is presented; and given the importance of having reliable high-yield methods for the purification of peroxisomes available, a review article on different methods for the isolation of peroxisomes from multiple sources is included.

In Part II, different approaches for the prediction of peroxisomal proteomes from mammals, *Drosophila*, fungi, and plants using bioinformatics approaches are presented, and mechanisms by which proteins can be targeted to multiple organelles with a focus on functional translational readthrough, a novel gene regulatory mechanism, are discussed. This part is complemented with a review on the current knowledge of the diversity, origin, and evolution of the peroxisomal proteome.

Research conducted on different peroxisome–proteome interaction networks is presented in Part III, including Pull Down strategies to analyze the interactome of peroxisomal membrane proteins in human cells, the identification of peroxisomal protein complexes in mammalian cells and the structure and function of peroxisomal protein import machineries. Studies of the proteome of peroxisomes from a fruit (sweet pepper), particularly the reactive oxygen species (ROS) proteome, and its relationship with the overall metabolism of the fruit are presented.

The importance of the connections between peroxisomes and other subcellular compartments are addressed in Part IV. Knowledge of the central role of peroxisomes in metabolic interaction networks in humans and the characterization of these regulation networks that can be useful for the treatment of peroxisomal biogenesis disorders and other diseases linked to peroxisomes is reviewed. The multi-localized proteins which are shared by peroxisomes and mitochondria in mammals and their key roles in the cooperative functions between these two organelles that impact on human health and disease are reviewed. Likewise, peroxisomes and mitochondria in plants also have a considerable overlap in their proteins, responses, and functions, and the interrelations between these two organelles are presented with results suggesting a more dynamic nature of their interactivity. The potential role of mammal peroxisomes as an intracellular redox signaling platform both in health and disease, and how protein redox modifications due to changes in the oxidant/antioxidant balance can impact on inter-organelle communication are examined. The cell death or survival against oxidative stress in mammalian cells is addressed in the light of the recent peroxisomal localization of BAK, a pro-apoptotic member of the Bcl-2 family proteins, which can release catalase to the cytosol to eliminate extra-peroxisomal hydrogen peroxide. Finally, the metabolism of reactive nitrogen species (RNS) in plant peroxisomes is reviewed and a role for peroxisome-derived RNS in the communication with other cellular organelles as well as in plant defense mechanisms is postulated.

We would like to thank all contributing colleagues for their enthusiastic collaboration and keen interest, as well as the reviewers of the different chapters for their valuable comments and suggestions. The helpful suggestions of Dr. Markus Islinger on the organization of this book are appreciated. We are convinced that in the upcoming years we will experience important methodological developments in proteomics and will witness its increasing importance to reveal novel peroxisomal functions and advance in the understanding of regulatory networks of these most intriguing subcellular organelles.

Granada, Spain — Prof. Luis A. del Río
Exeter, UK — Prof. Michael Schrader
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Contents

Part I
Mass Spectrometry-Based Organelle Proteomics

Proteome of Plant Peroxisomes

Ronghui Pan and Jianping Hu

Abstract Plant peroxisomes are required for a number of fundamental physiological processes, such as primary and secondary metabolism, development and stress response. Indexing the dynamic peroxisome proteome is prerequisite to fully understanding the importance of these organelles. Mass Spectrometry (MS)-based proteome analysis has allowed the identification of novel peroxisomal proteins and pathways in a relatively high-throughput fashion and significantly expanded the list of proteins and biochemical reactions in plant peroxisomes. In this chapter, we summarize the experimental proteomic studies performed in plants, compile a list of ~200 confirmed Arabidopsis peroxisomal proteins, and discuss the diverse plant peroxisome functions with an emphasis on the role of Arabidopsis MS-based proteomics in discovering new peroxisome functions. Many plant peroxisome proteins and biochemical pathways are specific to plants, substantiating the complexity, plasticity and uniqueness of plant peroxisomes. Mapping the full plant peroxisome proteome will provide a knowledge base for the improvement of crop production, quality and stress tolerance.

Keywords Plant peroxisomes · Proteomics · Peroxisome biogenesis
β-oxidation · Photorespiration · Detoxification · Peroxisome metabolism

R. Pan · J. Hu (✉)
MSU-Department of Energy Plant Research Laboratory,
Michigan State University, East Lansing, MI 48824, USA
e-mail: huji@msu.edu

J. Hu
Plant Biology Department, Michigan State University,
East Lansing, MI 48824, USA

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1 Introduction

Peroxisomes are small and dynamic organelles delimitated by a single lipid bilayer and carrying out a broad range of functions in eukaryotic cells. The physiological importance of peroxisomes is underscored by the fatal human disorders and plant embryonic lethality resulted from severe defects in peroxisomal function or biogenesis (Delille et al. 2006; Steinberg et al. 2006; Kaur et al. 2009; Fidaleo 2010; Hu et al. 2012). Peroxisomal functions vary significantly among different organisms, tissue types, developmental stages and prevailing environmental conditions. However, at least fatty acid β-oxidation and hydrogen peroxide (H_2O_2) degradation are universal across kingdoms. Plant peroxisomes also host a diverse and specialized set of metabolic reactions, such as photorespiration, biosynthesis of plant hormones and cofactors, and catabolism of various metabolites (Kaur et al. 2009; Hu et al. 2012). Peroxisomes are physically and metabolically linked to other organelles, such as chloroplasts, mitochondria, lipid bodies and the ER.

Since peroxisomes are devoid of a genome, the entire peroxisome proteome is nuclear-encoded and imported into the organelle mainly by the PEX proteins (peroxins). With some exceptions, peroxisome matrix proteins usually contain a C-terminal tripeptide Peroxisome Targeting Signal type 1 (PTS1), or a nonapeptide PTS2 in the N-terminal region. PEX proteins are largely conserved across kingdoms, although lineage-specific factors do exist (Hu et al. 2012; Fujiki et al. 2014; Kaur et al. 2014; Sibirny 2016).

Compared with animals and yeasts, plants seem to have an increased diversity and plasticity in peroxisome functions. Plant peroxisome proteins so-far identified significantly outnumber those reported for mammals and yeasts (Mi et al. 2007; Wiederhold et al. 2010; Kaur and Hu 2011; Gronemeyer et al. 2013). The proteome of plant peroxisomes changes across developmental stages. During seedling establishment, peroxisomes shift metabolically from the glyoxylate cycle to photorespiration, which is accompanied by the degradation of glyoxylate cycle enzymes; hence the name glyoxysome was given to the specialized peroxisomes in germinating seeds and fatty tissues (Beevers 1979). It was later suggested that peroxisomes behave somewhat similarly to glyoxysomes during senescence and should be classified as a subtype named gerontosomes (Vicentini and Matile 1993). Traditionally, plant peroxisomal proteins have been discovered through enzyme purification, genetic screens or sequence similarity with animal or yeast counterparts. In recent years, the exploration of plant peroxisomal proteome has been greatly boosted by advances in the high-throughput "omics" technologies, including genome sequencing, bioinformatics-based prediction, and equally if not more importantly, proteome analysis of isolated peroxisomes using mass spectrometry (MS).

2 MS-Based Peroxisome Proteome Studies in Plants

The full understanding of peroxisomal functions requires comprehensive cataloguing of the peroxisomal proteome. To achieve this goal, MS-based peroxisome proteomics studies have been carried out in various organisms (Mi et al. 2007; Wiederhold et al. 2010; Kaur and Hu 2011; Reumann 2011; Gronemeyer et al. 2013; Guther et al. 2014). These efforts were enabled by technological improvements on multiple fronts, including genome sequencing and annotation, high-sensitivity MS instrumentation, and organelle purification and protein separation methodologies. This unbiased approach has turned out to be very powerful in identifying new peroxisomal proteins and uncovering metabolic and physiological functions that had not been known to be associated with peroxisomes in a relatively high-throughput fashion.

A number of plant peroxisomal proteome studies have been performed using Arabidopsis, soybean and spinach, but mostly in Arabidopsis (Fukao et al. 2002; Fukao et al. 2003; Reumann et al. 2007; Arai et al. 2008a; Eubel et al. 2008; Reumann et al. 2009; Babujee et al. 2010; Quan et al. 2013). Due to the small size, high fragility, and low abundance of peroxisomes, and their adherence to several other subcellular compartments, it has been challenging to isolate peroxisomes with high yield and purity. Therefore, the authenticity of novel peroxisomal proteins identified in proteome studies normally needs to be verified by subcellular localization analyses using fluorescently tagged candidate proteins and a peroxisomal marker.

Early proteome studies were performed using greening and etiolated Arabidopsis cotyledons to enrich leaf peroxisomes and glyoxysomes (Fukao et al. 2002; Fukao et al. 2003). Peroxisomes were isolated using single density gradients, and peroxisomal proteins were separated on 2D-gels (2-DE) and identified using peptide mass fingerprinting (PMF), which resulted in the identification of 29 proteins in leaf peroxisomes and 19 proteins in glyoxysomes. The enrichment of specific subtypes of peroxisomes identified a number of photorespiratory pathway proteins from leaf peroxisomes and glyoxylate cycle enzymes from glyoxysomes. However, general coverage of the peroxisome proteome was fairly low.

Reumann et al. (2007) isolated peroxisomes from Arabidopsis green leaves using a two-successive density gradient centrifugation method. The combination of 2-DE-based and gel-free shotgun analyses identified 36 known peroxisome proteins and dozens of candidates for novel peroxisomal proteins, many of which were subsequently confirmed by in vivo targeting analysis using fluorescence microscopy. Novel peroxisomal proteins involved in NADP and glutathione metabolism and plant defense were discovered in this study.

Eubel et al. (2008) used non-green Arabidopsis cell suspension cultures for peroxisome isolation, with the goal to increase peroxisome purity by eliminating chloroplast contamination. Density gradient centrifugation followed by free-flow electrophoresis (FFE) helped to improve the separation of mitochondria from peroxisomes. Two-dimensional differential in gel electrophoresis (2D-DIGE) and quantitative proteomics were also employed to increase the chance of identifying

proteins enriched in peroxisomes relative to mitochondria, a major source of contamination. This study identified >20 possible novel peroxisomal proteins and validated the peroxisome localization of 5 of them by in vivo targeting analysis.

The Arabidopsis peroxisome 2010 project was aimed at comprehensively elucidating the Arabidopsis peroxisome proteome, using different peroxisomal subtypes. Leaf peroxisomes were isolated using a two-successive density gradient centrifugation method, followed by stringent selection of highly pure peroxisomes using post-preparative immunoblot analysis with organelle-specific antibodies. Peroxisomal proteins were then separated by 1-DE gel and identified by liquid chromatography-tandem MS (LC-MS/MS), which resulted in the identification of 65 known peroxisomal proteins, including nearly all established matrix proteins of plant peroxisomes and some membrane proteins, and 55 candidates for novel peroxisomal proteins. A large number of the candidate proteins were fluorescently tagged and analyzed by confocal microscopy in this study and a follow-up study, confirming the peroxisomal localization of ~20 candidate proteins and established two new PTS1 (SLM and SKV) and a new PTS2 (RVx5HF) (Reumann et al. 2009; Quan et al. 2010). This study provided evidence for new metabolic and regulatory peroxisomal pathways, such as methylglyoxal detoxification and phosphoregulation, and identified new putative auxiliary enzymes in β-oxidation, additional enzymes in polyamine catabolism and several nucleotide-binding proteins (Reumann et al. 2009; Quan et al. 2010).

In an attempt to decipher the proteome of glyoxysomes by the Arabidopsis peroxisome 2010 project, peroxisome isolated from etiolated Arabidopsis seedlings were subjected to three methods of protein separation before 1-DE-LC-MS/MS (Quan et al. 2013). In addition to using total peroxisome proteins and peroxisome membrane-enriched samples, a ZOOM® IEF (isoelectric focusing) Fractionator was also employed to separate total peroxisomal proteins into several subgroups based on isoelectric point (pI), with the goal to identify low abundance proteins that might have been masked by abundant proteins. A total of 77 peroxisomal proteins were detected, 11 of which were previously unknown to be peroxisomal but verified in this study by fluorescence microscopy. A substantial portion of the peroxisome proteome was found to overlap between etiolated seedlings and green leaves, including proteins involved in all the major peroxisomal functions—i.e. β-oxidation, detoxification and photorespiration, suggesting that the core peroxisome proteome is conserved among different peroxisome subtypes in plants. Through mutant analysis, a newly discovered peroxisomal protein, Response to drought 21A-like 1 (RDL1), was found to play a role in β-oxidation, seed germination, and plant growth. Proteins specific to or much more abundant in glyoxysomes include, not surprisingly, two major glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase (MLS), and small heat shock protein Hsp15.7, acetoacetyl-CoA thiolase 1.3 (AACT1.3), benzoyloxyglucosinolate 1 (BZO1), RDL1, serine carboxypeptidase-like protein 20 (SCPL20), and unknown protein 9 (UP9), suggesting that biochemical pathways other than the glyoxylate cycle may also be prevalent in glyoxysomes (Quan et al. 2013).

Systematic analyses of mutants of the newly discovered peroxisomal proteins were also conducted (Cassin-Ross and Hu 2014a; Cassin-Ross and Hu 2014b). Utilizing sucrose dependence and 2,4-dichlorophenoxybutyric acid (an analog to indole-3-butyric acid, precursor of indole-3-acetic acid) and 12-oxo-phytodienoic acid (OPDA) response assays, the mutants of 20 tested genes were found to be deficient to various degrees in β-oxidation-related processes. Some of these mutants showed further abnormality in seed germination, oil body accumulation and fatty acid degradation during seedling establishment. These proteins included enzymes expected to be associated with β-oxidation, such as acyl-CoA thioesterase 2 (ACH2), acyl-activating enzyme 1 (AAE1) and acyl-activating enzyme 5 (AAE5), as well as proteins previously unknown to be involved in β-oxidation, such as indigoidine synthase A (IndA), senescence-associated protein/B12D-related protein1 (B12D1), betaine aldehyde dehydrogenase (BADH), and unknown protein 5 (UP5) (Cassin-Ross and Hu 2014a). In addition, the OPDA response assay uncovered a potential role of two putative proteases, RDL1 and SCPL20, in the metabolism of OPDA, an intermediate in JA biosynthesis (Cassin-Ross and Hu 2014b).

Peroxisome proteome analyses were also conducted in other plant species. Using a percoll density gradient followed by an iodixanol density gradient, glyoxysomes were isolated from etiolated soybean cotyledons, separated by 2-DE PAGE, and subjected to PMF analysis, which detected 31 peroxisome proteins (Arai et al. 2008a). The four proteins verified by fluorescence microscopy to be peroxisomal include an adenine nucleotide transporter orthologous to the Arabidopsis peroxisomal adenine nucleotide carriers PNC1 and PNC2, a short-chain dehydrogenase/reductase orthologous to Arabidopsis indole-3-butyric acid response 1 (IBR1), an enoyl-CoA hydratase/isomerase orthologous to Arabidopsis monofunctional enoyl-CoA hydratase/isomerase A (ECHIA), and a 3-hydroxyacyl-CoA dehydrogenase-like protein orthologous to Arabidopsis hydroxybutyryl-CoA dehydrogenase (HBCDH) (Arai et al. 2008a; Arai et al. 2008b). Leaf peroxisomes were isolated from spinach and analyzed by 2-DE coupled with LC-MS/MS, which identified peroxisomal association of the short-chain dehydrogenase/reductase SDRa/IBR1, two enoyl-CoA hydratases/isomerases ECHIa and NS/ECHId, NS and acyl-CoA activating enzyme isoform 14 (AAE14); the latter two proteins revealed phylloquinone biosynthesis as a novel peroxisomal function (Babujee et al. 2010). Although MS-based peroxisomal proteome analysis has not been reported for monocot species, blast searches of the rice genome using Arabidopsis peroxisomal protein sequences revealed a strong conservation between the peroxisome proteomes of rice and Arabidopsis (Kaur and Hu 2011).

Plant peroxisome isolation and hitherto proteome analysis has been carried out in major plant organs and very few plant species. Contamination from other subcellular structures undermines the purity of peroxisome preparations. These factors made it difficult to identify bona-fide peroxisome proteins with very low abundance and those that are associated with peroxisomes in a spatio- or temporal-specific manner. Hence, in silico prediction of peroxisomal proteins becomes a vital complementary approach to catalog the peroxisome proteome. Plant-specific PTS1

prediction algorithms, including PredPlantPTS1 (Lingner et al. 2011) and PPero (Wang et al. 2017), predicted hundreds of plant peroxisome proteins. In vivo targeting validations using fluorescence microscopy confirmed the peroxisome association of 23 novel proteins predicted by PredPlantPTS1 (Lingner et al. 2011) and five predicted by PPero (Wang et al. 2017). However, existing algorithms still cannot accurately predict non-canonical PTS1 proteins and PTS2 proteins. Finally, identification of peroxisomal membrane proteins (PMPs) remains challenging for both experimental proteomics and bioinformatics. Plant peroxisome proteome may still be far from being completely decoded.

3 An Update on Arabidopsis Peroxisome Proteins and Their Functions

In this chapter, we use the list of >160 plant peroxisomal proteins that was compiled in an earlier review (Kaur and Hu 2011) as a template to provide an update that reflects advances in the past several years and the current understanding of the Arabidopsis peroxisomal proteome. The Arabidopsis peroxisomal proteins currently stand at about 199, including 144 PTS-containing matrix proteins, 45 membrane proteins, and 10 proteins lacking recognizable PTS (Tables 1 and 2). Here we also discuss briefly about the functions and pathways associated with the peroxisomal proteins and stress the identification of these proteins by Arabidopsis proteome studies. Due to space limitation, a complete description of all the current knowledge on protein functions and mutant phenotypes is not provided. There are several excellent review articles that contain more in-depth discussions of plant peroxisomal metabolism, biogenesis or turnover (Kaur et al. 2009; Hu et al. 2012; Bussell et al. 2013; Kaur et al. 2014; Reumann and Bartel 2016; Young and Bartel 2016; Corpas and Barroso 2018; Kao et al. 2018).

3.1 Biogenesis and Dynamics of Peroxisomes

Peroxisomes can arise by de novo formation from the ER and fission of existing peroxisomes. Newborn peroxisomes mature mainly by incorporating matrix and membrane proteins synthesized in the cytosol. Peroxisome protein import is mediated by peroxins (or PEX proteins), a core set of which is conserved across kingdoms. Many of these peroxins were originally identified in yeasts, and the plant homologs are believed or have been shown to carry out similar functions (Hu et al. 2012). In the cytosol, matrix proteins containing PTS1 and PTS2 are respectively recognized by the receptors PEX5 and PEX7. Cargo-receptor complexes dock at the peroxisome membrane via PEX13 and PEX14. After cargo release into the matrix, PEX5 and PEX7 are recycled for more rounds of protein import. PEX5

Table 1 Plant peroxisome matrix proteins

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
Proteins containing PTS					
ACH2[a]	At1g01710	Acyl-CoA thioesterase 2	Acyl-CoA hydrolysis	SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
sT4[a]	At1g04290	Small thioesterase 4	β-oxidation and related	SNL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009)
KAT1[a]	At1g04710	3-Ketoacyl-CoA thiolase 1	β-oxidation and related	RQx5HL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ACX3[a]	At1g06290	Acyl-CoA oxidase 3	β-oxidation and related	RAx5HI/SSV	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ACX6	At1g06310	Acyl-CoA oxidase 6	β-oxidation and related	RAx5HI/SSL	
ACD31.2[a]	At1g06460	Alpha-crystallin domain 32.1	Molecular chaperone	RLx5HF/PKL	Ma et al. (2006), Reumann et al. (2007), Reumann et al. (2009)
NDA1	At1g07180	NADPH dehydrogenase A1	NAD(P)H oxidation	SRI	
GAPC2[a]	At1g13440	Glyceraldehyde 3-phosphate dehydrogenase C2	NADH production	SKA	Reumann et al. (2009), Quan et al. (2013)
PP2A-B'θ	At1g13460	Protein Phosphatase 2A regulatory (B) subunit 'θ	Phosphoregulation	SSL	
UP6[a]	At1g16730	Unknown protein 6	Others	SKL	Reumann et al. (2009), Quan et al. (2010), Quan et al. (2013)
4CL3[a]	At1g20480	4-coumarate:CoA ligase 3	β-oxidation and related—JA biosynthesis	SKL	Eubel et al. (2008)
OPCL1[a]	At1g20510	OPC-8:0 ligase 1	β-oxidation and related—JA biosynthesis	SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009)

(continued)

Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
AAE1[a]	At1g20560	Acyl-activating enzyme 1	β-oxidation and related	SKL	Eubel et al. (2008), Reumann et al. (2009)
CAT3[a]	At1g20620	Catalase 3	Detoxification	QKL (internal)	Fukao et al. (2002), Fukao et al. (2003), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
CAT1[a]	At1g20630	Catalase 1	Detoxification	QKL (internal)	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ATF1[a]	At1g21770	Acetyl transferase 1	Others	SSI	Reumann et al. (2007), Reumann et al. (2009)
GGT1[a]	At1g23310	Glutamate-glyoxylate aminotransferase 1	Photorespiration	SKM	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
DEG15[a]	At1g28320	Deg/HtrA protease	Protease	SKL	Eubel et al. (2008)
UP9[a]	At1g29120	Unknown protein 9, Hydrolase-like protein family	Others	ASL	Quan et al. (2013)
AAE14[a]	At1g30520	Acyl-activating enzyme 14	Phylloquinone synthesis	SSL	Babujee et al. (2010)
PMK	At1g31910	5-phosphomevalonate kinase	Mevalonic acid (MVA) pathway	DVx5QL	
DHNAT1[a]	At1g48320	DHNA-CoA thioesterase 1	Phylloquinone synthesis	AKL	Reumann et al. (2009), Quan et al. (2013)
pxPfkB[a]	At1g49350	PfkB-type carbohydrate kinase family protein	Pseudouridine catabolism	SML	Eubel et al. (2008)
NQR[a]	At1g49670	NADH:quinone reductase	Others	SRL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
IndA[a]	At1g50510	Indigoidine synthase A	Pseudouridine catabolism	RIx5HL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ICDH[a]	At1g54340	NADP-dependent isocitrate dehydrogenase	NADPH production	SRL	Fukao et al. (2003), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

(continued)

Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
AAE18	At1g55320	Acyl-activating enzyme 18	β-oxidation and related	SRI	
NS[a]	At1g60550	Naphthoate synthase	Phylloquinone synthesis	RLx5HL	Reumann et al. (2007), Reumann et al. (2009), Babujee et al. (2010), Quan et al. (2013)
ECI1[a]	At1g65520	Δ3, Δ2-enoyl CoA isomerase 1	Auxiliary β-oxidation	SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009)
PAO4[a]	At1g65840	Polyamine oxidase 4	Polyamine oxidation	SRM	Eubel et al. (2008)
BZO1[a]	At1g65880	Benzoyloxyglucosinolate 1/ benzoate-coenzyme A ligase	β-oxidation and related– BA biosynthesis	SRL	Quan et al. (2013)
AAE12	At1g65890	Acyl-activating enzyme 12	β-oxidation and related	SRL	
AAE11	At1g66120	Acyl-activating enzyme 11	β-oxidation and related	SRL	
HPR1[a]	At1g68010	Hydroxypyruvate reductase 1	Photorespiration	SKL	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
RPK1	At1g69270	Receptor-like protein kinase 1	Phosphoregulation	SRL	
GGT2[a]	At1g70580	Glutamate-glyoxylate aminotransferase 2	Photorespiration	SRM	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ECH2[a]	At1g76150	Monofunctional enoyl-CoA hydratase 2	Auxiliary β-oxidation	SSL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ATF2[a]	At1g77540	Acetyl transferase 2	Others	SSI	Reumann et al. (2009), Quan et al. (2013)
NADK3	At1g78590	NADH kinase 3	NADPH production	SRY	
PAP7	At2G01880	Purple acid phosphatase 7	Phosphoregulation	AHL	
OPR3[a]	At2g06050	12-oxophytodienoate reductase 3	β-oxidation and related– JA biosynthesis	SRL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

(continued)

Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
SGT/AGT1[a]	At2g13360	Serine-glyoxylate aminotransferase/alanine:glyoxylate aminotransferase 1	Photorespiration	SRI	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009)
MDH1[a]	At2g22780	NAD^+-malate dehydrogenase 1	β-oxidation and related—NADH oxidation	RIx5HL	Fukao et al. (2002), Fukao et al. (2003), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
Uri[a]	At2g26230	Uricase	Urate degradation	SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
st5[a]	At2g29590	Small thioesterase 5	β-oxidation and related	SKL	Reumann et al. (2009)
NDA2	At2g29990	NADPH dehydrogenase A2	NAD(P)H oxidation	SRI	
CHY1H1	At2g30650	CHY1 homolog 1	Amino acid metabolism	AKL	
CHY1H2	At2g30660	CHY1 homolog 2	Amino acid metabolism	AKL	
UP3[a]	At2g31670	Unknown protein 3	Others	SSL	Reumann et al. (2007), Reumann et al. (2009), Quan et al. (2013)
KAT2[a]	At2g33150	3-Ketoacyl-CoA thiolase 2	β-oxidation and related	RQx5HL	Fukao et al. (2003), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ACX5[a]	At2g35690	Acyl-CoA oxidase 5	β-oxidation and related	AKL	Eubel et al. (2008)
GLH[a]	At2g38180	GDSL motif lipase/hydrolase family protein	Others	ARL	Eubel et al. (2008)
MVD	At2g38700	Mevalonate 5-diphosphate decarboxylase	Mevalonic acid (MVA) pathway	SVx5HL	
PM16[a]	At2g41790	Peptidase family M16	Protease	PKL	Eubel et al. (2008), Reumann et al. (2009)
CuAO[a]	At2g42490	Copper amine oxidase	Polyamine oxidation	SKL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

(continued)

Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
CSY3[a]	At2g42790	Citrate synthase 3	Glyoxylate cycle	RLx5HL/ SSV	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
PAO2	At2g43020	Polyamine oxidase 2	Polyamine oxidation	SRL	
SO[a]	At3g01910	Sulfite oxidase	Sulfite oxidation	SNL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
SDRc[a]	At3g01980	Short-chain dehydrogenase-reductase c	Auxiliary β-oxidation	SYM	Reumann et al. (2007), Reumann et al. (2009), Quan et al. (2013)
PGD2/ 6PGDH[a]	At3g02360	Phosphogluconate dehydrogenase 2	Oxidative pentose phosphate pathway	SKI	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
IPI2	At3g02780	Isopentenyl diphosphate isomerase 2	Mevalonic acid (MVA) pathway	HKL	
LACS6[a]	At3g05970	Long-chain acyl-CoA synthetase 6	β-oxidation and related	RIx5HL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
IBR3[a]	At3g06810	IBA-response 3	β-oxidation and related – IAA biosynthesis	SKL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
MFP2[a]	At3g06860	Fatty acid multifunctional protein 2	β-oxidation and related	SRL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
CML3	At3g07490	Calmodulin-like protein 3	Others	SNL	
PLL3	At3g09400	POL like phosphatase 3	Phosphoregulation	SSM	
SDRb/ DECR[a]	At3g12800	Short-chain dehydrogenase-reductase b/2,4-dienoyl-CoA reductase	Auxiliary β-oxidation	SKL	Reumann et al. (2007), Eubel et al. (2008), Quan et al. (2013)
HAOX2[a]	At3g14150	Hydroxy-acid oxidase 2	β-oxidation and related	SML	Reumann et al. (2009)
GOX1[a]	At3g14415	Glycolate oxidase 1	Photorespiration	PRL	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

(continued)

Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
GOX2[a]	At3g14420	Glycolate oxidase 2	Photorespiration	ARL	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
HBCDH[a]	At3g15290	Hydroxybutyryl-CoA dehydrogenase	β-oxidation and related	PRL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
AAE7[a]	At3g16910	Acyl-activating enzyme 7	β-oxidation and related	SRL	Eubel et al. (2008), Reumann et al. (2009)
GPK1[a]	At3g17420	Glyoxysomal protein kinase 1	Phosphoregulation	AKI	Fukao et al. (2003), Ma and Reumann (2008)
SCO3	At3g19570	Snowy cotyledon 3	Others	SRL	
PPK	At3g20530	Protein kinase superfamily protein/ peroxisomal protein kinase	Phosphoregulation	SKL	
ICL[a]	At3g21720	Isocitrate lyase	Glyoxylate cycle	SRM	Fukao et al. (2003), Quan et al. (2013)
GR1[a]	At3g24170	Glutathione reductase 1	Detoxification	TNL	Reumann et al. (2007), Eubel et al. (2008)
BADH[a]	At3g48170	Betaine aldehyde dehydrogenase	Polyamine oxidation	SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
MIF[a]	At3g51660	Macrophage migration inhibitory factor	Others	SKL	Reumann et al. (2007, Reumann et al. (2009)
ACX4[a]	At3g51840	Acyl-CoA oxidase 4	β-oxidation and related	SRL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
MDAR1[a]	At3g52880	Monodehydroascorbate reductase 1	Detoxification	AKI	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009)
GSTL2	At3g55040	glutathione S-transferase λ isoform 2	Detoxification	ARL	
MKP1	At3g55270	Mitogen-activated protein kinase phosphatase 1	Phosphoregulation	SAL	
SDRd[a]	At3g55290	Short-chain dehydrogenase-reductase d	Auxiliary β-oxidation	SSL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ZnDH[a]	At3g56460	Zinc-binding dehydrogenase	Others	SKL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

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Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
HIT3[a]	At3g56490	Histidine triad family protein 3	Nucleotide homeostasis	RVx5HF	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
CP	At3g57810	Cysteine protease	Protease	SKL	
CSY2[a]	At3g58750	Citrate synthase 2	Glyoxylate cycle	RLx5HL/SAL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
PAO3	At3g59050	Polyamine oxidase 3	Polyamine oxidation	SRM	
UP12	At3g60680	Unknown protein 12	Others	SKM	
sT3[a]	At3g61200	Small thioesterase 3	β-oxidation and related	SKL	Fukao et al. (2003), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
BGLU8	At3G62750	Beta glucosidase 8	Others	SSL	
ACH[a]	At4g00520	Acyl CoA thioesterase family protein	Acyl-CoA hydrolysis	AKL	Eubel et al. (2008)
EH3[a]	At4g02340	Epoxide hydrolase 3	β-oxidation and related	ASL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009)
MCD[a]	At4g04320	Malonyl-CoA decarboxylase	β-oxidation and related	SRL	Eubel et al. (2008), Reumann et al. (2009)
4CL1[a]	At4g05160	4-coumarate:CoA ligase 1	β-oxidation and related – JA biosynthesis	SKM	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
IBR1/SDRa[a]	At4g05530	Indole-3-butyric acid response 1/short-chain dehydrogenase-reductase a	β-oxidation and related - IAA biosynthesis	SRL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
UP10	At4g12735	Unknown protein 10	Others	SRL	
SCPL20	At4g12910	Serine carboxypeptidase-like 20	Protease	SKI	Quan et al. (2013)
ECI2/IBR10[a]	At4g14430	Δ(3), Δ(2)-Enoyl-CoA isomerase 2/Indole-3-butyric acid response 10	Auxiliary β-oxidation	PKL	Reumann et al. (2007), Reumann et al. (2009), Quan et al. (2013)

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Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
ECHIA[a]	At4g16210	Monofunctional enoyl-CoA hydratase/isomerase a	Auxiliary β-oxidation	SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
HIT1[a]	At4g16566	Histidine triad family protein 1	Nucleotide homeostasis	SKV	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ACX1[a]	At4g16760	Acyl-CoA oxidase 1	β-oxidation and related	ARL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
GOX3[a]	At4g18360	Glycolate oxidase 3	Photorespiration	AKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
4Cl5	At4g19010	4-Coumarate:CoA ligase 5	β-oxidation and related – JA biosynthesis	SRL	
NDB1	At4g28220	NADPH dehydrogenase B1	NAD(P)H oxidation	SRI	
AIM1[a]	At4g29010	Abnormal inflorescence meristem 1	β-oxidation and related	SKL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
CAT2[a]	At4g35090	Catalase 2	Detoxification	QKL (internal)	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
RDL1[a]	At4g36880	Cysteine proteinase/response to drought21A-like 1	Protease	SSV	Quan et al. (2013)
AGT2	At4g39660	Alanine: glyoxylate aminotransferase 2	Photorespiration	SRL	
PLL2	At5g02400	POL like phosphatase 2	Phosphoregulation	SSM	
UP11	At5g03100	F-box/RNI-like superfamily protein	Others	SKL	
MLS[a]	At5g03860	Malate synthase	Glyoxylate cycle	SRL	Fukao et al. (2003), Quan et al. (2013)
BIOTIN F	At5g04620	7-keto-8-aminopelargonic acid (KAPA) synthase	Biotin synthesis	PKL	

(continued)

Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
MDH2[a]	At5g09660	NAD^+-malate dehydrogenase 2	β-oxidation and related - NADH oxidation	RIx5HL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ASP3[a]	At5g11520	Aspartate aminotransferase 3	Amino acid metabolism	RIx5HL	Fukao et al. (2002), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
ELT1[a]	At5g11910	Esterase/lipase/thioesterase family 1	β-oxidation and related	SRI	Reumann et al. (2009)
AAE5[a]	At5g16370	Acyl-activating enzyme 5	β-oxidation and related	SRM	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
IPI1	At5g16440	Isopentenyl diphosphate isomerase 1	Mevalonic acid (MVA) pathway	HKL	
ATMS1[a]	At5g17920	Cobalamin-independent methionine synthase	Amino acid metabolism	SAK	Reumann et al. (2007), Reumann et al. (2009), Quan et al. (2013)
CSD3[a]	At5g18100	Copper/zinc superoxide dismutase 3	Detoxification	AKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
NUDT19	At5g20070	Nudix hydrolase homolog 19	NAD(P)H hydrolysis	SSL	
AAE17[a]	At5g23050	Acyl-activating enzyme 17	β-oxidation and related	SKL	Eubel et al. (2008), Reumann et al. (2009)
MIA40	At5g23395	Mitochondrial intermembrane space assembly machinery 40	Others	SKL	
PGL3/ 6PGL	At5g24400	6-phosphogluconolactonase 3	Oxidative pentose phosphate pathway	SKL	
LACS7[a]	At5g27600	Long-chain acyl-CoA synthetase 7	β-oxidation and related	RLx5HI/ SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
G6PD1	At5g35790	Glucose-6-phosphate dehydrogenase 1	Oxidative pentose phosphate pathway	SKY (internal)	

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Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
Hsp15.7[a]	At5g37670	15.7 kDa heat shock protein	molecular chaperone	SKL	Quan et al. (2013)
GSTT1[a]	At5g41210	Glutathione S-transferase θ isoform 1	Detoxification	SKI	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
GSTT3	At5g41220	Glutathione S-transferase θ isoform 3	Detoxification	SKM	
GSTT2	At5g41240	Glutathione S-transferase θ isoform 2	Detoxification	SKM	
SCP2[a]	At5g42890	Sterol carrier protein 2	β-oxidation and related	SKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
DCI[a]	At5g43280	Δ3,5-Δ2,4-enoyl-CoA-isomerase	Auxiliary β-oxidation	AKL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
UP5[a]	At5g44250	Unknown protein 5	Others	SRL	Reumann et al. (2009)
LON2[a]	At5g47040	Lon protease homolog 2	Protease	SKL	Reumann et al. (2009, Quan et al. (2013)
AACT1.3[a]	At5g47720	Acetoacetyl-CoA thiolase 1 splicing isoform 3	Mevalonic acid (MVA) pathway	SAL	Quan et al. (2013)
HIT2[a]	At5g48545	Histidine triad family protein 2	Nucleotide homeostasis	RLx5HL	Reumann et al. (2009)
KAT5[a]	At5g48880	3-Ketoacyl-CoA thiolase 5	β-oxidation and related	RQx5HL	Reumann et al. (2007), Reumann et al. (2009)
DHNAT2	At5g48950	DHNA-CoA thioesterase 2	Phylloquinone synthesis	SKL	
TLP[a]	At5g58220	Transthyretin-like protein	Urate degradation	RLx5HL	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009)
4CL2[a]	At5g63380	4-coumarate:CoA ligase 2	β-oxidation and related – JA biosynthesis	SKL	Reumann et al. (2009)
ACX2[a]	At5g65110	Acyl-CoA oxidase 2	β-oxidation and related	RIx5HL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

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Table 1 (continued)

Gene name	Locus	Annotation	Functional category	PTS	References (proteome analysis)
UP7[a]	At5g65400	Unknown protein 7	Others	SLM	Reumann et al. (2009)
CHY1[a]	At5g65940	3-hydroxyisobutyryl-CoA hydrolase 1	Amino acid metabolism	AKL	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

Gene Name	Locus	Annotation		Reference
Proteins lacking PTS				
PP2A-C2	At1g10430	Protein phosphatase 2A catalytic (C) subunit 2	Phosphoregulation	
GLX1[a]	At1g11840	Glyoxylase I homolog	Methylglyoxal detoxification	Reumann et al. (2009)
PP2A-C5	At1h69960	Protein phosphatase 2A catalytic (C) subunit 5	Phosphoregulation	
SOX	At2g24580	Sarcosine oxidase	Amino acid metabolism	
CoAE[a]	At2g27490	Dephospho-CoA kinase	CoA biosynthesis	Reumann et al. (2009)
PP2A-A2	At3g25800	Protein phosphatase 2A scaffolding (A) subunit 2	Phosphoregulation	
B12D1[a]	At3g48140	Senescence-associated protein/B12D-related protein 1	Others	Reumann et al. (2009)
NDPK1[a]	At4g09320	Nucleoside diphosphate kinase type 1	Nucleotide homeostasis	Reumann et al. (2009), Quan et al. (2013)
CPK1	At5g04870	Calcium dependent protein kinase 1	Phosphoregulation	
RabGAP22	At5g53570	Rab GTPase-activating protein 22	Defense	

[a]Detection in peroxisome proteome analyses

recycling depends on a suite of peroxins, including the ubiquitin conjugating enzyme PEX4 and its membrane anchor PEX22, three ubiquitin ligases PEX2, PEX10 and PEX12, and two AAA ATPases PEX1 and PEX6 that are tethered to their membrane anchor PEX26/APEM9 (reviewed in Reumann and Bartel 2016). Recently, the Arabidopsis RabE1c GTPase was found to be a PEX7 binding protein on the peroxisome membrane and facilitate the degradation of abnormally accumulated GFP-PEX7 (Cui et al. 2013).

A peroxisome membrane-associated E3 ubiquitin ligase SP1 negatively regulates matrix protein import by targeting PEX13 and PEX14 (Pan et al. 2016). SP1 belongs to a small protein family that contains two additional members, SPL1 and SPL2 (Pan and Hu 2017). SP1's closest homolog in Arabidopsis, SPL1, only shows weak, partial and possibly conditional targeting to peroxisomes, and plays a positive role in peroxisome biogenesis by reducing the function of SP1 (Pan et al. 2018), whereas SPL2 exhibits no peroxisome association (Pan et al. 2016). SP1, SPL1 and SPL2 also target to chloroplasts, where SP1 negatively regulates matrix protein import (Ling et al. 2012), and to mitochondria, where their functions are yet unknown (Pan et al. 2018; Pan and Hu 2018). It is possible that SP1 and SPL1 participate in coordinating the biogenesis of these three metabolically linked organelles essential in plant energy metabolism. The mammalian homolog of SP1, MAPL (mitochondria-associated protein ligase, also named MULAN/GIDE/MUL1/HIDES), localizes to mitochondria and peroxisomes; its transportation from mitochondria to peroxisomes is through vesicle structures and mediated by the retromer complex (Neuspiel et al. 2008; Braschi et al. 2010). It remains to be determined whether the peroxisome localization of SP1 and SPL1 also depends on the plant retromer complex.

The import of membrane proteins is poorly understood in plants. However, based on knowledge obtained from studies in animals and yeasts (Rottensteiner et al. 2004; Kim et al. 2006; Aranovich et al. 2014; Chen et al. 2014), three well conserved peroxins, PEX3 (AtPEX3A and AtPEX3B), PEX16 and PEX19 (AtPEX19A and AtPEX19B), should be involved in this process in Arabidopsis (Lin et al. 1999; Lin et al. 2004; Nito et al. 2007; McDonnell et al. 2016).

The multiplication of pre-existing peroxisomes can be generally divided into two phases: elongation/tubulation mediated by PEX11 proteins (Lingard and Trelease 2006; Nito et al. 2007; Orth et al. 2007; Lingard et al. 2008); and membrane constriction and fission mediated by dynamin-related proteins DRP3A, DRP3B and DRP5B (Mano et al. 2004; Fujimoto et al. 2009; Zhang and Hu 2009; Zhang and Hu 2010; Pan and Hu 2011; Aung and Hu 2012). FIS1A and FIS1B are believed to be membrane anchors for DRP3 (Scott et al. 2006; Lingard et al. 2008; Zhang and Hu 2008; Zhang and Hu 2009), while PMD1, a plant-specific protein localized to the membrane of both peroxisomes and mitochondria, appears to act independently from FIS1 and DRP3 in organelle fission (Aung and Hu 2011). Many proteins involved in peroxisome division are dual localized. Peroxisomes and mitochondria share DRP3A, DRP3B, FIS1A, FIS1B and PMD1, and peroxisomes and chloroplasts share DRP5B (Pan and Hu 2011; Hu et al. 2012; Pan and Hu 2015). Transcription factors, such as HY5 HOMOLOG (HYH) and Forkhead-Associated

Domain Protein 3 (FHA3), regulate the expression of *PEX11b* to impact peroxisome division (Desai and Hu 2008; Hu and Desai 2008; Desai et al. 2017).

The movement of plant peroxisomes involves the actin cytoskeleton. One myosin XI isoform, MYA2, partially localizes to peroxisomes in an actin-dependent manner, and is possibly involved in the movement of peroxisomes on actin filaments (Hashimoto et al. 2005). PMD1 physically interacts with F-actin, suggesting that PMD1 may affect peroxisome abundance and distribution via the cytoskeleton-peroxisome connection (Frick and Strader 2017).

Except for the five PEX11 isoforms, PEX7 and PEX14, most peroxisome membrane proteins involved in biogenesis and dynamics were not detected in proteome studies (Table 2). A comprehensive peroxisome membrane proteomic study has yet to be reported.

3.2 Fatty Acid Catabolism and Hormone Biosynthesis

Beta-oxidation is an essential metabolic pathway that occurs exclusively in peroxisomes in plants and is responsible for the catabolism of fatty acids to generate acetyl-CoA, as well as the biosynthesis of multiple key plant hormones, including jasmonic acid (JA), indole-3-acetic acid (IAA), and salicylic acid (SA). Prior to entering the β-oxidation cycle, the precursors such as fatty acids (FAs), 12-oxo-phytodienoic acid (OPDA), indole-3-butyric acid (IBA) and cinnamic acid (CA) must be imported into peroxisomes and activated to their CoA-esters. The import is at least partially mediated by the peroxisome ABC transporter PXA1/CTA1/PED3 (Hayashi et al. 1998; Zolman et al. 2001b; Theodoulou et al. 2005; Kunz et al. 2009; Nyathi et al. 2010; Zhang et al. 2011). The activation of β-oxidation substrates is catalyzed by the peroxisomal members of the acyl-activating enzyme (AAE) protein superfamily (Shockey et al. 2003). The β-oxidation cycle is composed of four enzymatic steps, all of which are mediated by multigene families: oxidation by acyl-CoA oxidase (ACX), hydration and dehydration by the multifunctional protein (MFP), and thiolysis by 3-ketoacyl-CoA thiolase (KAT) (reviewed in Kaur et al. 2009; Hu et al. 2012; Goepfert and Poirier 2007).

The degradation of cis-unsaturated fatty acids through β-oxidation requires auxiliary enzymatic activities. Δ3-Δ2-enoyl-CoA-isomerase (ECI), 2,4-dienoyl-CoA reductase (DECR) and Δ3,5-Δ2,4-enoyl-CoA-isomerase (DCI) are necessary for the degradation of fatty acids that are unsaturated on odd-numbered carbons (reviewed in Kaur et al. 2009). Short-chain dehydrogenase/reductase b (SDRb) shares high sequence similarity to yeast and mammalian DECRs, thus is a strong candidate for DECR activity (Reumann et al. 2007). The monofunctional type 2 enoyl-CoA hydratase ECH2 was shown to participate in the degradation of fatty acids unsaturated on even-numbered carbons (Goepfert et al. 2006). The two peroxisomal isoforms of malate dehydrogenase (MDH1 and MDH2) are also involved in β-oxidation, as they re-oxidize NADH, which can be produced by the hydroxyacyl-CoA dehydrogenase activity of MFP (Pracharoenwattana et al. 2007;

Cousins et al. 2008). Moreover, Sugar-dependent 1 (SDP1), the major lipase that converts triacylglycerol (TAG) into free fatty acids and glycerol (Eastmond 2006), was found to localize to peroxisomes in early seedling development and then move to oil bodies via peroxisome tubules (Thazar-Poulot et al. 2015).

As an intermediate in JA biosynthesis, OPDA is synthesized in the chloroplast and imported into the peroxisome, where it is reduced to 3-oxo-2-(2'-[Z]-pentenyl) cyclopentane-1-octanoic acid (OPC:8) by 12-oxophytodienoate reductase 3 (OPR3) (Sanders et al. 2000; Schaller et al. 2000; Stintzi and Browse 2000). OPC:8 is activated to its CoA ester by OPC-8:0 ligase 1 (OPCL1), a member of the 4-coumarate-CoA ligase-like (4CL) family of the acyl-activating enzyme (AAE) super family, before entering the β-oxidation cycle (Koo et al. 2006; Kienow et al. 2008). Other members of the 4CL (4-coumarate:CoA ligase) family, such as 4CL1 and 4CL2, may also contribute to OPDA activation (Kienow et al. 2008). In BA biosynthesis, imported CA is activated by benzoyloxyglucosinolate 1/benzoate-coenzyme A ligase (BZO1) to produce cinnamoyl-CoA for β-oxidation (Lee et al. 2012).

Other proteins that are presumably associated with β-oxidation and fatty acid degradation include hydroxy-acid oxidase 2 (HAOX2), epoxide hydrolase 3 (EH3), malonyl-CoA decarboxylase (MCD), sterol carrier protein 2 (SCP2) and esterase/lipase/thioesterase family 1 (ELT1). The human homolog of MCD localizes to peroxisomes, mitochondria and the cytosol, and has the enzymatic activity to convert malonyl-CoA to acetyl-CoA, thus it may function to degrade malonyl-CoA, a by-product of β-oxidation of odd chain-length dicarboxylic fatty acids (FitzPatrick et al. 1999; Zhou et al. 2004). HAOX2 was shown to have broad substrate specificities, with the highest activity for leucic acid (Esser et al. 2014).

ACH1 and ACH2 are two acyl-CoA thioesterase enzymes suggested to hydrolyze fatty acyl-CoAs and regulate the homeostasis of acyl-CoA, free fatty acids and CoA (Tilton et al. 2000; Tilton et al. 2004). Several small thioesterases are present in peroxisomes, but their substrate specificity has not yet been determined (Reumann et al. 2009). A systematic phenotypic screen of Arabidopsis peroxisomal mutants for defects in β-oxidation identified IndA, B12D1, BADH, and UP5, to possibly play a role in peroxisomal β-oxidation (Cassin-Ross and Hu 2014a).

Beta-oxidation is a major function of peroxisomes throughout the life cycle of plant. Thus, most proteins involved in β-oxidation have been identified through proteome studies (Table 1). There are several exceptions, such as AAE11, AAE12, AAE14, AAE18, 4CL5 and ACX6, which may have temporal and/or spatial specificities in gene expression and/or function, or are simply expressed at very low levels because members of these multigene families are functionally redundant and each does not need to accumulate to high levels.

3.3 Photorespiration

Photorespiration, the most prominent function of peroxisomes in photosynthetic tissues, spans across several organelles, including chloroplasts, mitochondria, peroxisomes, and the cytosol. This pathway salvages and recycles phosphoglycolate produced by the oxygenase activity of Rubisco during photosynthesis, and consumes O_2 while generating CO_2. Phosphoglycolate is first dephosphorylated in chloroplasts to glycolate, which enters peroxisomes to be oxidized to glyoxylate by glycolate oxidase (GOX1, GOX2 and GOX3). The peroxisomal aminotransferases, including one serine-glyoxylate aminotransferase (SGT/AGT1) and two glutamate-glyoxylate aminotransferases (GGT1 and GGT2), are responsible for transaminating glyoxylate to form glycine. Glycine enters mitochondria to be converted to serine, which then re-enters peroxisomes to be transaminated by SGT to form hydroxypyruvate. Hydroxypyruvate reductase 1 (HPR1) reduces hydroxypyruvate to yield glycerate, using NADH as the cofactor. Two more HPR isoforms are found in the cytosol and chloroplast respectively, but the peroxisome-localized HPR1 seems to play a major role. Finally, glycerate is transported to chloroplasts and phosphorylated prior to being fed into the Calvin-Benson-Bassham cycle (Bauwe et al. 2010).

Consistent with photorespiration being a major peroxisomal function in photosynthetic tissues, all the major enzymes in this pathway have been identified by MS-based proteomics (Table 1). In the green leaf peroxisome proteome, GGT1, GOX1, GOX2, SGT and HPR1 are among the most abundant proteins (Reumann et al. 2009). Interestingly, most photorespiratory enzymes are also detected in etiolated seedlings (Quan et al. 2013). GOX3 was found to catalyze the conversion of L-lactate to pyruvate in Arabidopsis roots, and was therefore hypothesized to sustain low levels of L-lactate (Engqvist et al. 2015).

3.4 The Glyoxylate Cycle

The glyoxylate cycle functions to fuel seedling establishment after germination and before photosynthesis starts, utilizing aconitase (ACO), malate dehydrogenase (MDH), citrate synthase (CSY), isocitrate lyase (ICL) and malate synthase (MLS) to convert acetyl-CoA derived from fatty acid β-oxidation into consumable 4-carbon compounds, which can be further used in gluconeogenesis and mitochondrial respiration. Arabidopsis MLS, CSY2, CSY3 and ICL play expected roles in the glyoxylate cycle (reviewed in Penfield et al. 2006; Graham 2008), whereas peroxisomal MDH1 and MDH2 seem to function mainly in β-oxidation (Pracharoenwattana et al. 2007). Thus, malate formed in the glyoxylate cycle was speculated to be oxidized to oxaloacetate by a cytosolic MDH (reviewed in Graham 2008). Since the glyoxylate cycle is a key peroxisomal pathway during

seed germination and becomes obsolete days after germination, MLS and ICL were only detected in proteome studies of peroxisomes from etiolated Arabidopsis seedlings (Fukao et al. 2003; Quan et al. 2013).

3.5 *Detoxification of ROS and Methylglyoxal*

Peroxisomes harbor numerous oxidative reactions that produce H_2O_2, which is scavenged mainly by peroxisomal catalase (reviewed in Kaur et al. 2009). Arabidopsis has three CAT isoforms (CAT1, CAT2 and CAT3), all of which could be detected by peroxisome proteome analyses (Table 1). CAT2 and CAT3 are among the most abundant proteins detected in Arabidopsis leaf peroxisomes (Reumann et al. 2009), consistent with the notion that catalase-catalyzed H_2O_2 degradation is highly active in leaf peroxisomes to reduce H_2O_2 produced in photorespiration. The ascorbate-glutathione cycle can also dispose H_2O_2 in plant peroxisomes, with enzymes including the ascorbate peroxidase APX3, two monohydroascorbate reductases MDAR1 and MDAR4, the dehydroascorbate reductase DHAR1, and the glutathione reductase GR1 (Kaur et al. 2009), all of which were detected in Arabidopsis peroxisome proteome studies (Tables 1 and 2). Peroxisomes contain several glutathione S-transferases (GSTs), including all three members of the theta family—GSTT1, GSTT2 and GSTT3 (Dixon et al. 2009). In addition, a lamda family member GSTL2 contains a PTS1 and partially localized to peroxisome-like structures when its N-terminal chloroplast targeting signal peptide was removed (Dixon et al. 2009). GSTT1 was first identified to be peroxisomal by a proteome study (Reumann et al. 2007), but GSTT2 and GSTT3 are not yet detected by proteomics. The presence of the enzyme superoxide dismutase in peroxisomes was first reported in pea (del Río et al. 1983), and Cu–Zn superoxide dismutase has been detected in peroxisomes from at least six different plant species (Corpas et al. 2017). The Arabidopsis Cu–Zn superoxide dismutase (CSD3) was detected by several peroxisome proteome studies (Table 1).

Methylglyoxal is a toxic by-product of glycolysis that is detoxified primarily by the glyoxalase system, which sequentially uses glyoxalase I (GLX1) and GLX2 to convert α-keto aldehydes into hydroxyacids in a glutathione-dependent manner (Thornalley 1990). That methylglyoxal detoxification might a peroxisome function was recently discovered by MS-based proteomics, where GLX1 was found in Arabidopsis leaf peroxisomes and later verified to be peroxisomal by fluorescence microscopy (Reumann et al. 2009; Quan et al. 2010). GLX2 has cytosolic and mitochondrial isoforms (Maiti et al. 1997; Zang et al. 2001), and it remains to be determined whether it has a peroxisomal isoform as well.

3.6 Biosynthesis of Phylloquinone, Biotin and CoA

Phylloquinone (or vitamin K1) is an important cofactor in electron transfer in photosystem I. Its biosynthesis starts in plastids with the synthesis of o-succinylbenzoate, which is subsequently activated to its CoA ester by AAE14 in peroxisomes (Kim et al. 2008). The activation is followed by two more steps in peroxisomes: (i) ring cyclization to form 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA) by naphthoate synthase (NS), and (ii) hydrolysis of DHNA-CoA to form DHNA by the thioesterase DHNAT1 or DHNAT2 (Widhalm et al. 2012). DHNA then re-enters chloroplasts to finalize phylloquinone biosynthesis (reviewed in Reumann and Bartel 2016). Phylloquinone biosynthesis was previously believed to occur solely in plastids until NS was identified by Arabidopsis peroxisome proteomics (Reumann et al. 2007). DHNAT1 was also detected in leaf peroxisome proteome (Reumann et al. 2009; Quan et al. 2013).

Biotin is a crucial cofactor in some carboxylation and decarboxylation reactions, and can only be synthesized by plants and some fungal species. In plants, the first committed step of biotin biosynthesis is mediated by the peroxisome-localized Biotin F, which converts pimeloyl-CoA to 7-keto-8-aminopelargonic acid (KAPA) (Pinon 2005; Tanabe et al. 2011). Steps leading to the formation of pimeloyl-CoA have not been elucidated in plants but was speculated to take place in peroxisomes as well (Tanabe et al. 2011). The last three steps of Biotin synthesis are believed to occur in mitochondria (Arnal et al. 2006; Muralla et al. 2008).

Coenzyme A (CoA) and its derivatives are involved in many metabolic reactions including β-oxidation. There are five enzymatic steps in the biosynthesis of CoA, last of which is mediated by Dephospho-CoA kinase (CoAE), which was first discovered by Arabidopsis leaf peroxisome proteomics (Reumann et al. 2009) but its physiological importance and metabolic effects have not been determined.

3.7 The Mevalonic Acid (MVA) Pathway

The MVA pathway provides isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), precursors for the biosynthesis of isoprenoids (McGarvey and Croteau 1995). The first step of this pathway is the generation of acetoacetyl-CoA from two acetyl-CoA molecules, catalyzed by acetoacetyl-CoA thiolase (AACT). Arabidopsis AACT1 was identified in peroxisomal proteome analysis of etiolated seedlings (Quan et al. 2013), and AACT2 was detected by leaf peroxisomal proteomics (Reumann et al. 2007; Reumann et al. 2009). One of its spliced forms, AACT1.3, localizes to peroxisomes, whereas AACT2 and the other spliced forms of AACT1 were found to be cytosolic (Carrie et al. 2007). The next three enzymatic steps occur sequentially in the cytosol and ER, resulting in the formation of mevalonate phosphate (Simkin et al. 2011). The last steps of the MVA pathway occur in peroxisomes, where mevalonate phosphate undergoes an

additional phosphorylation step catalyzed by 5-phosphomevalonate kinase (PMK) to yield mevalonate diphosphate, which is then decarboxylated into IPP by mevalonate 5-diphosphate decarboxylase (MVD) (Simkin et al. 2011). A reversible isomerization step catalyzed by IPP isomerase (IPI) converts IPP into DMAPP (Okada et al. 2008; Sapir-Mir et al. 2008). MS-based peroxisome proteomics studies have not yet detected these three enzymes, suggesting that the proteins are probably low in abundance or restricted to certain tissue types.

3.8 *Catabolism of Polyamines, Urate, Pseudouridine and Sulfite*

Polyamines have a variety of functions in plant development and defense (Alcázar et al. 2006). Peroxisome-localized flavin-containing polyamine oxidases (PAOs), copper-containing amine oxidases (CuAOs) and betaine aldehyde dehydrogenase (BADH) are proposed to participate in the catabolism of polyamines (Kaur et al. 2009). PAO4, CuAO and BADH have been detected by Arabidopsis proteome analyses (Table 1).

Uric acid is degraded in peroxisomes through the ureide pathway to produce the ureide, *S*-allantoin. This pathway recycles nitrogen released from purine catabolism for the synthesis of amino acids, and constitutes three enzymatic steps: (i) oxidation of uric acid by uricase (Uri) to yield 5-hydroxyisourate (5-HIU); (ii) hydrolysis of 5-HIU to 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) by HIU hydrolase; and (iii) decarboxylation of OHCU by OHCU decarboxylase to produce *S*-allantoin (reviewed in Kaur et al. 2009). Arabidopsis transthyretin-like protein (TLP) contains both HIU hydrolase and OHCU decarboxylase domains and catalyzes steps (ii) and (iii) (Hennebry et al. 2006; Lamberto et al. 2010). Both Uri and TLP were detected in Arabidopsis peroxisome proteome studies (Table 1).

Pseudouridine catabolism is a new plant peroxisomal function first discovered by MS-based proteome studies (Eubel et al. 2008; Reumann et al. 2009; Quan et al. 2013). Modified from uridine, pseudouridine is the most prevalent non-classical nucleoside in RNA. In *E. coli*, YeiC catalyzes the phosphorylation of pseudouridine to form pseudouridine 5'-phosphate, which is then cleaved by YeiN to produce uracil and ribose 5-phosphate (Preumont et al. 2008). The Arabidopsis orthologs of YeiC and YeiN, named PfkB and IndA respectively, are both localized to peroxisomes (Eubel et al. 2008; Reumann et al. 2009; Lingner et al. 2011), although their substrate specificities remain unknown. It was suggested that compartmentalization of pseudouridine catabolism in plant peroxisomes may help to avoid interference between RNA catabolism and biosynthesis (Reumann 2011).

The toxic effects of sulfite can be countered by the activity of sulfite oxidase (SO), which oxidizes sulfite into sulfate and is exclusively peroxisomal in plants (Nakamura et al. 2002; Nowak et al. 2004). This reaction produces H_2O_2, which can non-enzymatically oxidize an additional sulfite (Hänsch et al. 2006). SO was

detected in all the major Arabidopsis peroxisome proteome studies (Table 1), suggesting its constitutive role in Arabidopsis.

3.9 Amino Acid Metabolism

Branched-chain amino acids (BCAAs), such as valine, leucine and isoleucine, can be completely catabolized by peroxisomes isolated from mungbean hypocotyls (Gerbling and Gerhardt 1988; Gerbling and Gerhardt 1989). In Arabidopsis, the involvement of peroxisomes in BCAA catabolism is mainly supported by the identification of the peroxisome-localized 3-hydroxyisobutyryl (HIBYL)-CoA hydrolase 1 (CHY1) (Zolman et al. 2001a), which was later shown by biochemical and physiological evidence to play a role in valine catabolism (Zolman et al. 2001a; Lange et al. 2004). The CHY1 protein was detected by multiple peroxisomal proteome studies in Arabidopsis (Table 1), and has two Arabidopsis homologs, CHY1H1 and CHY1H2, which are also peroxisomal (Lingner et al. 2011).

Arabidopsis peroxisomes also contain sarcosine oxidase (SOX) (Goyer et al. 2004), whose homologs in bacteria and mammals catalyze the oxidation of secondary or tertiary amino acids (Reuber et al. 1997; Trickey et al. 1999). Aspartate aminotransferase isoform 3 (ASP3) was identified by peroxisome proteomics and found to localize to both chloroplasts and peroxisomes (Schultz and Coruzzi 1995; Fukao et al. 2002; Reumann et al. 2007; Eubel et al. 2008; Reumann et al. 2009; Quan et al. 2013). Cobalamin-independent Met synthase 1 (ATMS1), which is possibly involved in methionine regeneration (Ravanel et al. 2004), was detected in multiple Arabidopsis peroxisome proteome studies (Table 1). The metabolic roles of ASP3 and ATMS1 in peroxisomes and their physiological importance are not yet clarified.

3.10 Pathogen Response

Multiple studies link photorespiration and peroxisomal ROS metabolism to plant pathogen response, where CAT2, GOX1, GOX2, GOX3, HAOX2, HPR1 and SGT were found to play a role in plant-pathogen interaction (reviewed in Sørhagen et al. 2013). In addition, PEN2 is a glycosyl hydrolase required for plant resistance against a broad range of non-adapted pathogens (Lipka 2005), and was shown to be a tail-anchored protein dually targeted to the peroxisomal membrane and the mitochondrial outer membrane (Fuchs et al. 2016). PEN2 was proposed to hydrolyze a specific glucosinolate (I3G) and yield indole 3-yl methylamine (I3A) and raphanusamic acid (RA), which are toxic to fungi (Bednarek et al. 2009), and to hydrolyze 4-methoxy-indole 3-ylmethylglucosinolate (4MI3G) to provide signaling molecules or coactivators for callose deposition (Clay et al. 2009). In addition, RabGAP22 is a Rab GTPase-activating protein involved in plant defense against

the fungal pathogen *Verticillium longisporum*. Upon infection, *RabGAP22* gene expression is induced and its protein translocates from the nucleus to peroxisomes, where it interacts with peroxisomal SGT and possibly affects JA biosynthesis (Roos et al. 2014). CPK1 is a calcium-dependent protein kinase involved in innate immunity in Arabidopsis (Coca and San Segundo 2010).

3.11 Metabolism of NADPH and NADH

As a critical cofactor in plant peroxisomes, NADPH is consumed by many enzymatic reactions, such as those catalyzed by OPR3, ECI, SDRa, Monodehydroascorbate reductase (MDAR), Glutathione reductase (GR) and NADPH:quinone reductase (NQR), and several NADPH dehydrogenases (reviewed in Nyathi and Baker 2006; Kaur et al. 2009). Known peroxisomal NADPH dehydrogenases include NDA1, NDA2 and NDB1, which can also use NADH as substrate (Carrie et al. 2008). In addition, NADPH can be hydrolyzed to reduced nicotinamide mononucleotide (NMNH) and 2',5'-ADP by the peroxisome localized Nudix hydrolase homolog 19 (NUDT19), an enzyme that also hydrolyzes NADH to NMNH and AMP (Ogawa et al. 2008).

In plants, a primary source of NADPH is the plastid localized oxidative pentose phosphate pathway (OPPP) catalyzed by three enzymes: glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconolactonase (6PGL), and 6-phosphogluconate dehydrogenase (6PGDH) (Kruger and Von Schaewen 2003). Isoforms for all three enzymes in Arabidopsis (G6PD1, PGL3 and PGD2) are found to localize to peroxisomes (reviewed in Kaur and Hu 2011). The peroxisomal OPPP reactions have essential functions in fertilization, as loss of peroxisomal PGD2 activity inhibits the guided growth of male gametophytes and pollen tube-ovule interaction in Arabidopsis (Hölscher et al. 2016).

Other peroxisomal enzymes capable of generating NADPH include NADP-dependent isocitrate dehydrogenase (ICDH), NADH kinase 3 (NADK3) and possibly BADH. The peroxisomal pool of NADH can be augmented by the activity of Glyceraldehyde 3-phosphate dehydrogenase C2 (GAPC2), which catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and produces NADH from NAD^+ (reviewed in Kaur and Hu 2011).

Earlier studies using other plant species indicated the peroxisome localization of OPPP enzymes and ICDH (reviewed in Corpas and Barroso 2018). Arabidopsis PGD2, ICDH, BADH and GAPC2 were detected by peroxisomal proteomics followed by fluorescence microscopic validations (Table 1).

3.12 Peroxisomal Transporters for Metabolites and Cofactors

Numerous metabolites and cofactors need to be exchanged across the peroxisomal membrane, and many of them require membrane transporters (Linka and Esser 2012). As described earlier in this chapter, CTS/PXA1/PED3 imports various substrates, including precursors for β-oxidation and acetate, and was detected in multiple peroxisome proteome analyses (Table 2). CGI-58 is an α/β hydrolase that partially localizes to peroxisomes, interacts with PXA1 and positively regulates the function of PXA1 in fatty acid catabolism and hormone biosynthesis (Park et al. 2013).

Peroxisomal adenine nucleotide carrier 1 (PNC1) and PNC2, which were identified by sequence homology to yeast ATP carriers, can import ATP in exchange for ADP and AMP, presumably to support energy-consuming reactions, such as substrate activation in β-oxidation and protein phosphorylation by kinases (Linka et al. 2008). PNC2 and a soybean PNC homolog were detected by peroxisome proteome analyses (Eubel et al. 2008; Arai et al. 2008b).

PXN/PMP38 was identified as an NAD^+ carrier, which imports NAD^+ into peroxisomes in exchange for AMP (Bernhardt et al. 2012; van Roermund et al. 2016). Consistent with its constitutive role, PXN was detected by multiple peroxisome proteome studies (Table 2).

Several other integral membrane proteins with uncharacterized functions, including peroxisomal membrane protein of 22 kDa (PMP22) (Tugal et al. 1999; Murphy et al. 2003; Eubel et al. 2008), Ca^{2+}-dependent carrier (CDC) (Carrie et al. 2009) and short membrane protein 2 (SMP2) (Abu-Abied et al. 2009), may also possess transporter activities.

3.13 Nucleotide Homeostasis

Nucleoside diphosphate kinase type 1 (NDPK1), which catalyzes the interconversion of nucleoside diphosphates (NDPs) (Yegutkin 2008) and thus may be involved in the homeostasis of different NDPs in peroxisomes, was identified by leaf peroxisome proteome analysis and found to partially localize to the peroxisome (Reumann et al. 2009). Three Histidine Triad (HIT) family proteins (HIT1, HIT2 and HIT3) were discovered by leaf peroxisome proteomics and confirmed by fluorescence microscopy, making them the first reported peroxisomal members of this family (Reumann et al. 2009). HIT proteins are nucleotide binding proteins, and possess hydrolase or transferase activities on various nucleotide conjugate substrates (Brenner 2002). An in vitro study showed that all these three HIT proteins have sulfohydrolase activity, which converts adenosine 5'-phosphosulfate (APS) to AMP and sulfate (Guranowski et al. 2010), suggesting that these HITs may be involved in maintaining the peroxisomal pool of adenosine nucleotides.

Table 2 Plant peroxisome membrane-associated proteins

Gene Name	Locus	Annotation		Reference (proteome analysis)
PEX11C[a]	At1g01820	Peroxin 11C	Peroxisome division	Eubel et al. (2008), Reumann et al. (2009)
PEX6	At1g03000	Peroxin 6	Matrix protein import	
DHAR1[a]	At1g19570	Dehydroascorbate reductase 1	Detoxification	Reumann et al. (2009)
PEX7[a]	At1g29260	Peroxin 7	Matrix protein import	Eubel et al. (2008)
PEX11A[a]	At1g47750	Peroxin 11A	Peroxisome division	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
PEX3B	At1g48635	Peroxin 3B	Membrane protein import	
SPL1	At1g59560	SP1-like 1	Matrix protein import	
SP1	At1g63900	Suppressor of ppi1 locus 1	Matrix protein import	
PEX2	At1g79810	Peroxin 2	Matrix protein import	
SMP2	At2g02510	Short membrane protein 2	Transporter	
DRP3B	At2g14120	Dynamin-related protein 3B	Peroxisome division	
PEX10	At2g26350	Peroxin 10	Matrix protein import	
PXN/ PMP38/ PMP36[a]	At2g39970	Peroxisomal nicotinamide adenine dinucleotide carrier/ peroxisomal membrane protein 38/Peroxisomal membrane protein of 36 Kda	Transporter	Fukao et al. (2003), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)

(continued)

Table 2 (continued)

Gene Name	Locus	Annotation		Reference (proteome analysis)
PEN2	At2g44490	Penetration 2	Defense	
PEX16	At2g45690	Peroxin 16	Membrane protein import	
PEX11D[a]	At2g45740	Peroxin 11D	Peroxisome division and movement	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
PEX19A	At3g03490	Peroxin 19A	Membrane protein import	
PEX12	At3g04460	Peroxin 12	Matrix protein import	
PNC1	At3g05290	Peroxisomal adenine nucleotide carrier 1	Transporter	
PEX13	At3g07560	Peroxin 13	Matrix protein import	
APEM9/ PEX26	At3g10572	Aberrant peroxisome morphology 9	Matrix protein import	
PEX3A	At3g18160	Peroxin 3A	Membrane protein import	
DRP5B	At3g19720	Dynamin-related protein 5B	Peroxisome division	
PEX22	At3g21865	Peroxin 22	Matrix protein import	
MDAR4[a]	At3g27820	Monodehydroascorbate reductase 4	Detoxification	Reumann et al. (2009)
PEX11B[a]	At3g47430	Peroxin 11B	Peroxisome division	Reumann et al. (2009)
FIS1A	At3g57090	Fission 1A	Peroxisome division	

(continued)

Table 2 (continued)

Gene Name	Locus	Annotation		Reference (proteome analysis)
PMD1	At3g58840	Peroxisomal and mitochondrial division factor 1	Peroxisome division	
PEX11E[a]	At3g61070	Peroxin 11E	Peroxisome division	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
PMP22[a]	At4g04470	Peroxisomal membrane protein of 22 kDa	Transporter	Eubel et al. (2008)
DRP3A	At4g33650	Dynamin-related protein 3A	Peroxisome division	
APX3[a]	At4g35000	Ascorbate peroxidase 3	Detoxification	Fukao et al. (2002), Fukao et al. (2003), Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
PXA1/ CTS/ PED3[a]	At4g39850	Peroxisomal ABC transporter 1/comatose/peroxisome defective 3	Transporter	Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
SDP1	At5g04040	Sugar-dependent 1	Others	
PEX1	At5g08470	Peroxin 1	Matrix protein import	
FIS1B	At5g12390	Fission 1B	Peroxisome division	
PEX19B	At5g17550	Peroxin 19B	Membrane protein import	
PEX4	At5g25760	Peroxin 4	Matrix protein import	
PNC2[a]	At5g27520	Peroxisomal adenine nucleotide carrier 2	Transporter	Arai et al. (2008a, b), Eubel et al. (2008)

(continued)

Table 2 (continued)

Gene Name	Locus	Annotation		Reference (proteome analysis)
MYA2	At5g43900	Myosin XI isoform 2	Peroxisome division/ movement	
PEX5	At5g56290	Peroxin 5	Matrix protein import	
PEX14[a]	At5g62810	Peroxin 14	Matrix protein import	Reumann et al. (2007), Eubel et al. (2008), Reumann et al. (2009), Quan et al. (2013)
CDC	At3g55640	Ca^{2+} -dependent carrier	Transporter	
RabE1c	At3g46060	Ras-related protein RabE1c/Rab GTPase homolog 8A	Matrix protein import	
CGI-58	At4g24160	Comparative gene identification-58/ 1-acylglycerol-3-phosphate *O*-acyltransferase	Others	

[a]Indicates detection in peroxisome proteome analyses

3.14 Phosphoregulation

Several enzymes catalyzing protein phosphorylation or dephosphorylation are peroxisomal, indicating that peroxisomal functions may be subjected to phosphoregulation. Putative kinases include CPK1 (Coca and San Segundo 2010), glyoxysomal protein kinase 1 (GPK1) (Fukao et al. 2003; Ma and Reumann 2008), receptor-like protein kinase 1 (RPK1) (Narsai et al. 2011) and PPK (a Protein kinase superfamily protein/Peroxisomal protein kinase) (Wang et al. 2017). Protein phosphatase 2A B'θ subunit (PP2A-B'θ), PP2A-C5, PP2A-A2, PP2A-C2, POL like phosphatase 2 (PLL2), PLL3, purple acid phosphatase 7 (PAP7) and MAP kinase phosphatases 1 (MKP1) are putative phosphatases or phosphatase subunits identified by in silico analysis and confirmed to be peroxisomal by fluorescence microscopy (Matre et al. 2009; Kataya et al. 2015a; Kataya et al. 2015b; Kataya et al. 2016). MKP1 has a role in plant stress response by negatively regulating the production of reactive oxygen species (ROS) and salicylic acid (SA) (Bartels et al. 2009; Anderson et al. 2011), and is cytosolic in non-stressed conditions while peroxisomal when plants are under stress (Kataya et al. 2015b). With the exception of GPK1 (Fukao et al. 2003), none of these proteins were detected by proteome analyses.

3.15 Molecular Chaperones and Proteases

Hsp15.7 and Acd31.2 are small heat shock proteins (Hsps) with potential protective roles against unspecific abnormal protein aggregation in both normal and stressed conditions (Ma et al. 2006), and were detected in the peroxisome proteome in etiolated seedlings and green leaves (Table 1).

Peroxisome-associated endoproteases and peroxisome protein degradation were detected in pea leaves in earlier studies (Distefano et al. 1997; Distefano et al. 1999). In Arabidopsis, the LON2 protease is involved in peroxisome β-oxidation, PTS2 processing, matrix protein import and dynamics (Lingard and Bartel 2009; Farmer et al. 2013; Goto-Yamada et al. 2014). Degradation of periplasmic protein 15 (DEG15) has ATP-independent serine endopeptidase activity and removes *N*-terminal PTS2 of imported PTS2-containing proteins in peroxisomal matrix (Helm et al. 2007; Schuhmann et al. 2008). Three additional peroxisomal proteases—i.e. Serine carboxypeptidase-like protein 20 (SCPL20), Response to drought21A-like 1 (RDL1), and peroxisomal M16 metalloprotease (PM16), were identified through proteome analyses and confirmed by fluorescence microscopy (Eubel et al. 2008; Reumann et al. 2009; Quan et al. 2013). SCPL20 possesses serine hydrolase activity (Kaschani et al. 2009), and plays a role in β-oxidation (Quan et al. 2013) and plant defense (Floerl et al. 2012). RDL1, whose homolog in Daikon radish (*Raphanus sativus*) contains cysteine protease activity (Tsuji et al. 2013), is involved in β–oxidation, seed viability, and stress adaption (Quan et al. 2013).

In silico analysis followed by fluorescence microscopy identified a PTS1-containing ovarian tumor-like cysteine protease (CP), (Lingner et al. 2011).

3.16 Other Peroxisomal Proteins

Snowy cotyledon 3 (SCO3) was found to localize to the cytoskeleton and peroxisomes and suggested to be involved in chloroplast development (Albrecht et al. 2010). B12D1 was first identified by leaf peroxisome proteomics (Reumann et al. 2009) and later found to be involved in germination and β-oxidation (Cassin-Ross and Hu 2014a). CML3 is a calmodulin-like protein involved in Ca^{2+} signaling (Chigri et al. 2012), dimerization of DEG15, and peroxisome β-oxidation activity (Dolze et al. 2013). ATF1 and ATF2 are acetyl transferases first discovered in the peroxisome through MS-based proteomics (Reumann et al. 2007; Reumann et al. 2009). MIA40, a conserved protein in mitochondrial inter-membrane space and involved in mitochondrial protein import (Carrie et al. 2010), was also found to target to peroxisomes to help maintain the protein levels of CSD3 and AIM1, and thus may be involved in peroxisomal detoxification and β-oxidation (Carrie et al. 2010). NQR, which shares sequence similarity with human peroxisomal prostaglandin reductase 3, is a putative NADPH:quinone reductase identified first by peroxisome proteome analysis and confirmed by in vivo targeting analysis (Eubel et al. 2008; Reumann et al. 2007), and is involved in plant stress response (Babiychuk et al. 1995).

A number of peroxisome proteins with uncharacterized metabolic functions in peroxisomes or physiological roles were also identified through MS-based proteomics or PTS1 prediction. These include GDSL motif lipase/hydrolase family protein (GLH) (Eubel et al. 2008), Zinc-binding dehydrogenase (ZnDH) (Eubel et al. 2008; Reumann et al. 2009), Beta glucosidase 8 (BGLU8) (Wang et al. 2017), Macrophage migration inhibitory factor (MIF) (Reumann et al. 2007; Li et al. 2009; Reumann et al. 2009), and several so-called unknown proteins (UPs) (Reumann et al. 2007; Reumann et al. 2009; Quan et al. 2010; Quan et al. 2013; Wang et al. 2017). Mutants of *UP5*, *UP6* and *UP7* are partially disturbed in β-oxidation (Cassin-Ross and Hu 2014a). UP12 was identified by a transcriptomic screen for genes with induced expression during germination followed by in vivo targeting analysis (Narsai et al. 2011).

4 Conclusions and Perspectives

MS-based proteome analysis of plant peroxisomes is a powerful large-scale approach that has achieved remarkable successes in the past 15 years or so, leading to the discovery of many new peroxisomal proteins and pathways. Out of the 199 Arabidopsis peroxisomal proteins summarized here, 120 (60%) have been detected

in peroxisome proteome studies, and the coverage by proteomics is even higher for PTS-containing peroxisome proteins (102 out of 144, 71%).

The actual number of novel peroxisomal proteins detected by proteome studies may be higher than reported, as it is possible that some bona-fide peroxisomal proteins were identified by MS but mistakenly categorized as contaminants because they (i) lack an established PTS, (ii) are known to have a notable non-peroxisomal function, (iii) are well-known constituents of other subcellular compartments, and/or (iv) were not verified by fluorescence microscopy due to protein instability or conditional peroxisomal targeting.

The major challenges for peroxisome proteome studies are the detection of membrane proteins, protein post-translational modifications, proteins with extremely low abundance, and proteins transiently targeting to peroxisome under specific conditions. Quantitative MS methods such as LOPIT (localization of organelle proteins by isotope tagging), ICAT (isotope-coded affinity tag) and iTRAQ (isobaric tag for relative and absolute quantitation), may be necessary to expand the coverage of peroxisomal proteins (Reumann 2011). Peroxisome proteome analysis should extend to new tissue types such as roots and reproductive organs, and new plant species, such as cereal crops (Kaur and Hu 2011). Peroxisome isolation protocols need to be refined for higher efficiency and for adaptation to different tissues and plant species (Islinger et al. 2018). Deciphering the full and dynamic plant peroxisome proteome will provide a knowledge base for the improvement of crop production, quality and stress tolerance.

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Defining the Mammalian Peroxisomal Proteome

Eden Yifrach, Sven Fischer, Silke Oeljeklaus, Maya Schuldiner, Einat Zalckvar and Bettina Warscheid

Abstract The current view on peroxisomes has changed dramatically from being human cell oddities to vital organelles that host several key metabolic pathways. To fulfil over 50 different enzymatic functions, human peroxisomes host either unique peroxisomal proteins or dual-localized proteins. The identification and characterization of the complete peroxisomal proteome in humans is important for diagnosis and treatment of patients with peroxisomal disorders as well as for uncovering novel peroxisomal functions and regulatory modules. Hence, here we compiled a comprehensive list of mammalian peroxisomal and peroxisome-associated proteins by curating results of several quantitative and non-quantitative proteomic studies together with entries in the UniProtKB and Compartments knowledge channel databases. Our analysis gives a holistic view on the mammalian peroxisomal proteome and brings to light potential new peroxisomal and peroxisome-associated proteins. We believe that this dataset, represents a valuable surrogate map of the human peroxisomal proteome.

Keywords Peroxisome · Mass spectrometry · Protein databases
Mammals · Peroxisomal diseases

Eden Yifrach, Sven Fischer—contributed equally to this work.

E. Yifrach · M. Schuldiner (✉) · E. Zalckvar (✉)
Department of Molecular Genetics, Weizmann Institute of Science,
7610001 Rehovot, Israel
e-mail: maya.schuldiner@weizmann.ac.il

E. Zalckvar
e-mail: einat.zalckvar@weizmann.ac.il

S. Fischer · S. Oeljeklaus (✉) · B. Warscheid (✉)
Faculty of Biology and BIOSS Centre for Biological Signaling Studies,
Biochemistry and Functional Proteomics, Institute for Biology II,
University of Freiburg, 79104 Freiburg, Germany
e-mail: silke.oeljeklaus@biologie.uni-freiburg.de

B. Warscheid
e-mail: bettina.warscheid@biologie.uni-freiburg.de

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Abbreviations

MS Mass spectrometry
PTS1 Peroxisomal targeting signal 1

1 Introduction

Since their discovery in 1954 (Rhodin 1954), our view on peroxisomes has changed dramatically from being human cell oddities to vital organelles that host several key metabolic pathways (Vamecq et al. 2014). The essential role of peroxisomes in metabolism, health, and survival explains why an impairment in one or more of the metabolic functions of peroxisomes, not to mention the complete biogenesis, frequently leads to severe disorders with neurological dysfunction that often lead to early death (Wanders et al. 2015).

Human peroxisomes contain over 50 different enzyme activities, but the specific metabolic functions of the organelle can differ per organism, tissue and cell type and often are a reflection of metabolic needs and physiological conditions (Waterham et al. 2016). To fulfil this vast array of different enzymatic activities, peroxisomes host either unique peroxisomal proteins or dual-localized proteins that are also present in the cytosol or other organelles, such as mitochondria (Waterham et al. 2016).

The identification and characterization of the complete peroxisomal proteome in humans is important for diagnosis and treatment of patients with peroxisomal disorders as well as for uncovering novel peroxisomal functions and regulatory modules. Indeed, over the years, many studies have been undertaken to characterize the mammalian peroxisomal proteome, ranging from classical subcellular fractionation followed by mass spectrometry (MS), to DNA/RNA sequence-based strategies and protein-specific studies. However, while for the yeast *Saccharomyces cerevisiae* a list of *bona fide* peroxisomal proteins was recently published (Yifrach et al. 2016), such a list was not established for higher eukaryotes. Here we aim to provide such a list by integrating the presently available resources to a single coherent proteomic tally.

2 Mass Spectrometry-Based Studies of Mammalian Peroxisomes

The in-depth characterization of the peroxisomal mammalian proteome is a challenging task—peroxisomes are small and generally low abundance structures that have strong physical contact sites with other organelles (Shai et al. 2016).

Therefore, the subcellular fractions enriched in peroxisomes always contain contaminants from other organelles making it highly difficult to purify them well (Oeljeklaus et al. 2014). Moreover, the presence of dually localized proteins makes it difficult to unequivocally determine the peroxisomal proteome.

In the first comprehensive study, peroxisomes from rat liver were enriched by density gradient centrifugations followed by affinity purification of peroxisomes using antibodies recognizing the abundant peroxisomal membrane protein PMP70 (ABCD3) (Kikuchi et al. 2004). Later studies employed quantitative MS strategies including label-free (Gronemeyer et al. 2013; Wiese et al. 2007, 2012) or stable isotope labeling techniques (Islinger et al. 2007), which allow for the discrimination between true peroxisomal resident proteins and co-purified contaminants that are derived from other cellular compartments (Oeljeklaus et al. 2014).

While all the peroxisome-focused organellar proteomics strategies applied in the studies mentioned above proved to be highly effective for the identification of new peroxisomal proteins, they only provide a limited picture or even fail to detect proteins that are present in multiple subcellular compartments. To establish a more complete map of protein subcellular localization in mammalian cells, sophisticated proteomic profiling approaches based on quantitative MS have been applied to profile thousands of individual proteins across fractions of density gradients and allocate them to different compartments (Christoforou et al. 2016; Forner et al. 2006; Foster et al. 2006; Itzhak et al. 2016; Jadot et al. 2017; Jean Beltran et al. 2016).

While each of the studies mentioned above greatly contributed to our current knowledge of the mammalian peroxisomal proteome, a unified comprehensive list was not previously established from all of these studies as well as from other available sources of information on peroxisomal residents.

3 Definition of the Mammalian Peroxisomal Proteome

Here, we combined results of several quantitative and non-quantitative proteomic studies (Gronemeyer et al. 2013; Islinger et al. 2007; Jadot et al. 2017; Kikuchi et al. 2004; Wiese et al. 2007, 2012) (see Table 1) and entries in the UniProtKB

Table 1 List of quantitative and non-quantitative proteomic studies that were combined to curate the list of mammalian peroxisomal proteins

Proteomics study	Organism	Tissue/cell line	PubMed ID
Kikuchi et al. (2004), JBC, 279	Rat	Liver	14561759
Islinger et al. (2007), JBC, 282	Rat	Liver	17522052
Wiese et al. (2007), MCP, 6	Mouse	Kidney	17768142
Wiese et al. (2012), Int J Mass Spectrom, 312	Mouse, Human	Kidney, Huh7 cells	Not in PubMed
Gronemeyer et al. (2013), PLOS One, 8	Human	Liver, Huh7 cells	23460848
Jadot et al. (2017), MCP, 16	Rat	Liver	27923875

(The UniProt Consortium 2017) and Compartments knowledge channel (Binder et al. 2014) databases to curate the most comprehensive list of peroxisomal proteins and putative peroxisome-associated proteins in mammals. We chose the above proteomic studies because they analyzed purified peroxisomal fractions and most were performed in a quantitative manner. Entries in the databases were added in order to include peroxisomal proteins and further peroxisomal candidates that have been identified in single-protein studies or that for any reason were not detected in the MS-based studies. Our full list contains 196 proteins (Table 2). Since we relied on multiple sources of different specificity, we gave each protein a score, which represents how many times and in which experimental setup it came up as being a peroxisomal resident protein. Of course, the higher the score, the more it enforces its peroxisomal localization.

The scoring was as such: for identification as a peroxisomal protein in one of the quantitative or non-quantitative MS-based studies, a protein received four or two points, respectively. In addition, for annotation as a peroxisomal protein in one of the databases, the protein received one point. The final score is the sum of all points that each protein received. We set the threshold at equal to or higher than eight to consider a protein as one that was detected in high frequency in peroxisomal fractions. This threshold was chosen based on experimentally verified examples such that it maximizes inclusion of known peroxisomal proteins and minimizes inclusion of well-studied non peroxisomal ones. However, it is a subjective score and threshold and hence, the raw data is provided (Table 2) and can be utilized to determine independent scoring or threshold values. An extended version of Table 2 that includes further information extracted from the UniProtKB and Compartments can be downloaded from http://www.proteomics.uni-freiburg.de/downloads.

Overall, the majority of proteins that were detected with high frequency in our list were identified in at least two quantitative proteomic studies. Notably, two proteins (ABCD2 and MPV17) got a score of eight although they were only identified once in either a quantitative or a non-quantitative proteomic study. However, both proteins received high scores from their appearance in databases. Using a threshold of eight, the list contains 96 proteins that were detected in high frequency (Table 2, High detection frequency).

3.1 Proteins That Are Detected in High Frequency in Peroxisomal Fractions

Interestingly, seven proteins in the list of proteins that were detected in high frequency are currently not annotated as peroxisomal proteins in the databases. However, two of these proteins, malate dehydrogenase 1 (MDH1) and lactate dehydrogenase A (LDHA), were reported to be partially imported into peroxisomes using a translational read-through mechanism that exposes a hidden peroxisomal targeting signal 1 (PTS1) in the C-terminus of the extended proteins. While the

Table 2 List of frequently detected mammalian peroxisomal proteins

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
High detection frequency										
ABCD3	ATP-binding cassette sub-family D member 3	h, m, r	M[a,h], S[a]			Fatty acid transporter[c,e]	+	2–6	6	28
GNPAT	Dihydroxyacetone phosphate acyltransferase	h, m, r	M[h], S[a]	AKL[a,c]		Plasmalogen[c], glycerolipid[e] biosynthesis	+	2–6	6	28
PEX12	Peroxisome assembly protein 12	h, m, r	M[a]			Peroxisome biogenesis[c,d,e]	+	2–6	6	28
PEX3	Peroxisomal biogenesis factor 3	h, m, r	M[a,h], S[a]			Peroxisome biogenesis[c,d,e]	+	2–6	6	28
ACOT8	Acyl-coenzyme A thioesterase 8	h, m, r	S[a,h]	SKL[a,c]		β-oxidation[c,d,e]	+	2–6	6	28
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	h, m, r	S[a,h]	SKL[a,c,g]		β-oxidation[c,d,e,g]	+	2–6	6	28
ACOX2	Peroxisomal acyl-coenzyme A oxidase 2	h, m, r	S[a,h]	HKN[a], PKL[c]		β-oxidation[c,d,e]	+	2–6	6	28
CAT	Catalase	h, m, r	S[a,h]	ANL[a,g]		Antioxidant system[c], oxygen metabolism[d,e], auxiliary β-oxidation[g]	+	2–6	6	28
HACL1	2-hydroxyacyl-CoA lyase 1	h, m, r	S[a,h]	SNM[a,g]		α-oxidation[c,d,e,g]	+	2–6	6	28
PEX11A	Peroxisomal membrane protein 11A (HsPEX11p)	h, m, r	M[a,h]			Peroxisome biogenesis[c,d,e]	+	2–6	6	28
PHYH	Phytanoyl-CoA dioxygenase	h, m, r	S[a,h]		+[c,g]	α-oxidation[c,d,e,g]	+	2–6	6	28
PXMP4	Peroxisomal membrane protein 4	h, m, r	M[a,h]				+	2–6	6	28
ACBD5	Acyl-CoA-binding domain-containing protein 5	h, m, r	S[a]	KLN[a]			+	2–6	6	28
DECR2	Peroxisomal 2,4-dienoyl-CoA reductase	h, m, r	S[a,h]	AKL[a,c]		β-oxidation[c,d,e]	+	2–6	6	28
LONP2	Lon protease homolog 2	h, m, r	S[a,h]	SKL[a,c]			+	2–6	6	28

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
SCP2	Non-specific lipid-transfer protein	h, m, r	S[a,h]	AKL[a,c,g]		β-oxidation[c,d,e,g]	+	2–6	6	28
PIPOX	Peroxisomal sarcosine oxidase (PSO)	h, m, r	S[a,h]	AHL[a,g]		Amino acid metabolism[c,d,e,g]	+	2–6	5	27
PEX16	Peroxisomal membrane protein PEX16	h, m, r	M[a]			Peroxisome biogenesis[c,d,e]	+	2–6	5	27
PEX2	Peroxisome biogenesis factor 2	h, m, r	M[a],S[a]	NAL[g]		Peroxisome biogenesis[c,d,e,g]	+	2–6	5	27
CRAT	Carnitine O-acetyltransferase	h, m, r	M[h]	AKL[c]		β-oxidation[c,d], fatty acid transporter[e]		2–6	6	26
CROT	Peroxisomal carnitine O-octanoyltransferase	h, m, r	M[h]	AHL[c]		β-oxidation[c,d], fatty acid transporter[e]		2–6	6	26
DAO	D-amino-acid oxidase	h, m, r	S[h]	SHL[c,g]		Amino acid metabolism[c,d,g], oxygen metabolism[e]		2–6	6	26
EPHX2	Bifunctional epoxide hydrolase 2	h, m, r	S[h]	SKI[c,g]		Antioxidant system[c], oxygen metabolism[d], amino acid metabolism[g]		2–6	6	26
HSD17B4	Peroxisomal multifunctional enzyme type 2 (MFE-2)	h, m, r	S[a,h]	AKL[a,g]		β-oxidation[c,d,e]	+	2–5	6	24
PEX14	Peroxisomal membrane protein PEX14	h, m, r	M[a],S[a]			Peroxisome biogenesis[c,d,e]	+	2–5	6	24
ACOX3	Peroxisomal acyl-coenzyme A oxidase 3	h, m, r	S[a,h]	SQL[a,c]		β-oxidation[c,d,e]	+	2–5	6	24
ACSL1	Long-chain-fatty-acid–CoA ligase 1	h, m, r	M[a,h], S[a]	IKI[a]		β-oxidation[c,d], fatty acid transporter[e]	+	2–5	6	24

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
EHHADH	Peroxisomal bifunctional enzyme (PBE)	h, m, r	S[a,h]	SKL[a,c,g]		β-oxidation[c,d,e,g]	+	2–5	6	24
GSTK1	Glutathione S-transferase kappa 1	h, m, r	S[a,h]	ARL[a,c]		Antioxidant system[c], oxygen metabolism[d,e]	+	2–5	6	24
ACAD11	Acyl-CoA dehydrogenase family member 11	h, m, r	S[a]	ARM[a]			+	2–5	6	24
AMACR	Alpha-methylacyl-CoA racemase	h, m, r	S[a,h]	ANL[a,c,g]		α-oxidation[c,g], β-oxidation[d,e]	+	2–5	6	24
BAAT	Bile acid-CoA:amino acid N-acyltransferase	h, m, r	S[a,h]	SQL[a,c]		β-oxidation[c,d], bile acid synthesis[e]	+	3–6	6	24
FIS1	Mitochondrial fission 1 protein	h, m, r	S[a]	SKS[a]		Peroxisome biogenesis[e]	+	2–5	6	24
HAO2	Hydroxyacid oxidase 2	h, m, r	S[a]	SRL[a,c]		Oxygen metabolism[d]	+	2–4, 6	6	24
PECR	Peroxisomal trans-2-enoyl-CoA reductase	h, m, r	S[a,h]	ARL[a], AKL[c,g]		β-oxidation[c,d,e,g]	+	2–5	6	24
PXMP2	Peroxisomal membrane protein 2	h, m, r	M[a,h], S[a]				+	2–5	6	24
PEX6	Peroxisome assembly factor 2 (PAF-2)	h, m, r	M[a]			Peroxisome biogenesis[c,e]	+	2,3,5,6	6	24
PEX5	Peroxisomal targeting signal 1 receptor	h, m, r	M[a]			Peroxisome biogenesis[c,e]	+	2,3,5,6	6	24
ABCD1	ATP-binding cassette sub-family D member 1	h, m, r	M[a],S[a]			Fatty acid transporter[c,e]	+	2–5	5	23
SLC27A2	Very long-chain acyl-CoA synthetase (VLACS)	h, m, r	M[a]			Fatty acid transporter[c,e], β-oxidation[d]	+	2–5	5	23
PEX10	Peroxisome biogenesis factor 10	h, m, r	M[a]			Peroxisome biogenesis[c,e]	+	2,3,5,6	5	23
PEX11B	Peroxisomal membrane protein 11B	h, m, r	M[a]			Peroxisome biogenesis[c,d]	+	2–4, 6	5	23
DHRS4	Dehydrogenase/reductase SDR family member 4	h, m, r	S[h]	SKL[c], SRL[g]		Straight-chain fatty acid β-oxidation[g]		2–5	6	22

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
ECH1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	h, m, r	S[h]	SKL[c,g]		β-oxidation[c,d,e,g]		2–5	6	22
TMEM135	Transmembrane protein 135	h, m, r	M[h]					2–5	6	22
MARC2	Mitochondrial amidoxime reducing component 2 (mARC2)	h, m, r						2–5	5	21
NUDT12	Peroxisomal NADH pyrophosphatase NUDT12	h, m, r				β-oxidation[c]		2,3,5,6	5	21
AGPS	Alkyl dihydroxyacetone phosphate synthase	h, m, r	M[h], S[a]		+[c]	Plasmalogen biosynthesis[c]	+	2–4	6	20
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	h, m, r	S[a,h]	AKL[a]		Glyoxylate metabolism[c], oxidation of redox equiv.[e]	+	3–5	6	20
PRDX5	Peroxiredoxin-5	h, m, r	S[a,h]	SQL[a,c]		Antioxidant system[c], oxygen metabolism[d]	+	2–4	6	20
AGXT	Serine-pyruvate aminotransferase	h, m, r	S[a,h]	ARL[a], NKL[g]		Glyoxylate[d], amino acid[e,g] metabolism	+	4–6	6	20
ECI2	Enoyl-CoA delta isomerase 2	h, m, r	S[a,h]	PKL[a]		β-oxidation[d,e]	+	2,4,5	6	20
ISOC1	Isochorismatase domain-containing protein 1	h, m, r	S[a,h]	SKV[a]			+	3–5	6	20
SLC25A17	Peroxisomal membrane protein PMP34	h, m, r	M[a]				+	2,3,5	5	19
PEX1	Peroxisome biogenesis factor 1	h, m, r	M[a],S[a]			Peroxisome biogenesis[c]	+	2,3,6	5	19
HAO1	Hydroxyacid oxidase 1	h, m, r	S[a,h]	SKI[a,g]		Oxygen metabolism[d,e], α-oxidation[g]	+	4–6	5	19
ACOT4	Acyl-coenzyme A thioesterase 4	h, m, r	S[a]	CRL[a,c]		β-oxidation[c,e]	+	2,3,5	5	19
HMGCL	Hydroxymethylglutaryl-CoA lyase	h, m, r	S[h]	CKL[c,g]		Amino acid[c], ketone body[e] metabolism, isoprenoid biosynthesis[g]		3–5	6	18

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
ALDH3A2	Fatty aldehyde dehydrogenase	h, m	M[h], S[a]	DQL[a]			+	2–4	4	18
ZADH2	Prostaglandin reductase 3	h, m, r						2,3,5	5	17
PEX11G	Peroxisomal membrane protein 11C	h, m, r				Peroxisome biogenesis[c]		2,3,6	5	17
TYSND1	Peroxisomal leader peptide-processing protease	h, m, r						3,5,6	5	17
DDO	D-aspartate oxidase (DASOX)	h, m, r		SKL[c]		Amino acid metabolism[c]		2,3,6	4	16
PEX13	Peroxisomal membrane protein PEX13	h, m, r	M[a],S[a]			Peroxisome biogenesis[c]	+	2,3	5	15
ACAA1B	3-ketoacyl-CoA thiolase B	m, r			+[c,g]	β-oxidation[d,e]		2,3,5	3	15
IDE	Insulin-degrading enzyme	h, m, r	S[h]	AKL[c]		Amino acid metabolism[c]		3,5	6	14
MLYCD	Malonyl-CoA decarboxylase	h, m, r	S[h]	SKL[g]		straight-chain fatty acid β-oxidation[g]		3,4	6	14
UOX	Uricase (EC 1.7.3.3) (Urate oxidase)	m, r	S[a]	SRL[a,c,g]		Purine metabolism[e,g]	+	3,5	4	14
ATAD1	ATPase family AAA domain-containing protein 1	h, m, r						2,4	6	14
FAR1	Fatty acyl-CoA reductase 1	h, m, r				Plasmalogen biosynthesis[c]		2,3	6	14
HSDL2	Hydroxysteroid dehydrogenase-like protein 2	h, m, r						3,4	6	14
IDH2	Isocitrate dehydrogenase [NADP]	h, m, r			+[g]	straight-chain fatty acid β-oxidation[g]		2,4	6	14
NUDT19	Nucleoside diphosphate-linked moiety X motif 19	h, m, r		AHL[c,g]		β-oxidation[c,g]		2,3	6	14
ECI3	Enoyl-CoA delta isomerase 3	m, r	S[h]	PKL[c]	+[c]	β-oxidation[c]		3,5,6	2	14
ACAA1A	3-ketoacyl-CoA thiolase A	m, r	S[a]		+[c,g]	β-oxidation[c,g]	+	2,3	3	13
PAOX	Peroxisomal N(1)-acetyl-spermine/spermidine oxidase	h, m, r		PRL[c]		Amino acid metabolism[c]		3,6	5	13
NUDT7	Peroxisomal coenzyme A diphosphatase NUDT7	h, m, r		SKL[c]				3,6	5	13

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
ACNAT1	Acyl-coenzyme A amino acid N-acyltransferase 1	m, r						2,3	4	12
ACOT6	Putative acyl-coenzyme A thioesterase 6	h, m		SKL[c]		β-oxidation[c]		2,3	4	12
MPV17L	Mpv17-like protein	h, m						2,3	4	12
ACOT3	Acyl-coenzyme A thioesterase 3	m		AKL[c]		β-oxidation[c]		2,3	2	10
FABP1	Fatty acid-binding protein	h, m, r				Fatty acid transporter[e]		5	6	10
SOD1	Superoxide dismutase	h, m, r				Oxygen metabolism[e]		5	6	10
PEX5L	PEX5-related protein	h, m, r				Peroxisome biogenesis[c]		3	6	10
ACAA1	3-ketoacyl-CoA thiolase	h, m				β-oxidation[d]		3,4	2	10
SERHL	Serine hydrolase-like protein	m		ARL[c]				2,3	2	10
PEX26	Peroxisome assembly protein 26	h, m, r						2	5	9
ACSL5	Long-chain-fatty-acid–CoA ligase 5	h, m				β-oxidation[c,d]		3,4	0	8
MPV17	Protein Mpv17	h, m						3	4	8
GPD1	Glycerol-3-phosphate dehydrogenase	h, m	S[h]					2,4	0	8
DHRS7B	Dehydrogenase/reductase SDR family member 7B	m, r						3,5	0	8
ACOT1	Acyl-coenzyme A thioesterase 1	h, m				β-oxidation[d]		2,4	0	8
LDHA	L-lactate dehydrogenase A chain	h, r	S[h]			Oxidation of redox equivalents[e]		4,5	0	8
MDH1	Malate dehydrogenase	h, m						2,4	0	8
VWA8	von Willebrand factor A domain-containing protein 8	m						2,3	0	8
ABCD2	ATP-binding cassette sub-family D member 2	h, m, r	M[a]				+		6	8

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
Low detection frequency										
ACNAT2	Acyl-coenzyme A amino acid N-acyltransferase 2	m, r		SKL[c]	–	β-oxidation[c]		3	3	7
MGST1	Microsomal glutathione S-transferase 1	h, m, r	S[a]	LYL[a]	–		+		5	7
IDI1	Isopentenyl-diphosphate Delta-isomerase 1	h, m, r							6	6
ACSL4	Long-chain-fatty-acid-CoA ligase 4	h, m, r							6	6
PRDX1	Peroxiredoxin-1	r						5	2	6
IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	h, m, r							6	6
PEX19	Peroxisomal biogenesis factor 19	h, m, r							6	6
ACSL6	Long-chain-fatty-acid-CoA ligase 6	h, m, r							6	6
DNM1L	Dynamin-1-like protein	h, m, r							6	6
HSPD1	60 kDa heat shock protein	h, m, r			+[g]	Putative peroxisomal protein[g]			6	6
XDH	Xanthine dehydrogenase/oxidase	h, m, r							6	6
ACSL3	Long-chain-fatty-acid-CoA ligase 3	h, m, r							6	6
FNDC5	Fibronectin type III domain-containing protein 5	h, m, r							6	6
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase	h, m, r							6	6
MARF1	Meiosis regulator and mRNA stability factor 1	h, m, r							6	6
MAVS	Mitochondrial antiviral-signaling protein	h, m, r							6	6
MFF	Mitochondrial fission factor	h, m, r							6	6
NOS2	Nitric oxide synthase	h, m, r							6	6
PIK3C3	Phosphatidylinositol 3-kinase catalytic subunit type 3	h, m, r							6	6
PLA2G16	HRAS-like suppressor 3	h, m, r							6	6
RAB8B	Ras-related protein Rab-8B	h, m, r							6	6

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
SYT7	Synaptotagmin-7	h, m, r							6	6
TMEM173	Stimulator of interferon genes protein	h, m, r							6	6
TMEM35A	Transmembrane protein 35A	h, m, r							6	6
CYB5A	Cytochrome b5	m, r	S[a]	AED[a]			+	2	0	6
CYB5R3	NADH-cytochrome b5 reductase 3	m, r	S[a]	FTT[a]			+	2	0	6
URAH	5-hydroxyisourate hydrolase	m, r						6	2	6
PEX7	Peroxisomal targeting signal 2 receptor	h, m, r							5	5
PMVK	Phosphomevalonate kinase	h, m, r							5	5
TTC1	Tetratricopeptide repeat protein 1	h, m, r							5	5
VIM	Vimentin	h, m, r							5	5
ACOXL	Acyl-coenzyme A oxidase-like protein	h, m, r							5	5
AKAP11	A-kinase anchor protein 11	h, m, r							5	5
FAR2	Fatty acyl-CoA reductase 2	h, m, r							5	5
MUL1	Mitochondrial ubiquitin ligase activator of NFKB 1	h, m, r							5	5
PNPLA8	Calcium-independent phospholipase A2-gamma	h, m, r							5	5
PXT1	Peroxisomal testis-specific protein 1	h, m, r							5	5
SZT2	KICSTOR complex protein SZT2	h, m, r							5	5
TRIM37	E3 ubiquitin-protein ligase TRIM37	h, m, r							5	5
SOD2	Superoxide dismutase [Mn]	m				Antioxidant system[c]		3	0	4
CNOT1	CCR4-NOT transcription complex subunit 1	h, m							4	4
MAP2K2	Dual specificity mitogen-activated protein kinase kinase 2	h, m							4	4

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
ABCD4	ATP-binding cassette sub-family D member 4	h, m, r							4	4
ARF1	ADP-ribosylation factor 1	h, m							4	4
GBF1	Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	h, m, r							4	4
HRSP12	Heat-responsive protein 12, isoform CRA_a	h, m, r							4	4
IDI2	Isopentenyl-diphosphate delta-isomerase 2	h, m, r							4	4
LACC1	Laccase domain-containing protein 1	h, m							4	4
TKT	Transketolase (TK)	h, r							4	4
URAD	Putative 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase	h, m							4	4
HMGCS2	Hydroxymethylglutaryl-CoA synthase	r				Isoprenoid biosynthesis[g]		5	0	4
ACAT1	Acetyl-CoA acetyltransferase	h						4	0	4
ACOT12	Acyl-coenzyme A thioesterase 12	m	S[h]					2	0	4
SCCPDH	Saccharopine dehydrogenase-like oxidoreductase	r						5	0	4
DHRS2	Dehydrogenase/reductase SDR family member 2	m		SKL[c]				3	0	4
RHOA	Transforming protein RhoA	m						3	0	4
ADH1A	Alcohol dehydrogenase 1A	h						4	0	4
HSD3B3	3 beta-hydroxysteroid dehydrogenase/Delta 5–>4-isomerase type 3	m						2	0	4
ABHD14B	Protein ABHD14B	m						2	0	4
PAICS	Multifunctional protein ADE2	m						2	0	4

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
G6PD	Glucose-6-phosphate 1-dehydrogenase	r	S[h]			Oxidation of redox equivalents[e]		5	0	4
CMBL	Carboxymethylenebutenolidase homolog	r						5	0	4
LACTB2	Endoribonuclease LACTB2	r						5	0	4
SLC9A3R1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	r						5	0	4
TMEM5	Transmembrane protein 5	r						5	0	4
MARC1	mitochondrial amidoxime reducing component 1	r						5	0	4
SAMD4A	Protein Smaug homolog 1	r						5	0	4
HK2	Hexokinase-2	r						6	0	4
HK1	Hexokinase-1	r						6	0	4
ACBD4	Acyl-CoA-binding domain-containing protein 4	r						6	0	4
GLS	Glutaminase kidney isoform, mitochondrial	r						6	0	4
CCDC18	Coiled-coil domain-containing 18	r						6	0	4
OXCT1	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	r						6	0	4
ACSS1	Acetyl-coenzyme A synthetase	r						6	0	4
VPS13D	Vacuolar protein sorting 13 homolog D	r						6	0	4
RIDA	2-iminobutanoate/2-iminopropanoate deaminase	h, m, r	S[h]						3	3
MYO5A	Unconventional myosin-Va	m, r							3	3
ACOT5	Acyl-coenzyme A thioesterase 5	m							2	2
AOC1	Amiloride-sensitive amine oxidase	h							2	2

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
CAV1	Caveolin-1	r							2	2
CCDC33	Coiled-coil domain-containing protein 33	m							2	2
CRYM	Ketimine reductase mu-crystallin	h							2	2
GRHPR	Glyoxylate reductase/hydroxypyruvate reductase	h							2	2
HRASLS	Phospholipid-metabolizing enzyme A-C1	m							2	2
MVD	Diphosphomevalonate decarboxylase	r							2	2
POMC	Pro-opiomelanocortin	h							2	2
SERHL2	Serine hydrolase-like protein 2	h							2	2
SLC22A21	Solute carrier family 22 member 21	m							2	2
CPS1	Carbamoyl-phosphate synthase	r	S[a]	KAA[a]			+		0	2
PDIA6	Protein disulfide-isomerase A6	r	S[a]	DEL[a]			+		0	2
UGT1A1	UDP-glucuronosyltransferase 1-1	r	S[a]	KTH[a]			+		0	2
RAB2A	Ras-related protein Rab-2A	r	S[a]	GCC[a]			+		0	2
VAPA	Vesicle-associated membrane protein-associated protein A	r	S[a]	FIS[a]			+		0	2
VAPB	Vesicle-associated membrane protein-associated protein B/C	r	S[a]	IAL[a]			+		0	2
ACTG1	Actin, cytoplasmic 2	r	S[a]	KCF[a]			+		0	2
FDPS	Farnesyl pyrophosphate synthase	r							1	1
MVK	Mevalonate kinase	r							1	1
ACAT2	Acetyl-CoA acetyltransferase	r							1	1

(continued)

Table 2 (continued)

				Targeting signal			Detected in			
Gene name	Protein	Species	Loc.	PTS1[i]	PTS2	Pathway/function[l]	qual. study[a]	quant. study#	DB[j]	Score[k]
–	cDNA FLJ55296, highly similar to Homo sapiens WD repeat domain 42A (WDR42A), mRNA	h							1	1
G3V3G9	Uncharacterized protein	h							1	1

To increase the information content of our mammalian peroxisomal proteome index we have added several additional sources of information for each protein. Each protein in our list therefore comes with information on known function, classification as a soluble matrix or membrane protein, known targeting signal and related pathway, for all referencing the source of information (Gronemeyer et al. 2013; Islinger et al. 2007, Islinger et al. 2010; Kikuchi et al. 2004; Mi et al. 2007; Wiese et al. 2012)

h Human; *m* Mouse; *r* Rat; *Loc.* Localization; *M* Peroxisomal membrane; *S* Soluble fraction/peroxisomal matrix; *PTS* Peroxisomal targeting signal; *qual.* Qualitative; *quant.* Quantitative; *DB* Database

[a]Kikuchi et al. (2004)

[b]Wiese et al. (2007)

[c]Wiese et al. (2012)

[d]Gronemeyer et al. (2013)

[e]Islinger et al. (2007)

[f]Jadot et al. (2017)

[g]Mi et al. (2007)

[h]Islinger et al. (2010)

[i]Reference #1 reports the last three amino acids (C-terminal); please note that this does not necessarily implicate that these amino acids function as a PTS1 signal

[j]Number indicates the frequency of entries in UniProt (http://www.uniprot.org/) and/or the subcellular localization database Compartments (knowledge channel; https://compartments.jensenlab.org) for human, mouse, and/or rat (as of July 21st 2017)

[k]4 points per identification in quantitative MS study; 2 points per identification in the non-quantitative (i.e. 'qualitative') MS study; 1 point per entry and annotation as peroxisomal protein in a database

[l]Information listed are derived from references c–g

MDH1 protein itself is being extended to contain a PTS1 signal (Hofhuis et al., 2016), LDHA is piggy backing on extended lactate dehydrogenase B (LDHB) whose PTS1 is exposed (Schueren et al. 2014). Another protein that was detected in high frequency, but is not annotated as a peroxisomal protein in the databases is glycerol-3-phosphate dehydrogenase (GPD1). It was previously shown that rat liver peroxisomes contain GPD1 activity (Antonenkov et al. 1985), although the true identity of this enzyme activity has never been definitively resolved (Wanders et al. 2015). Intriguingly, the GPD1 homolog in the yeast *S. cerevisiae* is an abundant protein with partial localization to peroxisomes (Saryi et al. 2017). Therefore, our analysis, together with the previous finding and the localization of the yeast Gpd1, may suggest that the mammalian GPD1 is also localized, at least to some extent, to peroxisomes.

Other proteins that were identified in at least two quantitative proteomics studies, but are not currently annotated as *bona fide* peroxisomal proteins (ACSL5, DHRS7B, ACOT1 and VWA8), might be dually localized to peroxisomes and to another cellular compartment. A further possibility is that the identified proteins reside in another organelle at a specific site which physically interacts with peroxisomes like contact sites (Shai et al. 2016). Due to the close proximity of a partner organelle's membrane at contact sites and the strong physical tethering, such proteins, although not localized to peroxisomes, may be identified in peroxisomal fractions.

An interesting protein that was detected in high frequency in peroxisomal fractions is PEX5-like (PEX5L) protein. PEX5L was shown to be expressed preferentially in the brain (Amery et al. 2001) and to have distinct but overlapping substrate specificity from its paralog PEX5 (Fransen et al. 2008). It was recently discovered that the PEX5L homolog in the yeast *S. cerevisiae,* Pex9, is a condition-specific targeting receptor that binds a subset of PTS1 proteins, potentially to prioritize their targeting under specific metabolic needs (Effelsberg et al. 2016; Yifrach et al. 2016). Furthermore, it was shown that the targeting specificity of Pex9 is defined by the context of the PTS1 sequence (Yifrach et al. 2016). Hence, it would be intriguing to examine whether the same PTS1 context will define the targeting specificity of PEX5L in mammalian cells.

3.2 *Proteins That Are Detected in Low Frequency in Peroxisomal Fractions*

Looking at the peroxisomal proteins that were detected in low frequency in peroxisomal fractions, we could see the reverse—proteins that are well studied and verified peroxisomal proteins, which indeed appear as such in the databases, but were not detected in any of the proteomic studies in our dataset. Why then were these proteins not identified by MS studies? One possibility could be that these proteins are localized to the periphery of peroxisomes. Hence they are not tightly

attached to the peroxisome membrane and are therefore lost during sample preparation. One such obvious example is PEX19, which is a chaperone and an import receptor for peroxisomal membrane proteins. PEX19 is mainly localized to the cytosol and is attached to peroxisomes through transient interactions with PEX16 (Smith and Aitchison 2013). Therefore, most probably during the isolation steps required to obtain peroxisomal fractions, PEX19 gets diluted out.

A further explanation why characterized peroxisomal proteins were not detected in the MS-based studies could be their low abundance or tissue specificity. For example, fatty acyl-CoA reductase 2 (FAR2), which is a peroxisomal enzyme involved in the first step of wax biogenesis, has a very low copy number in the cell and seems to have tissue-specific expression (Wang et al. 2015). This is in contrast to its paralog, fatty acyl-CoA reductase 1 (FAR1), which is a more abundant protein (Wang et al. 2015) and was hence present in the list of proteins that were detected in high frequency in peroxisomal fractions.

An additional possibility for known peroxisomal proteins to be missed by the MS studies is if only a small fraction of them is localized to peroxisomes. In these cases these dual-localized proteins would not be enriched in peroxisomal fractions, and thus will most likely not be present in the list of proteins that were detected in high frequency. Such example is the enzyme glucose-6-phosphate 1-dehydrogenase (G6PD) that is an abundant protein in the cell, but only 10% of the cellular activity of G6PD was shown to associate with peroxisomal fractions (Patel et al. 1987).

4 Conclusions

In summary, we have assembled a high-confidence list of mammalian peroxisomal proteins by compiling information from many of the currently published studies. Our analysis brings to light potential new mammalian peroxisomal proteins that can now be further verified and studied for a possible physiological role in peroxisomes. For the above mentioned reasons, our list is still not final and more peroxisomal and peroxisome-associated proteins may be added in the future. However, it should provide an important foundation for annotating the complete peroxisomal proteome. This unified list gives an organized view on the current knowledge of the mammalian peroxisomal proteome and highlights the complexity and diversity of this fascinating organelle.

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Fungal Peroxisomes Proteomics

Xin Chen and Chris Williams

Abstract Peroxisomes in fungi are involved in a huge number of different metabolic processes. In addition, non-metabolic functions have also been identified. The proteins that are present in a particular peroxisome determine its metabolic function, whether they are the matrix localized enzymes of the different metabolic pathways or the membrane proteins involved in transport of metabolites across the peroxisomal membrane. Other peroxisomal proteins play a role in organelle biogenesis and dynamics, such as fission, transport and inheritance. Hence, obtaining a complete overview of which proteins are present in peroxisomes at a given time or under a given growth condition provides invaluable insights into peroxisome biology. Bottom up approaches are ideal to follow one or a few proteins at a time but they are not able to give a global view of the content of peroxisomes. To gain such information, top down approaches are required and one that has provided valuable insights into peroxisome function is mass spectrometry based organellar proteomics. Here, we discuss the findings of several such studies in yeast and filamentous fungi and outline new insights into peroxisomal function that were gained from these studies.

Keywords Proteomics · Peroxisome · Fungi · Yeast · Mass spectrometry Protein localization

Abbreviations

APEX	Ascorbate peroxidase
DDA	Data-dependent Acquisition
DIA	Data-independent Acquisition
ESI	Electro-spray Ionization
GFP	Green fluorescent protein
GPF	Gas Phase Fractionation

X. Chen · C. Williams (✉)
Molecular Cell Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747AG Groningen, The Netherlands
e-mail: c.p.williams@rug.nl

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ICAT	Isotope-coded affinity tags
MALDI	Matrix Assisted Laser Desorption Ionization
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
μLC	Micro Liquid chromatography
nHPLC	High performance liquid chromatography
nLC	Nano Liquid chromatography
PMP	Peroxisomal membrane protein
PNS	Post nuclear supernatant
PTS	Peroxisomal targeting signal
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGD	*Saccharomyces cerevisiae* Genome Database
SILAC	Stable Isotope Labelling by Amino acids in Cell culture

1 Introduction

Peroxisomes are eukaryotic organelles that are involved in a wide range of metabolic functions. Some general peroxisomal functions are the oxidation of fatty acids and detoxification of hydrogen peroxide (Smith and Aitchison 2013). Specific functions include the synthesis of plasmalogens, cholesterol and bile acids in mammals (Van den Bosch et al. 1992) while plant peroxisomes can house enzymes involved in amongst others, the glyoxylate cycle or photorespiration (Mano and Nishimura 2005). In this chapter, we will discuss on fungal peroxisomes, focussing particularly on peroxisomes in unicellular yeasts and filamentous fungi.

A very well-known species of yeast is *Saccharomyces cerevisiae*, which is used in the bakery, winery and brewery industries. *S. cerevisiae* is also widely used as model organism to study a huge range of biochemical, genetic and cell biological processes and much of our understanding on the biogenesis and function of yeast peroxisomes comes from studies in *S. cerevisiae*. However, the study of peroxisomes in yeast is not limited to this organism and a plethora of data are available from studies conducted with other yeast species, including *Cryptococcus neoformans*, *Candida albicans*, *Candida boidinii*, *Ustilago maydis*, *Yarrowia lipolytica*, *Hansenula polymorpha* and *Pichia pastoris*. Filamentous fungi are multicellular organisms that grow in a branched (filamentous) form, termed hyphae. A number of filamentous fungi are utilized for food production, such as certain species of *Aspergillus* that are used to produce Japanese Sake while another, *Penicillium chrysogenum*, is used to produce penicillin, as well as a range of bioactive secondary metabolites.

Like peroxisomes from other organisms, fungal peroxisomes also house a wide range of metabolic pathways, allowing them to be involved in many different

cellular functions. In many yeasts, fatty acid β-oxidation takes place exclusively in peroxisomes, as opposed to in higher eukaryotes (Kunau et al. 1988; Kindl 1993). In this respect, filamentous fungi are somewhat different. *Aspergillus nidulans*, supplementary to its peroxisomal β-oxidation system, is able to perform β-oxidation of fatty acids in the mitochondria (Flavell and Woodward 1971) while the closely related *Neurospora crassa* degrades fatty acids in glyoxysomes, a specialized form of peroxisome found in plants and certain fungi (Kionka and Kunau 1985). *N. crassa* (as well as several other filamentous fungi) is particularly interesting because it contains, additional to glyoxysomes, another specialized type of peroxisome called a Woronin body. In case of hyphal injury, Woronin bodies act as a plug to stop leakage of the cytoplasm (Jedd and Chua 2000), a fascinating, non-metabolic role for peroxisomes in cell vitality. Some additional peroxisomal functions in yeasts include the oxidation of methanol (Van Dijkan et al. 1982) and the metabolism of primary amines (Zwart et al. 1983) while peroxisomes from the filamentous fungus *P. chrysogenum* contain the enzymes that produce penicillin (Muller et al. 1991, 1992), one of the most important drugs of all time.

2 Organellar Proteomics on Peroxisomes in Fungi

In fungi peroxisome function is extremely diverse and depends heavily on species as well as the growth conditions. To obtain a complete understanding of the function(s) of the peroxisome in a given cell under a given condition, a comprehensive overview of the proteins present in these peroxisomes is crucial. The peroxisomal localization of many metabolic pathways has been determined through bottom up approaches, using cell fractionation methods or microscopy approaches (immunofluorescence, immunolabelling, genetic tagging with fluorescent proteins). However, these methods require prior knowledge of the protein in question, such as protein sequence, the presence of targeting signals, putative function etc. and may therefore not be applicable when the goal is to identify novel peroxisomal pathways. Furthermore, cells sometimes house certain enzymes of a metabolic pathway in different compartments, potentially making it a challenge to say that the localization of one protein from the pathway is representative for the entire pathway.

Such situations call for the use of top down approaches and mass spectrometry (MS) based proteomics methods have proved invaluable when studying the peroxisomal proteome. Here, we summarize the findings of several MS based organellar proteomics studies in yeast and filamentous fungi, outlining the new insights into peroxisomal metabolism and function gained from these studies.

2.1 *Organellar Proteomics on Peroxisomes from* S. Cerevisiae

The earliest characterization of *S. cerevisiae* peroxisomes using proteomics was performed by Schäfer et al. (2001). Peroxisomes were isolated from oleate grown cells through the use of differential centrifugation, followed by sucrose and Accudenz density gradient centrifugation. Peroxisomes were lysed by osmotic shock and peroxisomal membrane fractions were extracted and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Following in gel digestion, peptides were extracted and analysed with three different types of mass spectrometry: matrix assisted laser desorption ionization (MALDI) MS, micro liquid chromatography electrospray ionization (μLC-ESI) MS and nano liquid chromatography ESI-MS (nLC-ESI-MS). A total of 6 known peroxisomal membrane proteins (PMPs) were identified, as well as 19 known peroxisomal matrix proteins, even though peroxisomal membrane fractions were analysed. The authors did not comment further on this aspect, but it may suggest that certain peroxisomal matrix proteins are associated with the membrane. Indeed, a previous report suggested that the matrix protein Fat2p (also known as Pcs60p), one of the proteins identified in this proteomics approach, is membrane associated (Blobel and Erdmann 1996). What this could mean in terms of peroxisome function remains unclear.

Another interesting observation in this work was the identification of a phosphorylation site at Threonine 711 in the long chain fatty acid CoA ligase 2 (Faa2p). Peptides corresponding to both the phosphorylated and unphosphorylated forms were identified, with the phosphorylated form displaying a lower signal. This could suggest that the unphosphorylated form is the major species in vivo, although this difference in signal intensity may be due to poor ionisation of phosphorylated peptides compared to unphosphorylated peptides, as has been reported before (Steen et al. 2006). In a later high throughput study, the phosphorylation status of Faat2 was confirmed (Albuquerque et al. 2008) yet the function of this post-translational modification remains unknown.

The success of proteomics approaches is influenced by protein abundance, with highly abundant proteins providing the majority of peptides present in a given sample, which may in turn potentially mask lower abundant ones. Since peroxisomal matrix proteins are likely to be much more abundant that most PMPs, approaches that regress this balance can be extremely helpful when studying the proteome of peroxisomes. In the above-mentioned study, Schäfer and colleagues analysed membrane fractions, rather than whole organelles, to aid in the identification of low abundant PMPs. A similar approach was utilised in (Yi et al. 2002). However, in order to enhance the recovery of peptides for proteomics analysis, the authors performed tryptic digestion directly on the isolated peroxisomal membrane fractions, rather than on gel pieces after electrophoresis. Their approach was further enhanced through the use of gas-phase fractionation (GPF) in combination with nLC-ESI-MS/MS. GPF relies on the separation of peptide ions in the gas-phase of

the mass spectrometer, according to their m/z value, which allows for increased peptide coverage and reproducibility (Spahr et al. 2001; Davis et al. 2001). Yi et al. identified 181 proteins, including 38 known peroxisomal proteins. At this time, 41 proteins were either identified or predicted to be peroxisomal, demonstrating that the authors has a coverage of ~90% with their analysis. Of note is the identification of Pex5p in their study. Pex5p only transiently associates with peroxisomes during the matrix protein import cycle (Kragt et al. 2005), indicating the sensitivity of their approach.

While these two publications (Schafer et al. 2001; Yi et al. 2002) clearly established that it was possible to isolate peroxisomes for proteomics based study, they also demonstrated one of the major drawbacks when it comes to such approaches, namely that of contamination. Indeed, ~45% of the proteins identified in peroxisomal membrane fractions by Schäfer et al. (2001) and ~75% of those identified in Yi et al. (2002) were not described as peroxisomal, based on experimental evidence or prediction programs, which raises the question whether these proteins are previously uncharacterised peroxisomal proteins or contaminants? In the case of proteins such as the mitochondrial membrane proteins Cyt1p and Tom40p, contamination is very probably the explanation for their presence. However, the situation is less clear for other proteins. For example, both studies identified Cat2p, a carnitine acetyl transferase, in their analysis. Cat2p displays dual localisation in the peroxisome and mitochondria (Elgersma et al. 1995), raising the question whether this protein can be considered a genuine peroxisomal protein or a contaminant when identified in proteomics approaches? Furthermore, Schäfer et al. classified the glycerol-3-phosphate dehydrogenase 1 protein (Gpd1p) as a cytosolic contaminant while later studies demonstrated that this protein does indeed localise to peroxisomes (see below) (Jung et al. 2010; Kumar et al. 2016; Marelli et al. 2004). Hence, methods that allow for the discrimination of contaminants from *bona fide* peroxisomal proteins can aid enormously in organellar proteomics approaches by narrowing down the number of potential peroxisomal proteins that require further validation. With this in mind, the chapter by Islinger et al. in this book is of interest (Islinger et al. 2018).

To tackle this, Marelli et al. utilized quantitative mass spectrometry to identify novel peroxisomal proteins in *S. cerevisiae* (Marelli et al. 2004). In this study, the authors combined isopycnic density gradient fractionation with isotope-coded affinity tags (ICAT) to discriminate between peroxisomal proteins and contaminants. ICAT is an approach that relies on the chemical labelling of proteins from two different fractions with chemically identical but isotopically different tags (Gygi et al. 1999). The two fractions are then mixed and the relative abundance of isotopically labelled peptides can be determined using MS analysis. The relative ratio between peptides in the two fractions will give information on whether these peptides, and hence the proteins from which they are derived, are enriched in one fraction compared to the other. Marelli et al. took two approaches; in the first (ICAT I), membrane fractions isolated from peroxisomes and mitochondria derived from oleate grown cells were differentially treated with ICAT reagents and subjected to μLC-ESI-MS/MS analysis (Fig. 1). In the second approach (ICAT II),

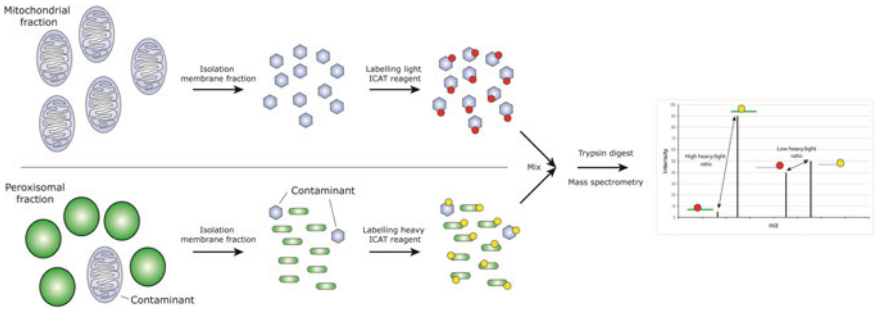

Fig. 1 Schematic depiction of the use of isotope-coded affinity tag (ICAT) reagents to identify mitochondrial contaminants in peroxisomal fractions, as described in (Marelli et al. 2004). Peroxisomes (depicted in green) and mitochondria (in blue) were individually isolated and a membrane fraction was prepared. Proteins isolated from the mitochondrial fraction (blue hexagons) were treated with light ICAT reagent (red), while those isolated from the peroxisomal fraction (green bars) were treated with heavy ICAT reagent (yellow). Next the treated fractions were mixed and subjected to trypsin digest and mass spectrometry and the ratio between heavy and light versions of peptides were calculated. A high heavy to light ratio indicates that the peptide is much more abundant in the peroxisomal fraction compared to the mitochondrial fraction, identifying this peptide as likely originating from a *bona fide* peroxisomal protein. A low heavy to light ratio indicates that the peptide is not enriched in the peroxisomal fraction, identifying it as likely contaminant

a peroxisomal membrane fraction was isolated from oleate grown cells that produced a Protein-A tagged version of the PMP Pex11p. This fraction was split into two and one fraction was subjected to affinity purification using IgG beads. Both this affinity-purified fraction, together with the untreated membrane fraction were differentially treated with ICAT reagents and subjected to μLC-ESI-MS/MS. ICAT I identified a total of 346 proteins, of which 23 were known peroxisomal components according to the *Saccharomyces* genome database (SGD) while 134 were described as mitochondrial. However, comparison of the relative peptide ratios suggested that 57 of the 346 were in fact peroxisomal proteins. Of these 57, 18 were previously described as peroxisomal and none as mitochondrial, demonstrating that the ICAT I approach can be effectively used to discriminate between genuine peroxisomal proteins and mitochondrial contaminants. ICAT II identified 365 proteins but when the peptide ratios were taken into consideration, 98 proteins were suggested to be peroxisomal, with 28 annotated as peroxisomal in the SGD. These data indicate that ICAT II was able to identify more proteins than ICAT I, likely because the mixture was less complex due to the affinity purification step. However, the authors state that ICAT I would not help in identifying proteins that target to both peroxisomes and mitochondria because such proteins would be considered mitochondrial contaminants in this approach. ICAT II on the other hand would be able to identify such dually localised proteins but was less efficient at identifying mitochondrial contaminants.

The authors integrated the ICAT I data into the list of 98 proteins suggested to be peroxisomal based on the ICAT II approach and split the list of candidate proteins into three groups. Group 1 contained 25 proteins with high peroxisomal abundance ratios based on ICAT I. Group 2 consisted of 27 proteins with high peroxisomal abundance ratios in ICAT I but low ratios in ICAT II. The authors concluded that these were in fact mitochondrial contaminants. Group 3 contained 46 proteins that were predicted to be peroxisomal in ICAT II but were not identified in ICAT I. To validate their findings the authors tagged three proteins from Group 1 (Ybr159w, Rho1p and Faa1p) and five from Group 3 (Erg6p, Emp24p, Gdp1p, Erg1p and Spf1p) with Protein-A and determined their sub-cellular localisation using isopycnic density gradient fractionation. These eight candidates were chosen because they are known to localise to different cellular compartments, including the cytosol (Gdp1p), the ER (Spf1p, Ybr159w and Emp24p), lipid bodies (Faa1p, Erg1p and Erg6p) and plasma/endo-membranes (Rho1p). The fractionation data clearly demonstrated that all eight proteins targeted partially to peroxisomes while additional fluorescence microscopy analysis of green fluorescent protein (GFP) fusions of Rho1p, Gdp1p and Emp24p further confirmed that these proteins can partially localise to peroxisomes. The localisation of Erg1p-GFP was unclear but appeared to be close to peroxisomes.

Localisation is one thing, but the question remained as to what the function of these proteins in or at peroxisomes could be. To address this aspect, the authors chose to study the role of Rho1p, a small, ras-related GTPase, in peroxisome biology. Rho1p functions in signal transduction and has been shown to regulate actin reorganisation (Fujiwara et al. 1998; Yamochi et al. 1994; Nonaka et al. 1995). The authors demonstrated that Rho1p targets to peroxisomes in cells grown on oleate and not on glucose, which led them to suggest that the reason Rho1p was not previously localised to peroxisomes was because most studies of Rho1p were performed with glucose grown cells. Peroxisomes were smaller and lower in number in cells containing a temperature sensitive mutant form of *rho1* while Rho1p interacts with the peroxisome biogenesis factor Pex25p and requires Pex25p for its peroxisomal localisation, suggesting a link between Rho1p and peroxisome fission/biogenesis. Finally, the authors demonstrated that actin disassembly at peroxisomes is controlled by Rho1p and Pex25p, leading to the suggestion that Rho1p plays a role in peroxisome fission by dissembling actin at fission sites in order to allow Pex11p and other proteins involved in peroxisomal fission to finalise the fission event. Taken together, this report elegantly demonstrates that quantitative proteomics can be used to identify previously unknown peroxisomal proteins in order to shed new light onto peroxisome biogenesis.

It is worthy to note here that isotope labelling of proteins can also be performed metabolically, using a method called Stable Isotope Labelling by Amino acids in Cell culture (SILAC). Rather than using a chemical approach to modify proteins or peptides after isolation, as ICAT does, SILAC relies on the cells themselves to incorporate the isotopically labelled amino acids Lysine and Arginine residues into proteins. Cells are grown in the presence of “heavy” or “light” versions of these amino acids, samples are mixed and subjected to MS and the relative ratios of the

heavy and light forms of the peptides can be used to identify contaminants. Although this method has not been used in organellar proteomics on fungi, it has been successfully used when investigating the interaction partners of yeast peroxisomal proteins (Oeljeklaus et al. 2012; Piechura et al. 2012; David et al. 2013).

Because peroxisomes are metabolic organelles, the protein content of peroxisomes depends very much on the metabolic needs of the cell. Peroxisomes contain an import system that can react to the metabolic needs of the cell (Effelsberg et al. 2016; Yifrach et al. 2016), which means that peroxisomal protein content is dynamic and condition specific. Measuring the change in subcellular localisation with bottom up approaches such as live cell imaging can provide invaluable information on the dynamic properties of a given protein. However, such properties are challenging to measure in top down approaches that seek to characterise global changes in protein localisation. One reason for this is the difficulty that is encountered when comparing samples of different origins, such as peroxisomes isolated from cells grown on glucose compared to peroxisomes isolated from cells grown on oleate. Classical MS approaches often rely on the data-dependent acquisition (DDA) method, a semi-random process that effectively selects ionized peptides with high signal to noise ratios for further analysis. Because of this, ions of low signal to noise ratio may be "ignored" by the detector. Hence, a given peptide ion may have a low signal to noise ratio in one sample, meaning that it is underrepresented, while the same peptide ion may have a high signal to noise ratio in another sample, meaning that it is overrepresented. Because of this bias in sampling, valuable data may become lost. To address this issue, Jung et al. (2010) employed a data independent acquisition (DIA) approach to investigate global differences in protein distribution in cells grown on glucose compared to those grown on oleate. Although they did not focus on peroxisomes in this report, their data did elegantly demonstrate that protein redistribution can occur on many different levels. Enzymes involved in metabolic processes associated with fatty acid processing underwent strong upregulation and redistribution to organelles in response to exposure to oleate whereas proteins involved in peroxisomal organisation (such as the docking factors Pex3p and Pex14p) also redistributed to organelles in response to oleate treatment yet they were not strongly upregulated. While this may not seem surprising at first glance, it does provide a very interesting insight into the behaviour of these different classes of proteins on a global scale and it also provides a benchmark that can be utilised to assess the role of proteins with unknown functions.

2.2 *The Proteome of Peroxisomes in* N. Crassa

The filamentous fungus *N. crassa* possesses two types of peroxisomes; glyoxysomes and Woronin bodies. Glyoxysomes in *N. crassa*, like peroxisomes in many organisms, house enzymes required for β-oxidation. However, they also contain enzymes of the glyoxylate cycle, a metabolic pathway that allows for the

conversion of acetyl-CoA to succinate, which is then used further for carbohydrate production (Flavell and Woodward 1971). The β-oxidation pathway in glyoxysomes is somewhat different from that in other yeasts. Rather than relying on an acyl-CoA oxidase to perform the dehydration of the fatty acyl-CoA species, the first step in the β-oxidation pathway, glyoxysomes instead use acyl-CoA dehydrogenase to perform this function. This alternative mechanism does not generate hydrogen peroxide and subsequently, glyoxysomes from *N. crassa* do not contain catalase, the major detoxifier of hydrogen peroxide in peroxisomes (Schliebs et al. 2006). Woronin bodies, on the other hand, perform a non-metabolic function. They stop the loss of cytoplasm upon hyphal injury by acting as a plug (Jedd and Chua 2000). Interestingly, Woronin bodies seem to form from glyoxysomes. First the protein Hexagonal 1 (HEX1) is imported to glyoxysomes through its Peroxisomal targeting signal type 1 (PTS1), after which it forms a large, hexagonal crystal. The Woronin body, complete with HEX1 crystal, then buds off from the glyoxysome through fission (Managadze et al. 2007; Liu et al. 2008).

In order to gain a better understanding of the protein content of these two specialized forms of peroxisomes, Managadze et al. performed organellar proteomics upon isolated glyoxysomes and Woronin bodies from *N. crassa* (Managadze et al. 2010). Woronin bodies were purified from a post nuclear supernatant (PNS) isolated from sucrose grown cells through the use of a linear sucrose gradient. Samples were subjected to SDS-PAGE and fractions that contained the most amount of the Woronin body marker protein HEX1 and the least amount of glyoxysomal and mitochondrial contaminants were pooled and subjected to SDS-PAGE and coomassie staining. The authors observed 15 protein bands on the gel after these steps and these 15 bands were excised, subjected to in gel digestion and peptides were analysed by nano High-Performance Liquid Chromatography (nHPLC) ESI-MS/MS. As could be expected, the major component identified was HEX1. The authors did identify a number of additional proteins but since these corresponded to ribosomal and mitochondrial proteins, they concluded that these likely represented contaminants. However, this approach did identify NCU00627, a protein of unknown function with homologues in other filamentous fungi. Whether this is a *bona fide* Woronin protein and if so, what its role might be, are questions that remain to be answered.

The authors had more luck with their organellar proteomics approach on glyoxysomes. Glyoxysomes were isolated from a PNS derived from oleate grown cells and subjected to "density barrier centrifugation". In this approach, the organellar pellet was mixed with iodixanol to a final concentration of 23.5% and this mix was layered onto a denser solution of iodixanol (35%). After centrifugation, glyoxysomes concentrate at the interface between the two densities; the "barrier". Glyoxysomes were then disrupted with SDS and heating and the resulting protein fraction was subjected to reverse phase chromatography, SDS-PAGE analysis and coomassie staining. Visible protein bands were excised and subjected to in gel digestion and the peptides were analysed by nHPLC-ESI-MS/MS. This approach led to the identification of 191 proteins. Amongst this list, the authors noted that 16 proteins contained a putative PTS1 sequence while 3 contained a putative PTS2

sequence and although the rest lacked a recognisable targeting sequence, they noted a number of proteins that were functionally linked to glyoxysomes, such as isocitrate lyase (ICL), which was shown to be peroxisomal in *Aspergillus nidulans* (Valenciano et al. 1998).

The authors validated their results through the use of fluorescence microscopy, showing that three candidates from the list of PTS1 proteins indeed targeted to peroxisomes. The three proteins they chose were NCU02287, NCU08924 and NCU04803. The first two are putative acyl-CoA dehydrogenases (which the authors named ACD1 and ACD2) and confirmation of their glyoxysomal localisation was significant because up until this point, the identity of the acyl-CoA dehydrogenase required for β-oxidation in glyoxysomes was unknown. Interestingly, the authors also identified a fumarate reductase homologue in the list of the 191 putative glyosysomal proteins. As mentioned, the first step of β-oxidation in glyoxysomes is a dehydration reaction, performed by acyl-CoA dehydrogenase. The authors speculate that this fumarate reductase enzyme could be involved in the re-oxidation of the co-factor that is required by acyl-CoA dehydrogenase.

The third candidate which the authors tagged with GFP for localisation studies was a 2-nitropropane dioxygenase (now referred to as nitronate monooxygenase), which they named NPD1. Another protein (NCU09931), which possessed similar domain structure as well as a PTS1, was identified in the proteomics screen. The authors termed this candidate NPD2 and propose that these enzymes are involved in the detoxification of nitroalkanes, which may play a role in protecting *N. crassa* against nitroalkanes excreted by other organisms (Hipkin et al. 1999; Alston et al. 1977). Taken together, these data identified a novel enzymatic activity housed within peroxisomes, expanding the role of peroxisomes in cell metabolism.

2.3 *Identification of Peroxisomal Matrix Proteins in* P. Chrysogenum

Peroxisomes in the filamentous fungus *P. chrysogenum* are important to the medical and industrial sectors because they house the enzymes that produce penicillin (Muller et al. 1991, 1992). With this in mind, knowledge on the protein content of peroxisomes in this organism can help in understanding how penicillin and other secondary metabolites are produced. This led Kiel and colleagues to investigate the proteome of peroxisomes in *P. chrysogenum* (Kiel et al. 2009). Peroxisomes were isolated with a sucrose density gradient from a PNS, lysed by osmotic shock and the matrix protein fraction was analysed by SDS-PAGE, coomassie staining and in gel digestion. The subsequent peptide mix was subjected to nHPLC-MS/MS, leading to the identification of more than 500 proteins. A significant portion of these (119) were involved in translation, which could represent a large amount of ribosomal contamination. However, the authors demonstrated with electron microscopy analysis that ribosomes sometimes

associated with isolated peroxisomes. The authors therefore suggested that rather than representing contamination, these may be ribosomes translating peroxisomal proteins at the peroxisomal membrane. Since this study, Zipor et al. demonstrated that mRNA translation of peroxisomal proteins can indeed occur in close proximity to peroxisomes in *S. cerevisiae* (Zipor et al. 2009), which could validate the authors theory.

The remaining proteins were manually annotated and classified based on their potential localisation within the cell and the authors listed a total of 89 putative peroxisomal proteins. Many proteins contained a PTS1 (69) while 10 more contained a putative PTS2, strongly suggesting that they indeed target to peroxisomes. A further 10 proteins that lacked a PTS were deemed likely to be peroxisomal, either because they were particularly abundant in the preparation, because of function (e.g. several were involved in β-oxidation, which is often a peroxisomal process) or because of previous data on their localisation, such as ICL (see above).

Of the 89 putative peroxisomal proteins, many were enzymes that take part in metabolic pathways, such as penicillin production, fatty acid β-oxidation, the glyoxylate cycle and nitrogen metabolism. Furthermore, a number of enzymes involved in the detoxification of ROS were identified, as were several thioesterases. Finally, the peroxisomal role of around 35 of the 89 enzymes identified in the approach was not clear from their putative function, which was defined by their homology to other enzymes. It is worthy to note that the authors identified one acyl-CoA oxidase and four acyl-CoA dehydrogenases in their proteomic approach. As mentioned, fatty acid β-oxidation generates hydrogen peroxide when acyl-CoA oxidase catalyses the first step in the cascade whereas this is not the case when the first step is catalysed by acyl-CoA dehydrogenase. This high number of acyl-CoA dehydrogenases could suggest that fatty acid β-oxidation occurs via a dehydration step in *P. chrysogenum* peroxisomes. In support of this, the author also identified a Fumarate reductase homologue (Pc12g0390) in their proteomic approach and confirmed its localisation using GFP tagging and fluorescence microscopy. This enzyme may play a role in the re-oxidation of co-factors required by acyl-CoA dehydrogenase for fatty acid β-oxidation, as was suggested for the *N. crassa* orthologue (see above). However, the authors also identified several catalase like enzymes in their proteomics approach, indicating that *P. chrysogenum* peroxisomes are a likely site of hydrogen peroxide production. Furthermore, later studies demonstrated that additional acyl-CoA oxidases target to peroxisomes and that these enzymes are involved in fatty acid β-oxidation (Veiga et al. 2012). Clearly further studies are required to investigate the intricate nature of peroxisomal fatty acid β-oxidation in *P. chrysogenum*.

The wide range of functions displayed by the 89 putative peroxisomal proteins led the authors to conclude that peroxisomes in *P. chrysogenum* are not simply penicillin production factories but are instead multi-purpose organelles that house many different metabolic pathways. Nevertheless, since the peroxisomes used in this study were isolated from cells growing on media that stimulates the production of penicillin, these data laid down a solid basis for further study into the role of peroxisomes in the production of penicillin.

3 Perspectives

Advances in the sensitivity and speed of mass spectrometers, the development of methods to identify contaminants, as well as in statistical methods to analyse the huge amount of data generated by these approaches have allowed organellar proteomics to make invaluable contributions to peroxisomal research. However, a number of PMPs are relatively low in abundance (Reguenga et al. 2001), making their detection using MS still tricky, while it remains a challenge to investigate proteins displaying a dual localisation using MS because the issue of contamination still arises (Schäfer et al. 2001; Yi et al. 2002). Finally, the preparation of peroxisomal fractions for MS analysis remains a long and often challenging process (as discussed in Saleem et al. 2006 and Islinger et al. 2018). Because the success of an organellar proteomics approach depends heavily on the quality and purity of the samples being analysed, we will end by discussing two recent developments that may allow isolation procedures for future MS based studies to be simplified.

Recently, Peikert et al. reported a method they termed ImportOmics, which relies on RNA inhibition (RNAi) of the docking factor Pex14p in the parasite *T. brucei* (Peikert et al. 2017). The inhibition of Pex14p production blocked the import of matrix proteins into the glycosome, a specialized type of peroxisome involved in the breakdown of glucose in this organism (Bauer and Morris 2017). The authors elegantly demonstrated that matrix proteins became mistargeted in cells where Pex14p was targeted with RNAi, allowing them to directly compare the levels of certain proteins in the organellar pellet fraction of untreated cells versus an organelle pellet isolated from cells where Pex14p was targeted. This was possible using a simple differential centrifugation approach, negating the requirement for density centrifugation and greatly shortening and simplifying the isolation procedure. Although RNAi has not been used extensively in yeast or filamentous fungi, alternative approaches such as the Degron based system have been used successfully to down-regulate peroxisomal proteins (Nuttall et al. 2014; Motley et al. 2015; Knoops et al. 2015), which would allow such experiments to be performed in these organisms. Furthermore, the authors noticed that blocking the import of proteins into mitochondria through the same RNAi based approach not only resulted in mitochondrial protein mistargeting to the cytosol but also to their proteasomal based degradation, allowing the authors to gain information on whether a given protein targets to the mitochondria or not based on their absolute levels in total cell lysates. Therefore the authors could identify proteins that target to mitochondria using a single step isolation procedure. While this may not be applicable for peroxisomal matrix proteins, because proteasomal degradation of mistargeted matrix proteins has not, to the best of our knowledge, been reported for yeast matrix proteins, this would certainly be an interesting possibility when PMPs are being studied. Several PMPs are degraded when mistargeted (Knoops et al. 2014) meaning that in principle downregulating Pex19p, the receptor protein for PMPs (Rucktaschel et al. 2009; Neufeld et al. 2009), would result in decreased levels of

proteins that require Pex19p for targeting to peroxisomes. This could allow for the identification of novel PMPs using a single step isolation procedure.

The second development we mention concerns the use of proximity labelling, a chemical biology based approach that utilises a labelling enzyme to modify proteins with an affinity tag in vivo (Kim and Roux 2016). This affinity tag can then be employed to fish out modified proteins for further analysis. Targeting the labelling enzyme to a particular compartment (through the use of a targeting signal or by fusing it to an abundant protein present in that compartment) results in the specific modification of proteins in that compartment (Fig. 2). A commonly used labelling enzyme is an engineered version of ascorbate peroxidase (APEX) from plants (Martell et al. 2012) and this enzyme was successfully employed by Rhee and co-workers to identify novel mitochondrial proteins in mammalian cells (Rhee et al. 2013). Another recent report demonstrated that a similar system can be used in yeast (Hwang and Espenshade 2016). APEX oxidizes biotin-phenol in the presence of hydrogen peroxide, which generates short-lived biotin-derivative radicals that can covalently react with tyrosine residues in proteins in the near vicinity. Both biotin-phenol and hydrogen peroxide are added externally, meaning that the amount as well as the time at which protein labelling occurs can be regulated. Because the isolation of organelles is not required, proteins (or peptides resulting from tryptic digestion) modified with biotin can be isolated directly from cell lysates with streptavidin beads and analysed by MS, speeding up and simplifying extraction procedures. Furthermore, since streptavidin can bind to biotin under denaturing conditions, the isolation of biotinylated proteins can be performed under denaturing conditions, which dramatically reduces the loss of material due to the action of cellular proteases. Finally, such approaches have the potential to identify transient residents of an organelle, which is still highly challenging with alternative approaches (Jung et al. 2010). Needless to say, we eagerly await the first report on the use of proximity labelling in the study of the proteome of fungal peroxisomes.

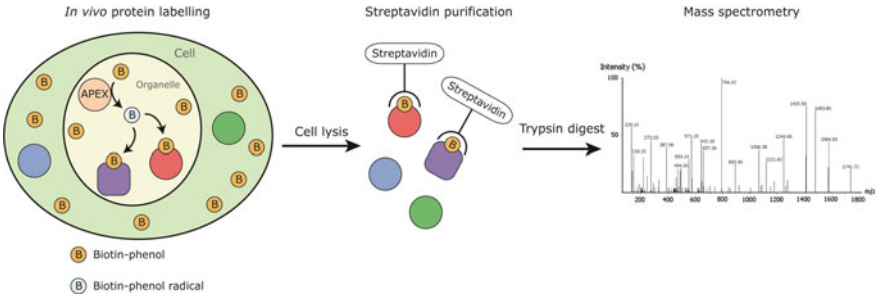

Fig. 2 Schematic depiction of *in vivo* proximity labelling of proteins using an engineered form ascorbate peroxidase (APEX). Targeting of APEX to an organelle will result in the modification of proteins present in the organelle. APEX converts biotin-phenol substrates into highly reactive biotin-phenol radicals that become covalently attached to neighbouring proteins on tyrosine residues. Following cell lysis, modified proteins can be extracted using streptavidin beads (if required, under denaturing conditions), the samples can be subjected to trypsin digest and the resulting peptides can be analysed with mass spectrometry

To conclude, the use of organellar proteomics to study fungal peroxisomes has provided valuable insights into the role of peroxisomes in the cell. The new developments listed above, together with others not mentioned here, will help to make isolation procedures both quicker and easier, allowing organellar proteomics approaches to continue to make important contributions to the study of peroxisome function in the future.

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The Craft of Peroxisome Purification—A Technical Survey Through the Decades

Markus Islinger, Andreas Manner and Alfred Völkl

Abstract Purification technologies are one of the working horses in organelle proteomics studies as they guarantee the separation of organelle-specific proteins from the background contamination by other subcellular compartments. The development of methods for the separation of organelles was a major prerequisite for the initial detection and characterization of peroxisome as a discrete entity of the cell. Since then, isolated peroxisomes fractions have been used in numerous studies in order to characterize organelle-specific enzyme functions, to allocate the peroxisome-specific proteome or to unravel the organellar membrane composition. This review will give an overview of the fractionation methods used for the isolation of peroxisomes from animals, plants and fungi. In addition to "classic" centrifugation-based isolation methods, relying on the different densities of individual organelles, the review will also summarize work on alternative technologies like free-flow-electrophoresis or flow field fractionation which are based on distinct physicochemical parameters. A final chapter will further describe how different separation methods and quantitative mass spectrometry have been used in proteomics studies to assign the proteome of PO.

Keywords Centrifugation · Gradient media · Peroxisomes · Free flow electrophoresis · Flow field-flow fractionation

Abbreviations

Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
MOPS	4-Morpholinepropane sulfonic acid
Tricine	N-[Tris(hydroxymethyl)methyl]glycine

M. Islinger (✉) · A. Manner
Institute for Neuroanatomy, Centre for Biomedicine and Medical Technology Mannheim, Medical Faculty Mannheim, University of Heidelberg, Heidelberg, Germany
e-mail: markus.islinger@medma.uni-heidelberg.de

A. Völkl
Department of Medical Cell Biology, Institute of Anatomy, University of Heidelberg, Heidelberg, Germany

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EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethanesulfonyl fluoride,
DTT	1,4-Dithiothreitol
LM fraction	Light mitochondrial fraction
HM fraction	Heavy mitochondrial fraction
PTS	Peroxisome targeting sequence
FFE	Free flow electrophoresis
FlFFF	Flow field-flow fractionation
DIGE	Difference Gel Electrophoresis

1 Introduction

Organelle proteomics studies allow allocating individual proteins to specific subcellular compartments thus placing them into the functional context of a defined subcellular compartment. In this regard, novel proteins have been assigned to peroxisomes (PO) during the last decade using proteomics technologies, thus expanding our view on the organelle's function and biology (Islinger et al. 2012). However, the significance of organelle proteomics experiments is closely correlated to the sample purity, which is crucial to discriminate correctly allocated proteins from contaminants. The elimination of contaminants is of fundamental significance since there is growing evidence that a substantial number of proteins are located to more than one subcellular compartment (Thul et al. 2017). Thus, sophisticated purification technologies are a prerequisite for any organelle proteomics experiment and build the fundament for the acquisition of meaningful data.

The biochemical and functional characterization of cell organelles distinguishing the beginning of modern cell biology in the middle of the past century is hardly conceivable without cell fractionation. Citrate cycle, β-oxidation of fatty acids and oxidative phosphorylation might have been not located to mitochondria without this technical approach and hence, mitochondria not been recognized as powerhouses of the cell. It was A. Claude in his pioneering studies (Claude 1946a, b) inquiring in which manner enzymes or other biochemical components were distributed between all the fractions from a homogenate, who constituted "quantitative" centrifugal fractionation. Hogeboom and Schneider (Hogeboom et al. 1948; Schneider 1948) championed this idea "What Is In" this or that subcellular entity, thereby setting in collaboration with Claude the still valid standards of tissue fractionation.

With the extension of Claude's four-fraction to a five-fraction scheme, and the observation that subcellular entities could be characterized by exclusively localized marker enzymes, De Duve and collaborators complemented Claude's concept of quantitative cell fractionation (De Duve and Baudhuin 1966; De Duve et al. 1955). Beyond that, and in context with the various technical achievements and accessories becoming available at that time,—Spinco ultracentrifuges and SW-39

swinging bucket rotor, gradient media like Ficoll etc.—they definitely established quantitative cell fractionation as the third pillar of modern cell biology.

Even after more than 50 years of PO research the fractionation methods developed in these ground-breaking studies still apply for the purification of this organelle. Nevertheless, centrifugation techniques and gradient media have been continuously improved to guarantee the isolation of preferably pure PO fractions from a variety of organisms and tissues. In this review we will give an overview of the different methods developed for the isolation of PO from animals, plants and fungi. Most purification schemes rely on the combination of a series of differential centrifugation steps followed by a final density gradient centrifugation, using a variety of gradient media, namely sucrose, Metrizamide, Percoll, Nycodenz and Optiprep. Thus, the first chapters of this review will give an overview on the various centrifugation-based methods used for the isolation of PO from different sources. Sequentially, novel separation techniques have been developed through the decades, which offer the opportunity to use alternate separation principles such as organellar surface charge or epitope affinity for PO purification. The combination of separation techniques, which are based on different physicochemical separation parameters, finally allows designing multidimensional separation schemes, which can further increase the resolution of the purification. Thus, the latter part of this manuscript will describe how several alternative techniques used for PO isolation were combined with centrifugation methods to increase the purity of the samples for subsequent proteome analysis.

2 Isolation of PO from Liver and Kidney

2.1 Liver Peroxisomes

The story on the isolation of PO, the very last of the classic cell organelles identified, impressively reflects the inventions and the progress in tissue fractionation at that time, both on the technical and conceptional level. Described for the first time by Rhodin in his doctoral thesis (Rhodin 1954), and termed "microbodies", PO were identified shortly after by Bernhard and Rouiller (1956) as regular components of hepatocytes, yet not recognized as cell organelles *sui generis*, but rather considered to be precursors of mitochondria. Subsequent quantitative centrifugal fractionation of mouse and rat liver, analyzing the fractions for urate oxidase, catalase and D-amino acid oxidase (De Duve et al. 1955; Paigen 1954; Thomson and Klipfel 1957; Baudhuin et al. 1964; Tsukada 1974), not only revealed the consistent location of these enzymes in the very same subcellular particle clearly differing from mitochondria as well as lysosomes (De Duve 1960; Beaufay et al. 1964) but also provided the unequivocal proof that microbodies are identical with these particles (Baudhuin et al. 1965a). The recognition of L-α-hydroxyacid oxidase as another enzyme that is exclusively located in microbodies (Baudhuin et al.

1965b) fully confirmed these results. After all, Leighton et al. were the first succeeding to purify the new subcellular compartment (Leighton et al. 1968), which had been renamed "peroxisome" in the meantime accommodating the concept of an organelle concerned with hydrogen peroxide formation by oxidases and its consumption by catalase (De Duve and Baudhuin 1966). In their ground-breaking experiments the authors used female rats, which were chosen because of the lower content of their liver in soluble catalase (Adams and Burgess 1957). The animals were injected intraperitoneally with Triton WR-1339 reportedly causing a considerable and selective decrease in the equilibrium density of lysosomes in a sucrose gradient (Wattiaux et al. 1963), thereby facilitating their separation from PO. The animals were fasted overnight, decapitated and the liver homogenized by means of a motor-driven pestle in 0.25 M sucrose containing 0.1% ethanol to prevent the formation of the inactive catalase compound II (Chance 1950). Differential centrifugation of the homogenate according to De Duve's five-fraction scheme enriched PO in the so-called λ-fraction corresponding to the L-fraction of De Duve. Subfractionation was performed by isopycnic centrifugation on a linear sucrose/dextran density gradient, employing the newly designed automatic Beaufay rotor, specially adapted to isopycnic zonal centrifugation (Beaufay 1966). This special rotor differed from the common zonal rotors in several aspects: (i) it had an annular separation chamber near the outer wall, thus the separation path was extremely short and only low hydrostatic pressure developed; (ii) the time to achieve a result was only about one-fourth of that required with the SW-39 swinging-bucker rotor running at the same speed; (iii) the fractions were delivered automatically into a fraction collector; (iv) the rotor was suitable for large-scale preparations.

The morphological examination of the preparations collected by differential and density gradient centrifugation (Leighton et al. 1968), their biochemical analysis for enzyme activities, the kind of calculation of rates of recovery, purity and mutual contamination of the particulate fractions, as well as the presentation of the results in histogram form, have unequivocally set the benchmark for appropriate studies in the following decades. In consequence, in the succeeding research on PO, the conditions provided by this publication were widely adapted to isolate PO preferentially from liver using the light mitochondrial (λ-) fraction for subsequent density gradient centrifugation.

Three aims have been mainly focused on in these studies: (i) to complement the family of peroxisomal enzymes and proteins; (ii) to unveil their activities and metabolic role; (iii) to get an idea of the putative biological significance and clinical impact of PO. A few observations, yet of extensive relevance, had an remarkable impetus on these studies: (i) the number of hepatic POs had been shown to considerably increase by the pretreatment of the animals with some distinct drugs (Hess et al. 1965; Svoboda and Azarnoff 1966); (ii) rat liver PO were shown to house a β-oxidation system, which could be induced by the application of the hypolipidemic drug clofibrate (Lazarow and De Duve 1976); (iii) hepatic and renal proximal tubule cells in children suffering from the Zellweger syndrome were shown to lack PO (Goldfischer et al. 1973).

Tackling the issues just outlined, researchers took advantage of new gradient media and a refined rotor technology trying to overcome in their isolation experiments the overt drawbacks of the Leighton method in their isolation experiments: (i) the pretreatment of the animals with Triton WR-1339, which is obligatory when sucrose is used as gradient medium; (ii) the high viscosity and hyperosmolality of sucrose impeding experiments on intact PO; (iii) the unavailability of a Beaufay rotor. Metrizamide and Nycodenz, iodinated deoxy-D-glucose derivatives, have been the first of the new media used to replace sucrose in density gradients, generating higher densities at lower viscosity and osmolality. Isopycnic centrifugation of a rat liver light mitochondrial fraction in a linear Metrizamide gradient was shown by Wattiaux et al. (1978) to allow a good separation of PO from lysosomes and the other constituents, and to purify lysosomes extensively and with satisfactory yield. Since the rats had not been injected with Triton WR-1339, the quite distinct buoyant densities of POs compared to the other organelles were supposed to be related to a pore-forming protein in the membrane of PO but not of lysosomes or mitochondria, allowing the free diffusion of molecules up to 3000 Da. This could explain the permeability of PO to Metrizamide and other gradient media. Relying on the observations of Wattiaux et al., Hajra and collaborators consequently used linear Metrizamide gradients to isolate POs from livers of rat, guinea pig and mouse as well as of rat kidney (Hajra and Bishop 1982; Hajra and Wu 1985), however, coming up with a few modifications: (i) EDTA was included in the Tris-HCl/0.25 M sucrose homogenization buffer to complex Ca^{2+}-ions, and more important, (ii) a vertical-tube rotor was employed to fractionate the light mitochondrial fractions. It should be noted in this context, that this type of rotor apparently meets best the integrity and considerable fragility of PO. Since the entire content of the tube, i.e. gradient plus top-loaded sample are slowly tilted at the beginning of the run from an upward-down to an equatorial position, particles have to travel only a short distance. Moreover, the hydrostatic pressure during centrifugation is also much lower than exerted e.g. in a swing-out rotor, and last but not least, the running-time can be drastically reduced (Neat and Osmundsen 1979). According to the distribution profiles of different marker enzymes and also to the morphological examinations reported by Hajra and Wu (1985), and presented earlier by Hajra and Bishop (1982), PO isolated by the Metrizamide gradient technique from the tissues mentioned above proved to be free from lysosomes, mitochondria or microsomes. More important, dihydroxyacetone-phosphate acyltransferase, a key-enzyme of the acyl-dihydroxcyacetone phosphate pathway, could be definitely and exclusively localized to PO, pointing to a strong association of PO with ether lipid biosynthesis. A rapid preparative method to isolate PO from the untreated rat liver was published by Ghosh and Hajra (1986) following the Metrizamide report. Replacing the vertical rotor by an angular head type, using Nycodenz, a similar but less expensive density gradient material, in a simple one-step barrier PO of high purity and yield were obtained at less time and less material needed.

Continuous Metrizamide density gradients, yet spun in the swinging-bucket rotor SW65 at 151,000×g for 90 min, were used by Bronfman et al. (1979) to clarify the role of PO in human lipid metabolism, and to evaluate their contribution

to the hypolipidemic action of some drugs. To this end, homogenized liver biopsies from patients with normal liver function were fractionated, and the fractions collected assayed for catalase as well as lysosomal, microsomal and mitochondrial marker enzyme activities. Moreover, the distribution of the peroxisomal fatty acid oxidizing system was monitored. The findings presented, established the presence of the oxidase in human hepatic PO confirming that the organelle is involved in the metabolism of fatty acids in humans.

To precisely localize palmitoyl-CoA oxidase (ACOX1) in the mucosa of the guinea pig small intestine, a post-nuclear supernatant prepared by centrifugation of the homogenate at 500×g for 10 min, was loaded onto a linear Metrizamide gradient and centrifuged in a M.S.E. swinging bucket rotor 180 min at $1.3 \times 10^5 \times g_{max}$. The distribution of the oxidase across the gradient was similar to that of catalase and different to that of markers for the other subcellular organelles (Small et al. 1980) implying that the particles histochemically identified as micro-peroxisomes are identical with PO.

PO of the mouse liver were the first isolated in the own lab by means of a vertical rotor and a linear Metrizamide gradient (Waechter et al. 1983). The biochemical analysis of the gradient fractions revealed a clear-cut co-localization of epoxide hydrolase with peroxisomal marker enzymes. Interestingly, besides the PO-bound hydrolase a soluble isoform was detected in the final supernatant collected by differential centrifugation of the corresponding liver homogenate, indicating a bi-modal distribution of the enzyme. It should be pointed out in this context, that a quite similar distribution pattern was also observed for catalase in guinea pig liver (Bulitta et al. 1996) as well as lactate dehydrogenase and superoxide dismutase in rat liver (Baumgart et al. 1996; Islinger et al. 2009). Being aware of the remarkable fragility of PO and the leaking out of soluble peroxisomal enzymes like catalase during centrifugation, the conditions established for the isolation of mouse hepatic PO (Waechter et al. 1983) were refined, to purify PO from the untreated rat liver (Völkl and Fahimi 1985, 1996). Minced livers of normal female rats starved overnight prior to the experiment were homogenized in ice-cold 5 mM MOPS buffer pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol, for 2 min at 1000 rpm using a Potter-Elvehjem homogenizer and applying only a single stroke. Fractions pelleted during differential centrifugation were resuspended in the homogenization buffer using a glass-rod, and the light mitochondrial fraction was adjusted to 5 ml/g liver. A pre-formed linear Metrizamide gradient prepared in gradient buffer composed of 5 mM MOPS pH 7.4, 1 mM EDTA, 0.1% ethanol, 2% (w/v) Dextran T10 was employed for density gradient centrifugation of the light mitochondrial fraction. It was spun in a VTi 50 vertical rotor (Beckman) at an integrated force of $1252 \times 10^6 \times g \times min$, corresponding to a maximal relative centrifugal force of 33,000×g, for 45 min (acceleration and deceleration included), to minimize the hydrostatic pressure during centrifugation as far as possible. The biochemical, morphometric and morphological examination of the peroxisomal fraction thus purified by rate-dependent banding, revealed that 98% of the particles consisted of PO, contributing 95% of the total protein content of the fraction. The cytochemical reaction product of catalase was distributed uniformly over the matrix

of most PO, confirming the low level of extraction of the enzyme. This was also reflected by the 90% latency of catalase, indicating the integrity of the isolated PO. Continuing studies on the biogenesis of PO, the regenerating rat liver was picked up as model (Luers et al. 1990), and PO were purified from control and regenerating livers after pulse-labeling newly synthesized proteins with 35S-methionine (Luers et al. 1993). For that purpose, the approach just described (Völkl and Fahimi 1985) was modified as follows: (i) the composition of the homogenization buffer was altered and 0.2 mM PMSF, 1 mM ε-aminocaproic acid, 0.2 mM DTT were included in addition; (ii) the homogenates were fractionated by differential centrifugation and two crude peroxisomal preparations were pelleted; (iii) the latter were subsequently subjected to rate-dependent density gradient centrifugation according the conditions given before (Völkl and Fahimi 1985), yet employing a shallow sigmoidal continuous Metrizamide gradient without Dextran instead of the linear one used in the preceding experiments. The gradients were pre-formed in advance by freezing and thawing appropriate step-gradients. Two PO-fractions banding at 1.20 and 1.24 g/mL were collected (Fig. 1a). They differed in their mean diameters and in respect to the activities of the β-oxidation enzymes as well as the polypeptide pattern of the membranes. More important, the less dense population clearly showed a higher rate of 35S-methionine incorporation than the heavier one. In summary, evidence was provided for the existence of peroxisomal subpopulations in rat liver, differing in morphological and biochemical properties as well as in the import rates of newly synthesized proteins.

The report on PO from human liver samples published by Alvarez et al. (1992) deserves special notion, dealing with frozen biopsies from patients undergoing surgery for uncomplicated gallstone disease. Homogenates of fresh and frozen tissue were subjected to differential centrifugation, and the corresponding light mitochondrial preparations were layered on top of linear Nycodenz gradients. Density gradient centrifugation was performed at 32,640×g for 60 min in a Sorvall SV 288 vertical rotor. Gradient fractions enriched in PO were assayed for marker enzyme activities, and examined by electron microscopy and catalase cytochemistry. PO from frozen and fresh tissue were recovered in quite the same high densities (≈1.22 g/mL), and proven to be enriched about 15 times (rat liver about 30 times). Contamination with other subcellular organelles was found higher in PO from frozen than fresh tissue. Particles from the frozen biopsies appeared reasonable well preserved, yet more extracted than the "fresh" ones, and catalase was not as uniformly distributed but more concentrated in the center of the particle. Despite of some inconsistencies, the results shown in this paper indicate that frozen liver samples might be used for the isolation of PO.

With Iodixanol, commercialized under the tradename Optiprep, a novel non-ionic gradient medium came into operation in the mid-1990s (Ford et al. 1994). Originally developed as an X-ray contrast medium like Metrizamide, it consists effectively of two Nycodenz molecules with a Mr of 1550 Da, can form iso-osmotic dense solutions up to 1.32 g/ml and is capable of forming self-generating gradients. Due to its higher Mr compared to Metrizamide or Nycodenz it should be less permeable, and particles should band at lower densities on gradients and hence,

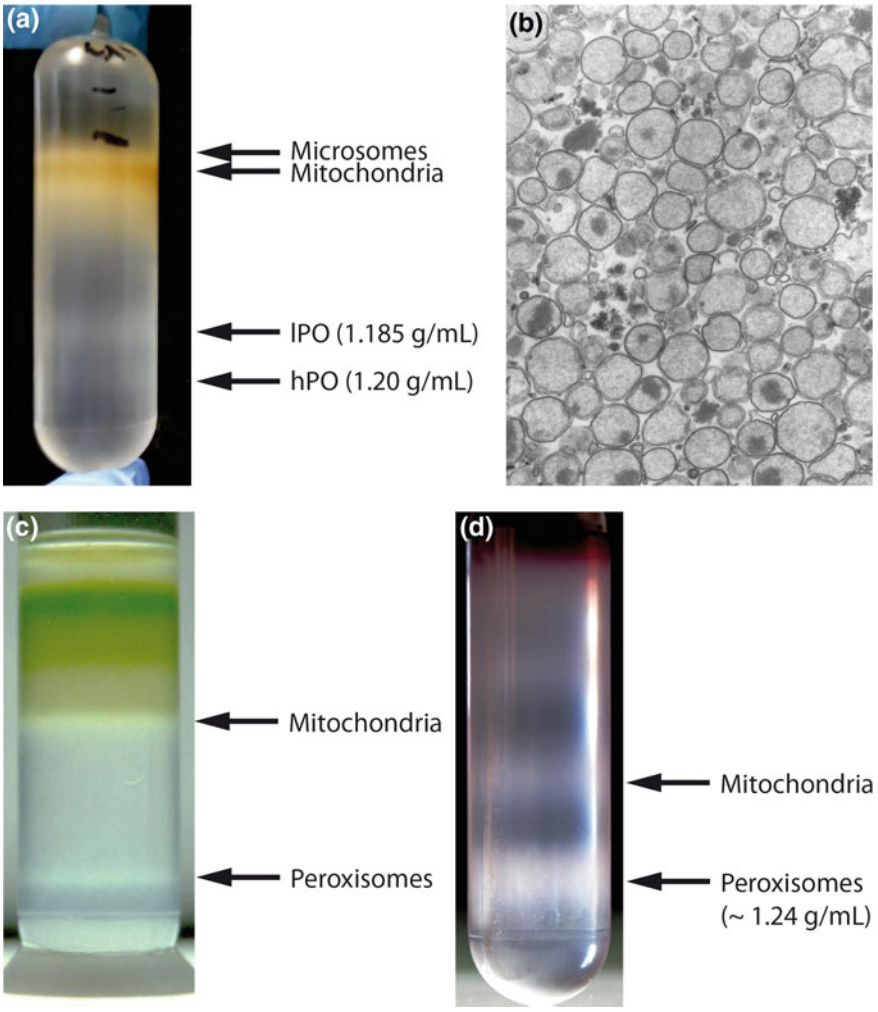

Fig. 1 Peroxisome isolation from rodent liver and plant tissues. **a** Typical separation pattern of a light mitochondrial (L-)fraction in a sigmoidal iodixanol gradient (1.12–1.26 g/mL). PO focus in two distinct bands (lPO and hPO) at densities of 1.18 and 1.20 g/mL, respectively. Note that the equilibrium densities of the PO fractions in iodixanol are somewhat lower than in metrizamide (1.20 and 1.24 g/mL, respectively; Lüers et al. 1993). **b** EM image of PO isolated from rat liver. The organelles show a distinct and preserved limiting membrane and a fine granular matrix. Many organelles contain crystalline cores of urate oxidase typical for rodent liver PO. **c** PO isolation from pea leaves. After a differential centrifugation series, PO were isolated by centrifugation in a 15–53% Percoll-gradient. Like in animals, PO migrate to the densest parts of the gradient (Corpas 1992). **d** PO isolation from olive fruits. Separation pattern of 12.000×g pellet fraction in a 35–60% sucrose step gradient (López-Huertas and del Río 2014). PO assemble at a density between 1.23 and 1.25 g/mL and are well separated from mitochondria

being exposed to a reduced hydrostatic pressure. Self-generated Optiprep gradients have been first initially used for the isolation of subcellular organelles from mouse liver (Graham et al. 1994). However, spun in a fixed-angle rotor at 180,000×g for 3 h might have affected the integrity of POs known to be sensitive to mechanical stress. Van Veldhoven et al. (1996) used continuous Optiprep gradients for the iso-osmotic isolation of rat liver PO, conditions for centrifugation in the fixed-angle rotor, however, were set at 145,000×g for only 1 h. PO banded at a density of ≈1.17 g/mL and were minimally contaminated by other organelles.

Due to its properties Percoll should in fact be predestinated for preparing density gradients. Yet, PO purified accordingly proved to be somewhat more contaminated than those from other gradients. Banding as the lightest organelle at a density around 1.05–1.08 g/ml, an overlapping with the other particles was reported, due to a variable tendency to trail into dense parts of the gradient (Graham et al. 1994). Moreover, Percoll interferes with peroxisomal enzyme activities (Appelkvist et al. 1981). Nevertheless, PO successfully purified by Percoll density gradient centrifugation under various conditions valuably contributed to the biochemical and metabolic features of the organelle. Peroxisomal fatty acid β-oxidation activities were shown to be enhanced not only by hypolipidemic drugs but also by fat diets (Neat et al. 1980). Evidence was provided that the PO membrane is permeable to small molecules (sucrose, Metrizamide etc.), most likely due to a non-selective porin-like pore-forming protein (Lemmens et al. 1989; Rokka et al. 2009; Antonenkov et al. 2004b; Van Veldhoven et al. 1987).

In most of the reports discussed before, PO were isolated to study their biochemical composition, morphological appearance and metabolic role. Yet, also experiments have been conducted dealing primarily with parameters of the isolation procedure other than the type of rotor or gradient medium. Applekvist and collaborators crosslinked PO with glutaraldehyde before gradient loading to stabilize the peroxisomal membrane (Appelkvist et al. 1981). Different rates of contamination were observed using either Percoll or sucrose gradients, yet no effect of the crosslinking was observed. Hartl et al. compared total homogenate and corresponding light mitochondrial fraction of rat liver as starting material used for density gradient centrifugation (Hartl et al. 1985). At first, homogenate (TH) and light mitochondrial preparation (L) were subjected to rate zonal centrifugation on a linear sucrose gradient for 11 min at 7000×g. Gradient fractions mostly enriched in PO were subsequently fractionated by isopycnic centrifugation on linear Nycodenz gradients spun in a swinging-bucket rotor at 70,000×g for 2 h. To this end, fractions were bottom-loaded to allow separation by floating rather than sedimentation. L-PO consisted of ≈98% peroxisomal protein with catalase activity 49-fold enriched. TH-PO represented about 55% of the entire PO population and were enriched 43-fold in catalase activity. Differences were observed in the average particle size with TH-POs more heterogeneous than L-POs as well as in the polypeptide patterns of TH- and L-PO.

2.2 Kidney Peroxisomes

PO have been first described in the mouse kidney (Rhodin 1954), and have been isolated a decade later by isopycnic sucrose density centrifugation from rat kidney (Baudhuin et al. 1965b). Comparing their enzyme composition to hepatic particles it was realized that both shared catalase and some oxidases providing support for the hypothesis that PO are centers of hydrogen metabolism. However, the significantly higher activities of D-amino acid oxidase and the lack of urate oxidase in rat kidney PO clearly pointed to somewhat distinct metabolic roles of hepatic and renal PO. Fractionating a homogenate prepared from the cortex tissue of dog kidney by discontinuous sucrose density gradient respective zonal centrifugation (Kitano and Morimoto 1975) widely confirmed the results of Baudhuin. PO isolated by zonal centrifugation were clear-cut separated from mitochondria, lysosomes and microsomes as could be demonstrated by marker enzyme analysis, with catalase and D-amino acid oxidase predominantly associated with PO. Further support in this regard was provided by Small et al. (1981) reporting the co-localization of palmitoyl-CoA oxidase, catalase and D-amino acid oxidase in renal PO isolated from albino mice, and the significant increase of the palmitoyl-CoA oxidase by the dietary clofibrate treatment. PO had been isolated on a stepper Metrizamide gradient centrifuged in a swinging bucket rotor and shown by biochemical analysis to be reasonable well separated from other organelles. Finally, dihydroxyacetone-phosphate acyltransferase an enzyme involved in the biosynthesis of ether lipids could be exclusively allocated to kidney PO. The PO studied had been purified from rat and mouse kidneys by Metrizamide respective Nycodenz gradient centrifugation in a vertical rotor (Hajra and Wu 1985; Mackness and Connock 1985).

PO in the convoluted tubule cells of the kidney cortex are nearly as abundant as in hepatocytes. In addition, they display some striking features in shape and ultrastructure, e.g. marginal plates, of hitherto unknown nature. Trying to get to the bottom of these properties, experiments were started to isolate the organelle as pure as possible from the cortex of beef, sheep and cat kidneys (Zaar et al. 1986b). Metrizamide density gradient centrifugation of the light mitochondrial fractions according the protocol established for hepatic PO (Völkl and Fahimi 1985) resulted in PO fractions banding at a mean density of 1.22 g/mL. The ultrastructural and marker enzyme analysis revealed that PO made up 97–98% of the isolated particles. PO differed species-specific in their ultrastructure; while cat PO were round to oval-shaped, sheep and particular beef PO exhibited an angular shape and showed structural membrane associations with the smooth ER. Marked variations were also noted concerning the activities of peroxisomal marker enzymes; in particular urate oxidase was exclusively restricted to beef kidney PO.

D-amino acid oxidase characterizes PO in the rat kidney, which lack urate oxidase as already described by Baudhuin (Baudhuin et al. 1965b). Since D-amino acids, the "native" substrate for the oxidase, are not common in higher organisms, the metabolic role of the enzyme is not fully understood. To tackle this question,

PO were isolated from rat liver and rat, bovine and sheep kidney cortex as described (Zaar et al. 1986b). D-amino acid oxidase activities were assayed using the known substrates D-proline, D-alanine and the D- and L-stereoisomers of pipecolate (Zaar et al. 1986a). According to the specific activities, D-pipecolate was almost exclusively oxidized compared to the L-isomer with rates matching those of D-alanine. Since increased levels of pipecolic acid have been reported in patients with Zellweger syndrome, which are devoid in functionally competent PO, the absence or deficiency of peroxisomal D-amino acid oxidase might be implicated in the pathogenesis of hyperpipecolatemia in Zellweger patients.

D-aspartate oxidase is a flavo-protein catalyzing the oxidative deamination of dicarboxylic D-amino acids. Although it was known that the kidney cortex exhibits the highest activities of the oxidase in mammals (Davies and Johnston 1975), it was so far not assigned to PO. Highly purified POs were therefore isolated from rat liver as well as from rat, bovine and sheep cortex and assayed for the oxidase in comparison to D-amino acid oxidase (Zaar et al. 1989). Both the enzymes were alternately inhibited by the enzyme-specific inhibitors sodium benzoate and meso-tartrate, respectively, proofing that D-amino acid and D-aspartate oxidase are distinct enzymes. Since D-aspartate oxidase co-fractionated without exception with peroxisomal marker enzymes during the isolation procedure, and was recovered in the supernatant after disrupting PO, it has to be considered a peroxisomal matrix enzyme.

The close association of PO with membranes of the smooth ER has been repeatedly demonstrated, inter alia in freeze-etch preparations of rat liver and kidney (Kalmbach and Fahimi 1978). In own experiments on the isolation of PO from kidney corteces of beef and sheep, a small amount of the microsomal marker enzymes esterase and glucose-6-phosphatase consistently co-sedimented with the peroxisomal markers. Consequently, PO from the bovine cortex which showed this association most conspicuously, were purified and examined biochemically and morphologically (Zaar et al. 1987). A striking association of smooth membrane elements with the isolated PO (membrane distance 10–15 nm) was indeed noted by electron microcopy. In line with these ultrastructural observations, the biochemical analysis corroborated the esterase activity in the purified PO preparation. The activity forming a small peak coinciding with that of the peroxisomal enzymes, was clearly separated from the bulk of esterase activity, thus ruling out a mere microsomal contamination of the peroxisomal fraction.

The most common and conspicuous inclusions in kidney PO are membrane associated plate-like elements named “marginal plates”. They are most prominent in bovine kidney PO causing the peculiar angular shape of the latter. To elucidate the composition of marginal plates, highly purified PO from this source (Zaar et al. 1986b) were treated with Triton X-100 to release these inclusions, and the mixture was further subfractionated by differential and two-step sucrose gradient centrifugation. Marginal plates accumulated at the sucrose interface (Zaar et al. 1991). Prompted by the distinct yellow color of the fraction pointing to a flavo-protein, various substrates related to peroxisomal oxidases were tested. By far the highest activities were observed using L-hydroxybutyrate, which is known to be the

preferential substrate for L-α-hydroxyacid oxidase 2 (HAOX2). Immunoblotting using antibodies raised against HAOX1 as well as urate oxidase confirmed the biochemical results clearly demonstrating that marginal plates are mainly composed of HAOX2.

3 Peroxisome Isolation from Brain Tissue—A Still Unsolved Problem

While PO from liver and kidney can be isolated with high purity applying a combination of differential centrifugation and a single density gradient, isolation from brain tissue is not as straight forward. On the one hand, PO abundance is significantly lower than in hepatocytes or kidney proximal tubule epithelia (Islinger et al. 2010a) requiring higher enrichment factors to achieve sufficient purities; on the other hand PO from the CNS do not represent a homogenous pool but are specialized according to different cell type functions and brain regions(Ahmad et al. 1988). Moreover, the myelin enveloping axons lead to high lipid concentrations in brain homogenates and are a major obstacle in the purification process.

An early attempt to isolate PO from the brain of adult rats was performed by separating homogenates into typical crude subcellular fraction using differential centrifugation (Singh et al. 1989). Subsequently the microsomal fraction (between 16,900 and 100,000×g) in 0.32 M sucrose was layered on a 17% Nycodenz layer to yield a PO-enriched fraction at the bottom after centrifugation. Comparison of different PO marker enzyme activities, however, shows inconsistent results. While glyceronephosphate O-acyltransferase (GNPAT) activities point to an 16 fold enrichment if compared to the homogenate, enzyme activities for alkylglycerone phosphate synthase (AGPS) and lignoceric acid β-oxidation point to a mere 3-fold and 5-fold enrichment. As a consequence the PO enriched fractions revealed were significantly contaminated with microsomal material. Interestingly a comparable enrichment of C24 β-oxidation was also associated with purified mitochondrial fraction (Singh et al. 1989), which suggests that considerable PO amounts were contained in several of the organelle fractions. This is in line with the heterogeneous pattern of PO in brain tissue slices or subcellular fractionation experiments, when PO are detected by immunodetection of different marker proteins (see Fig. 2).

A major problem for organelle isolation from brain is the presence of large amounts of myelin in the tissue homogenates impeding an efficient organelle separation. To alleviate myelin contamination, most available fractionation protocols use rats or mice at an age where myelinization is still incomplete (P10–P15). Moreover, PO are required for the synthesis of plasmalogens and polyunsaturated fatty acids, which are contained in myelin sheaths in high concentrations (Wanders and Waterham 2006). Correspondingly, PO enzyme activities for etherlipid synthesis and β-oxidation maximize during the period of myelinization (Lazo et al. 1991), thus facilitating the detection of PO in the subcellular fractions.

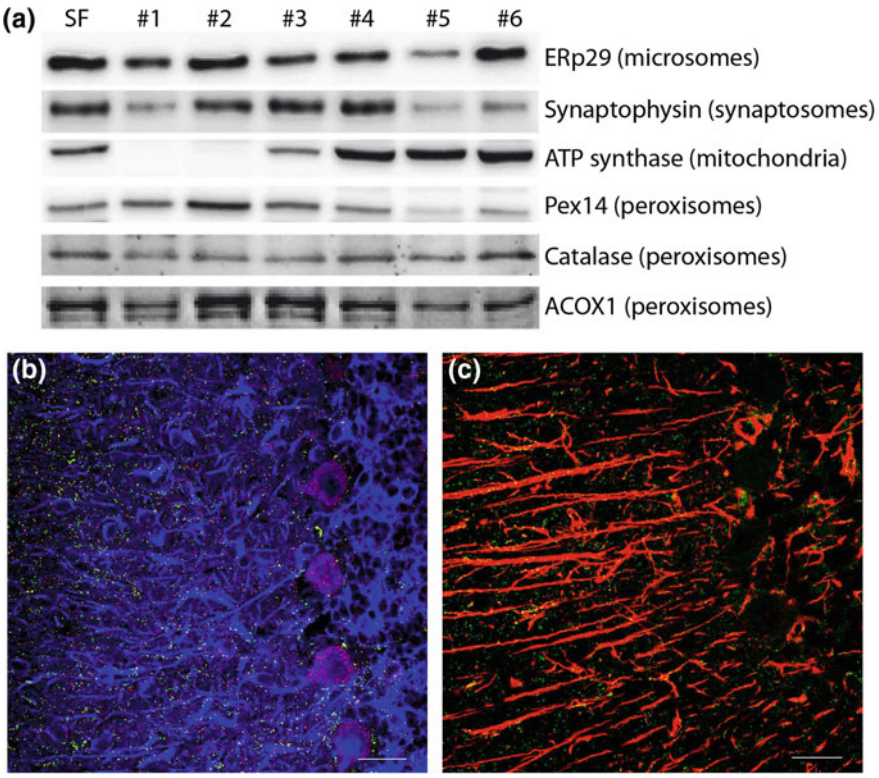

Fig. 2 Heterogeneity of PO in the rodent brain. **a** Separation of brain PO in a sucrose step gradient reveals an incongruent distribution of PO marker proteins. For PO purification mouse brain post nuclear supernatants were separated with a 1st discontinuous sucrose gradient (0.5,0.7, 0.9 and 2 M). Material migrating below 0.9 M sucrose (1.113 g/ml, SF) was collected and further purified by a 2nd gradient of 0.7, 0.9, 1.1, 1.2, 1.3, 1.4 and 2 M sucrose layers. Organelle bands (#1–#6) enriched at the interfaces of the sucrose layers were subsequently analyzed by immunoblotting. Note that PEX14 and ACOX1 peak in different fraction than catalase. **b**, **c** Immunostaining of PO in the cerebellum of adult rats. PO were stained by antibodies against the Pex14 (red) and catalase (green). **b** While PO found in Purkinje cells (prominent somata marked by Map2, blue) were not stained by the catalase antibody **b**, radial extensions of the Bergmann glia are stained by both PO markers **c**

In such purification schemes, PO are isolated from brain by a combination of differential centrifugations, a demyelinization step and density gradient centrifugation. Available protocols differ in the order of these three steps and the separation media used for density gradient centrifugation. Demyelinization is usually achieved by centrifugation in a two-step gradient consisting of a 0.25 M/0.32 M and a 0.85 M sucrose layer, which was adapted according to the protocol for crude synaptosome isolation (Gray and Whittaker 1962). After centrifugation myelin floats above or at the interface of the 0.25 M and 0.85 M solutions, while organelles can be found in the 0.85 M solution and as a pellet at the bottom of the tube.

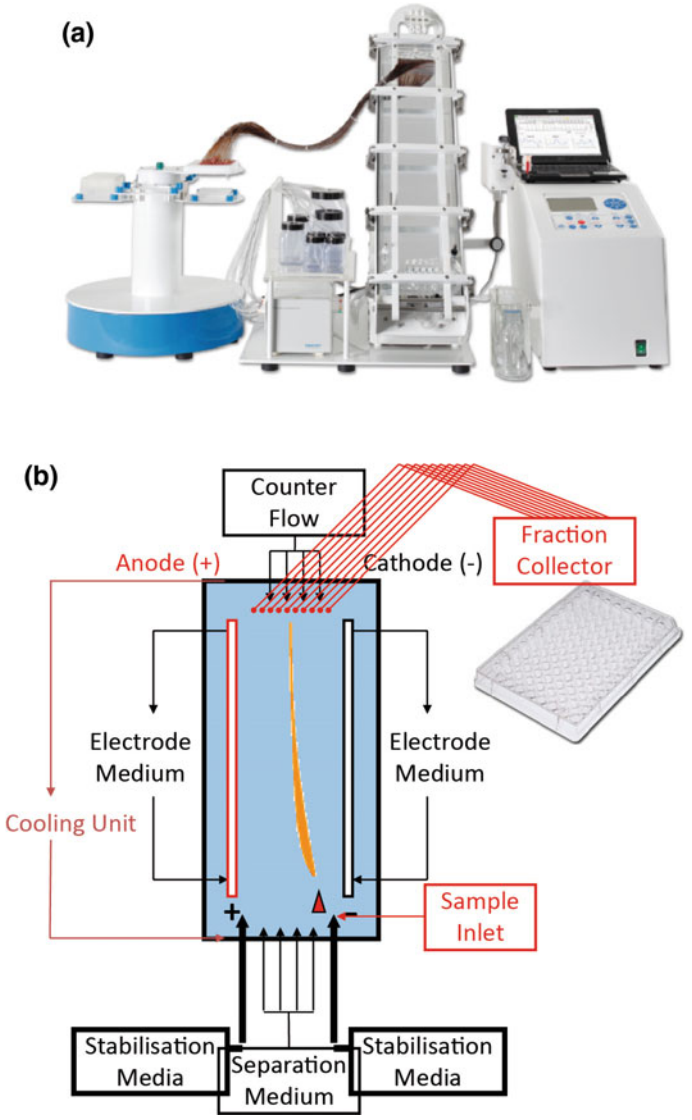

Fig. 3 Separation of organelles by FFE. **a** Image of a current instrument consisting of a power supply/control unit, separation chamber, media pumps and automated fraction collector. **b** Principle of a zonal free flow electrophoresis (ZE-FFE). Media pump inlets at the bottom of the instrument generate a laminar buffer flow in the separation chamber. At the outlet of the chamber 96 openings collect the analytes for subsequent analyses. Electrodes generate an electric field perpendicular to the buffer flow. To prevent electrolysis in the separation buffer stabilization media shield the zones close to the instrument's electrodes. The sample to be separated is pumped into the chamber at an inlet near the cathode of the instrument to guarantee a maximum migration distance for the different analytes

Testing two alternative purification schemes Lazo and colleagues used whole brains of 10-day old rats (Lazo et al. 1991). In one method (Method A), myelin removal was performed after two differential centrifugation steps pelleting nuclei and large mitochondria. After demyelinization a 39,191×g pellet was finally separated in a 0–35% Nycodenz gradient containing 0.25 M sucrose. In the alternative purification scheme (Method B) the brains were homogenized in 0.85 M sucrose buffer and myelin was immediately removed. A light mitochondrial fraction (between 12,096 and 39,191×g) was produced in a subsequent differential centrifugation series. Finally, PO were purified in a self-generating 13% Percoll-gradient in 0.25 M sucrose. Both gradients were analyzed by enzyme marker assays and relative PO enzyme revealed a 3–5fold enrichment from the initial homogenate with the Nycodenz method and 4–6.5fold enrichment with the Percoll method (Lazo et al. 1991). These results imply that PO constituted a minor proportion of the total organelle amount in the brain PO peak fractions.

In a subsequent publication the authors performed a complete differential fractionation producing a heavy mitochondrial (12,096×g), light mitochondrial (39,191×g) and microsomal pellet (190,479×g) in advance to the demyelinization step (Singh et al. 1993). Each fraction was individually demyelinated as described for Method B above. Each of demyelinated organelle preparations were finally separated on linear 0–30% Nycodenz-gradients containing 0.25 M sucrose to yield mitochondria, PO and microsomes from the respective HM, LM and microsomal prefractions. Measurements of catalase and GNPAT enzymatic activities revealed a 7.5–12-fold purification of PO from the LM gradient, respectively, which still implies a significant contamination with other subcellular compartments.

Kovacs and colleagues restricted the brain material used for PO isolation to brainstem, cerebellum and spinal cord of 15 day old mice, as these regions of the CNS show lowest myelination at this age (Kovacs et al. 2001). After homogenization, a differential centrifugation series was performed to produce heavy (5500×g), light mitochondrial (LM, 18,000×g) and microsomal pellets (105,000×g). The LM fraction was demyelinated as described for Method B above and finally separated in a 0.25 M sucrose containing 0–40% linear Nycodenz gradient. According to catalase activities and immunoblots for ambient marker proteins, PO assembled at the high density region of the gradient and were well separated from microsomes and mitochondria. According to catalase activities the authors reported a 40-fold enrichment from the homogenate.

Using brainstem, cerebellum and spinal cord of 14 day old rats, Cimini and colleagues performed the same organellar prefractions as described in the publication by Kovacs (Cimini et al. 2003). However, instead of a demyelination procedure the LM fraction was subjected to 7 freezing/thawing cycles in order to release entrapped PO from artificial synaptosome-like vesicles probably produced during initial homogenization (Hajra and Bishop 1982). The resulting organelle suspension was cleared from larger aggregates using a 10,000×g centrifugation and ultimately pelleted 105,000×g. Finally this pre-peroxisomal fraction was separated on a linear Nycodenz gradient of 1.1–1.2 g/ml. According to catalase activity measurements the authors reported a >100fold enrichment of PO from the

homogenate, which would stand for the most successful isolation procedure so far published (Cimini et al. 1993). Nevertheless, ultrastructural images from the isolated PO revealed that these final fractions were considerably contaminated with undefined vesicles and membranes whereas PO appear to constitute still a minor proportion of the fraction. Thus, for a preparation suitable for a proteomic characterization of brain PO, still further technical improvements have to be developed.

Compared to the quite similar protocols applied for the isolation of liver and kidney PO, the strategies and procedures developed to purify PO are highly diverging and often hardly comparable. Apparently, according to the age, species and brain regions used for the isolation, PO were observed to sediment at remarkably different densities. The more successful purification protocols concordantly use animals with ongoing cerebral development. During this period, catalase positive PO were observed to be notably abundant in oligodendrocytes and axon terminals (Arnold and Holtzman 1978) and in all likelihood provide lipid material required for the formation of myelin sheaths and axon growth. In brains of adult animals PO in oligodendrocytes, astrocytes and neurons show PO with significantly differing protein composition and likely fulfill distinct functions (Ahlemeyer et al. 2007). Thus, our current knowledge on the role of PO in the adult brain was primarily gained from the investigation of cell type specific conditional knockout mice (Bottelbergs et al. 2010; Kassmann et al. 2007), while a profound characterization of the distinct PO proteomes and associated traits of adult astrocytes, oligodendrocytes and neurons is still missing. In this respect, novel sophisticated purification methods have still to be developed to isolate PO with sufficient purity suitable for subsequent proteomic analysis.

4 Peroxisome Isolation from Yeast and Other Fungi

When grown on glucose as a carbohydrate source, many yeast species contain only a few PO, thus precluding an efficient isolation applicable for subsequent functional analyses (Szabo and Avers 1969). However, PO massively proliferate when the cells—depending on the species—are grown on carbon sources such as oleate or methanol, which are metabolized in PO (van Dijken et al. 1975; Sahm et al. 1975). Yeast species used in subcellular fractionation experiments are the methylotrophic genus *Candida* (Kamiryo et al. 1982; Goodman et al. 1984) and *Pichia* (Wriessnegger et al. 2007) as well as *Saccharomyces* (Roggenkamp et al. 1975; Erdmann and Blobel 1995) and *Yarrowia* (Uchiyama et al. 1982; Smith et al. 1997).

In order to facilitate successful PO purification increasing recovery and sample purity, isolations are usually performed after PO-proliferation. During methanol- or oleate induction PO not only increase in number but as well in size and can constitute up to 20–80% of the cellular interior (Sahm et al. 1975; Veenhuis et al. 1987). Generally, for organelle preparation, initially cell walls have to be removed by a zymolase treatment yielding osmotically labile spheroblasts; subsequently the cells are lysed by incubation in a hypoosmolar buffer or by Dounce/Potter Elvehjem

homogenization (Roggenkamp et al. 1975; McCammon et al. 1990; Erdmann and Blobel 1995; Wriessnegger et al. 2007; Cramer et al. 2015a). Compared to animal cells the homogenization buffers used for the isolation procedure have a higher osmolarity containing usually 1 M sorbitol or mannitol at a pH 5.5–6.0, which was reported to stabilize the integrity of the organelle membranes (Goodman et al. 1984). Like in animal tissue, PO are usually first enriched by differential centrifugation preparing a light mitochondrial fraction. Depending on species and separation protocol sedimenting forces between 5000×g and 30,000×g have been used for preparation of this PO and mitochondria-enriched pellet (Roggenkamp et al. 1975; Wriessnegger et al. 2007). If a higher contamination of the isolated PO fraction can be tolerated, post-nuclear fractions are alternatively directly subjected to density gradient centrifugation (Thoms et al. 2008; Cramer et al. 2015b). Media used for density gradient centrifugation are Ficoll (Roggenkamp et al. 1975), sucrose (Kamiryo et al. 1982; Goodman et al. 1984; Moreno de la Garza et al. 1985; McCammon et al. 1990), Nycodenz (Wriessnegger et al. 2007; Yi et al. 2002; Small et al. 1987), and Optiprep (Cramer et al. 2015b) and were used in continuous linear or discontinuous step gradients. Buoyant densities for PO isolated from methanol-grown *Candida tropicalis* were observed to be 1.23–1.24 g/mL in sucrose and 1.19 g/mL in Nycodenz (Kamiryo et al. 1982; Nuttley et al. 1990; Small et al. 1987), which is quite similar to the values reported for liver PO (see above). Purities of the PO fractions obtained in such combined differential and density gradient approaches were reported to be above 95% (Nuttley et al. 1990; Kamiryo et al. 1982; McCammon et al. 1990; Small et al. 1987). A direct comparison between sucrose and Nycodenz-gradients, consistently revealed a better separation from PO and mitochondria in the latter gradient medium.

In order to improve the purity of PO fractions from *S. cerevisiae* for subsequent membrane protein analysis Erdman and Blobel developed an isolation method combining differential centrifugation with two subsequent density centrifugation steps (Erdmann and Blobel 1995). A 25,000×g light mitochondrial fraction was first separated in a continuous 36–68% sucrose gradient; the PO enriched fractions from this gradient were subsequently pooled and further purified in a 20–40% Nycodenz gradient containing an inverse 4.25–8.5% sucrose gradient. While substantial amounts of the PO matrix was according to EM observations lost during the extensive isolation procedure, the fractions served successfully for the analysis of the integral components of the yeast PO and led to the identification of the Peroxin Pex11. The same isolation procedure was further used in subsequent proteomic studies, which in addition to a high coverage of the known PO membrane proteome led to the identification of novel PO constituents (Schafer et al. 2001; Thoms et al. 2008) PO can multiply by growth and division but as well by budding of pre-peroxisomal vesicles from the ER, which later fuse into mature functional PO (Erdmann 2016). Using a multistep fractionation assay Titorenko and colleagues separated a set of different PO fractions from the oleic acid-induced yeast *Yarrowia lipolytica*, which were interpreted as different PO sub-forms from a multistep assembly pathway (Titorenko et al. 2000). Compared to mature PO (P6), which were prepared by the established methods from the 20,000×g pellet, these

sub-forms constitute of 5 fractions (P1–P5), which were purified from a 200,000×g high-speed pellet. To this end this high-speed organelle fraction was subjected to centrifugation on a discontinuous gradient ranging from 18–53% (w/w) sucrose. Immunoblot analysis of the gradient revealed four PO peaks at 1.18 g/mL (P5), 1.14 g/mL (P3+P4), 1.11 g/mL (P1), and 1.09 g/mL (P2) with different compositions of PO proteins. Individual peak fractions were pooled, recovered in 50% sucrose buffer and further purified by flotation density centrifugation using individual discontinuous sucrose gradients. The authors used these purified fractions for fusion and pulse-chase labeling experiments for further characterization. Based on this data the authors hypothesized a maturation pathway were P1 and P2 pre-PO fuse to yield P3 which consecutively matures via P4 and P5 stages into mature P6-PO (Titorenko et al. 2000). Up to the present age the existence of ER-derived pre-PO could be further shown by budding assays using purified microsomal membranes from different yeast Pex-mutant strains (Agrawal et al. 2011; Lam et al. 2010; Agrawal et al. 2016). In this respect, it is likely that the purified P1–P5 fractions may represent pre-peroxisomal maturing vesicles released from the ER, however, the different patterns of peroxins and matrix proteins cannot be convincingly explained by our current models of PO biogenesis and protein import (Hettema et al. 2014) and would require intensive further characterization of the different PO sub-forms to understand their physiological meaning.

PO from filamentous ascomycetes such as *Neurospora crassa, Magnaporthe oryzae, Aspergillus fumigatus* and *Penicillium chrysogenum* came more recently into the scientific focus, since the organelles appear to be relevant for the process of host invasion or play a role in the synthesis of antibiotics (Kubo 2013; Bartoszewska et al. 2011) These ascomycetes inhabit specialized peroxisome-derived structures termed Woronin bodies (WB), which are required for the sealing of septal pores and arise from PO through fission (Steinberg et al. 2017). PO in addition to β-oxidation contribute majorly to the glyoxylate cycle and were thus historically termed glyoxisomes (Breidenbach and Beevers 1967). *Neurospora crassa* is an ascomycete with particularly exceptional PO with a unique protein composition, which was unraveled in a series of subcellular fractionation studies. Homogenization buffers used for the fractionation of organelles from *N. crassa* contain 150 mM Tricine and 440 mM sucrose and possess a near neutral pH of 7.4 (Managadze et al. 2010; Kionka and Kunau 1985). In order to associate specific enzyme functions with PO, crude extracts from glucose-acetate and oleate-grown hyphae were fractionated in a 30–60% sucrose gradient in an early study (Kionka and Kunau 1985). Remarkably, catalase separated well from the PO-associated activities of the glyoxylate cycle and β-oxidation enzymes and migrated to a higher density (1.21 vs. 1.19 g/mL). Moreover, the PO-enriched fractions at 1.21 g/mL exhibited acyl-CoA dehydrogenase instead of acyl-CoA oxidase activities, which could partially explain the lack of catalase in PO. In an independent study, separating a dense vacuolar fraction, in Metrizamide-gradients, Tenney and coworkers detected that catalase activities co-migrate with the PTS-1 containing dominant WB protein Hex1 (Tenney et al. 2000). To clarify this unexpected subcellular catalase distribution, Schliebs and colleagues analyzed the

distribution of 4 *Neurospora* catalase genes in the 30-60% linear sucrose gradient described above using isoform-specific antibodies. Unexpectedly, all catalases were found in the cytosol but neither in PO nor in WB-enriched fractions suggesting that the WB associated catalase activities were generated by another enzyme, probably a peroxidase (Schliebs et al. 2006). In an attempt to characterize the proteomes of WB and PO from *N. crassa*, both organelles were isolated by individual protocols in order to obtain highly pure fractions for subsequent MS (Managadze et al. 2010). For WB isolation, 10,000×g organelle pellets from sucrose-grown hyphae were centrifuged through 52% sucrose cushions (1.25 g/mL) thus removing most mitochondria and PO, which remain on top of the gradient. The resulting WB-enriched pellet was further purified in a linear 30–60% sucrose gradient, resulting in increasingly pure WB fraction at a density of 1.26 g/mL. MS analysis of the WB showed that the organelles are almost entirely composed of Hex1 whereas no other WB-associated candidates were identified. For PO purification a "classic" 25,000×g LM pellet was initially prepared. PO were further purified by density-barrier centrifugation using iodixanol (Optiprep) as separating medium (Graham and Eastmond 2002). To this end the LM pellets were suspended in an iodixanol solution adjusted to the density of 1.145 g/mL and loaded on top of 1.199 g/mL iodixanol. In this method PO concentrate at the interface of both iodixanol solutions while other organelles float in the less dense separation medium. MS analysis of the purified PO again revealed that catalase is neither associated with PO or WB but led to the identification of several novel PO protein candidates. Three candidates are predicted acyl-CoA dehydrogenases thus confirming the enzymatic observations from the early fractionation studies. In this respect the studies on *N. crassa* are an intriguing example how subcellular fractionation combined with different analytical techniques can reveal unexpected organelle functions, which may lead to a better understanding of the mechanisms of organelle evolution.

5 Peroxisome/Glyoxysome Purification from Plants

Like fungi, plants exploit the glyoxylate cycle to metabolize fatty acids into carbohydrates—a pathway which is required to convert stored lipids into cellulose for the growing plant. Subcellular fractionation experiments performed to unravel the organelle localization of this pathway using castor bean endosperm led to the discovery of a microbody-like compartment termed glyoxysome (Breidenbach and Beevers 1967). One year later, a study of PO in the ciliate *Tetrahymena pyriformis* revealed that the enzymes of the glyoxylate cycle were contained in the same compartment as PO oxidases and catalase (Muller et al. 1968). Stimulated by this publication, plant microbodies were also purified from spinach leafs and revealed the presence of FAD-dependent oxidases and catalase typical for PO involved in the process of photorespiration (Tolbert et al. 1968). Step by step the discovery of more enzyme functions in microbodies purified from both tissues brought to light that

glyoxysomes and PO are indeed specialized populations of the same organelle but adapted to a tissue-specific physiology (Tolbert 1981). Indeed, glyoxysomes and leaf PO represent most specialized PO subspecies and photorespiratory as well as glyoxylate cycle enzymes have been shown to co-exist in PO, which were isolated from e.g. pepper fruits (Mateos et al. 2003). To date PO were purified from roughly 20 higher plant species and a variety of different tissues including whole seedlings, endosperm, hypocotyls, cotyledons, green leafs, petals, roots and root tubers as well as fruits (Tolbert et al. 1968; Schwitzguebel and Siegenthaler 1984; Mateos et al. 2003; Reumann et al. 1997; Corpas et al. 1991; Huang and Beevers 1971). Such plant tissues according to their function contain a quite heterogeneous organelle composition (e.g. leaves contain massive amounts of chloroplasts while oil seed cotyledons are enriched in lipid droplets). Moreover, the tissues can vary considerably in water or oil content (e.g. fruits from pepper vs olive). Hence, even if PO from different plant tissues and species have been successfully purified using even the same protocol (Huang and Beevers 1971; Gerhardt 1983), methods for tissue extraction and organelle prefractionation have usually to be adapted to the specific tissue requirements.

Technically most of the fractionation studies performed with plant material apply the classic three steps of organelle purification: homogenization, differential centrifugation and final density gradient centrifugation. For the subsequent gradient centrifugation most of the isolation protocols used sucrose as a separation medium. Unlike mammalian tissues, plant cells contain a cell wall of cellulose necessitating a more vigorous mincing and grinding to yield the initial homogenate.

Glyoxysomes have been initially isolated from the endosperm of Castor beans (*Ricinus communis*) (Breidenbach and Beevers 1967). To this end, endosperm was homogenized in a buffer containing i.a. 50 mM Tricine, pH 7.5, 0.4 M sucrose and 10 mM DTT as a reducing agent. As the endosperm tissues is devoid of chloroplasts the post nuclear supernatant was directly used to produce a 10,000×g pellet, which was finally separated on a 32–60% sucrose gradient. Glyoxysomes were found to be well separated from mitochondria and migrated to the high density of 1.25 g/mL as it is characteristic for PO. Only slightly altered fractionation schemes were used two subsequent publications of the group, which characterized the basic enzyme inventory of the organelle including the pathway of fatty acid β-oxidation (Cooper and Beevers 1969; Gerhardt and Beevers 1970). Very similar buffer systems and isolation procedures were also used for the isolation of glyoxysomes from cotton, pea and pumpkin cotyledons, which were as well purified from an medium-density organelle pellet using 30/35–60% sucrose gradients (Huang and Beevers 1971; Yamaguchi and Nishimura 1984; Gerhardt 1983). Moreover, Huang and Beevers used the very same protocol to purify PO and glyoxysomes from very different plant tissues such as including tubers, fruits, roots, shoots, petals and leaves (Huang and Beevers 1971). In all experiments microbodies assembled in the densest regions of the gradient (1.20–1.25 g/mL) and could be successfully separated from mitochondria. With regard to gradient shape both continuous linear and discontinuous step gradients have been used for isolation. While linear gradients provide a useful overview of the distribution of different organelles across the

gradient, step gradients allow band sharpening of individual organelle fractions at the interface of two gradient medium concentrations leading to improved purities (Gerhardt 1983; Cooper and Beevers 1969). Enzymatic characterization of the individual PO fractions confirmed the functional heterogeneity of PO in plants being specialized on the glyoxylate cycle in fat-containing seed tissue, the glycolate pathway in chloroplast-containing tissue and more general PO enzymes in less specialized tissues.

Leaves, as photosynthesizing tissue, contain massive amounts of chloroplasts. In early protocols, PO were purified from leaves from spinach and other species directly from an initial particle pellet produced from the PNS (Tolbert et al. 1968, 1969; Gerhardt 1981). However, the homogenization buffers and G-forces used in these initial fractionation schemes vary considerably (0.5 M/1 M sucrose in the homogenization buffer, 3000, 6000, 12,500×g pellet for PO enrichment). In concordance with the g-forces used for pelleting of the PO pre-fractions the authors used different sucrose density concentration ranges of 44–85% sucrose (Tolbert) and 35–60% (Gerhardt) in the final gradient centrifugation. Despite the obvious in the isolation methodology, both isolation strategies were able to successfully separate PO from mitochondria and chloroplasts and isolated fractions with sufficient purity to associate enzymatic activities of the glycolate pathway and fatty acid β-oxidation with leaf PO. To increase the purity of isolated leaf PO, subsequent studies included an additional centrifugation step in order to remove the bulk of chloroplasts from the organelle fraction subjected to density gradient centrifugation. For the purification of leaf PO from different plant species del Río and colleagues homogenized the leaves in a 1 M sucrose, 170 mM Tricine buffer, pH7.5 and removed chloroplasts by a differential centrifugation step at 2000/2600×g (Corpas et al. 1991, 1998; López-Huertas et al. 1995). Subsequently, PO-enriched pre-fractions were pelleted by a g-force of 12,000 and subsequently separated on sucrose step gradients ranging from 30–60%. As PO sediment at a sucrose concentration of approx. 55% sucrose, they usually enrich at the interface of 51 and 57% sucrose solutions, which are commonly used as steps in discontinuous gradients. Thus, despite their obvious functional differences, leaf PO sediment at comparable equilibrium densities (1.24 g/mL, see Fig. 1c) as oil seed PO (glyoxysomes), hence, indicating that the membranes of both PO subspecies are equally permeable to the sucrose gradient medium. In this respect the observable density shift of PO in gradient media appears to be a general trait preserved across the major domains of eukaryotic life and may be explained by evolutionary conserved pore forming proteins or a remarkable fragility of the PO membranes thus permitting entry of gradient medium into the organelle interior (Rokka et al. 2009; Antonenkov et al. 2004a).

As an alternative for sucrose, Percoll has been used for leaf PO isolations. In contrast to other gradient media, Percoll is not a homogenous solution but consists of colloidal silica particles (diameter: 15–30 nm), which minimizes osmotic pressure and viscosity. Thus PO incorporate Percoll less efficiently than sucrose or Metrizamide derivatives resulting in lower buoyant densities. While PO from liver or kidney do not separate well from mitochondria in Percoll, the medium has been

successfully used in plant PO isolation (Schwitzguebel and Siegenthaler 1984; Sandalio et al. 1987). Both publications used an alternative 0.35 M mannitol, 30 mM MOPS buffer system for homogenization. Chloroplasts were pelleted at 2000/5000×g and a PO-enriched fraction (20,000/12,000×g pellet) was separated in discontinuous Percoll 4-step gradients covering slightly different concentration ranges of 21–60% and 15–53% Percoll in 0.25 M sucrose, respectively (Schwitzguebel and Siegenthaler 1984; Sandalio et al. 1987). PO were found to sediment at comparable densities of approx. 1.095 g/mL and were well separated from the mitochondrial peak (~1.07 g/mL).

In addition to leaves and endosperm, plant PO have been purified from a variety of other tissues. For root tubers such as potatoes and carrots the protocols largely resemble the purification methods used for the purification of leaf PO (Huang and Beevers 1971; Schwitzguebel and Siegenthaler 1984). Likewise PO from pepper and olive fruits have been isolated with an only slightly adjusted protocol for leaf PO isolation (Mateos et al. 2003; López-Huertas and del Río 2014). Otherwise the protocol for PO isolation is largely identical with the method for the isolation of pea leaf PO published by the same group (López-Huertas et al. 1995). In the final sucrose step gradient PO sediment at an equilibrium density between 1.23 and 1.25 g/ml well separated from other organelles such as microsomes and mitochondria (Fig. 1d).

Compared to higher plants, PO from algae have been functionally significantly less characterized. Isolated PO from green algae (chlorophyta) were often acquired directly from the post nuclear supernatant or filtered homogenates using a single density gradient centrifugation in a linear 30–60% sucrose gradient (Stabenau and Beevers 1974; Stabenau 1976; Stabenau et al. 1984a; Gross et al. 1985). For the isolation of PO from *Euglena*, Becker and coworkers utilized a more sophisticated isolation protocol removing the bulk of mitochondria and chloroplasts by differential centrifugation at 3000×g prior to the final purification in a linear 25–48% sucrose gradient (Graves et al. 1972; Graves and Becker 1974).

According to the enzyme activities measured across these gradients, the PO from the different species exhibit a remarkable heterogeneity in protein composition and function. While PO from *Spirogyra* and *Mougeotia* appear to be functionally similar to the leaf-type PO from higher plants (Stabenau 1984), the PO from *Euglena* are more similar to glyoxysomes (Graves et al. 1972); those from *Chlorogonium* or *Eremosphaera* in contrast have been reported to possess neither glyoxylate nor glycolate pathway activities (Stabenau and Beevers 1974; Stabenau et al. 1984b). Remarkably, the subcellular localization of fatty acid β-oxidation appears also to vary among different algae. In *Mougetia* the pathways was reported to be, like in higher plants, solely located in PO, while *Eremosphaera* appears to possess a mitochondrial and a PO β-oxidation systems as it is common for animals (Stabenau et al. 1984a). *Bumilkropsis* seems to even lack PO β-oxidation activity and the pathway was entirely detected in mitochondrial fractions (Gross et al. 1985). Red algae and green algae evolutionary separated from each other more than 1000 mio years ago. Thus, the characterization of PO from red algae (Rodophyta)

might shed light on the evolution of PO β-oxidation in plants. A method for PO isolation was published for the rodophyte *Cyanidium caldarium* (Gross and Beevers 1989). PO were isolated from a 1500×g supernatant which was further separated via a flotation centrifugation step using a linear 30–57% linear sucrose gradient and a prolonged centrifugation time of 15 h at 60,000×g. Enzymatic characterization of the gradient fractions revealed that PO from this species inhabit, like higher plants, enzymes of the glycolate pathway (Gross and Beevers 1989); β-oxidation activities, however, were only found in mitochondrial fractions (Gross 1989).

The striking heterogeneity of PO in algae points to a functional diversification, which might give clues on the evolution of PO in the plant phylum. Nevertheless, PO in the different species might also alter their enzyme composition according to environmental conditions and energetic status. Thus, future studies would be required to unravel the proteome variability of PO in lower plants under different conditions using high-purity PO fractions combined with modern proteomics techniques.

Isolated PO gained by the purification procedures described above have been successfully used for the description of organelle-specific enzymes activities and the characterization of the main PO protein composition, however, a profound proteomic characterization requires organelle fractions as highly pure as possible, to eliminate low abundant contaminants. Tolbert in 1971 already published an improved PO purification protocol for spinach leaf PO combining two consecutive sucrose gradient centrifugation steps (Tolbert 1971). After preparing a PO enriched fraction by differential centrifugation (6800×g pellet), PO are further enriched on a discontinuous sucrose gradient trapping PO at an interface between 1.75 and 2.3 M sucrose. Finally, the PO peak fractions are pooled and subjected to another round of centrifugation using a continuous sucrose gradient (Tolbert 1971). For the proteomic characterization of *Arabidopsis* leaf PO, Reumann and colleagues combined Percoll and sucrose gradients in two consecutive purification steps (Reumann et al. 2007; Reumann and Lisik 2017). Initially mainly chloroplasts were removed by differential centrifugation using a g-force of 5000. The resulting supernatant was top-loaded on a discontinuous gradient combining different Percoll (15/38% in 0.75 M sucrose) and pure sucrose solutions (1.2 M). While mitochondria and chloroplasts were predominately retained in the Percoll layers of the gradient, PO were recovered at the bottom of the gradient in the 1.2 M sucrose layer. This PO-enriched fraction was subsequently washed and top-loaded on a step gradient covering a range from 41 to 60% sucrose. After centrifugation PO were found to enrich at the interface of 51 and 55% sucrose concentrations. The addition of this second density centrifugation run allowed reducing contaminating organelles in the final PO fraction by the factor of 3 if compared to the PO pre-fraction gained from the Percoll gradient (Reumann et al. 2007). In this respect, the combination of two separation media in consecutive centrifugation steps allowed to produce PO fractions with sufficiently high purities for a subsequent MS-based proteome analysis (see below).

Even if the combination of different gradient media in consecutive centrifugation runs can be used to improve the purity of PO, such double purification steps still rely on a single fractionation parameter, namely density, limiting separation capacities. Consequently, multistep purification protocols would benefit from purification techniques combining separation principles, which are based on two independent physicochemical properties. Indeed, non-centrifugation-based separation techniques were consecutively developed during a later phase of PO research in the late 1980s and 1990s and are described in the next paragraph.

6 The Limits of Density Gradient Centrifugation—The Development of Alternative Purification Techniques

As described above, PO have been successfully purified by density gradient centrifugation from yeast, plant and animal tissues. However, while these purification methods work well for tissues or cells with high PO abundance, they do not permit the characterization of PO from more complex tissues or the purification of distinct PO subpopulations. Therefore, a survey for alternative techniques, which would allow PO isolation at high purities, began in the 1990s.

Centrifugation methods separate particles according to their individual density. Thus, alternative separation principles should rely upon different physicochemical parameters in order to allow the separation of organelles with highly similar density. The surface of membrane-surrounded organelles is covered with the cytosolic parts of integral and/or peripherally attached membrane proteins. Generally, the proteins on organelles sum up to negative surface charge which enforces their migration towards the anode in an electric field (Islinger et al. 2010b). Different organelle species, however, exhibit distinct membrane proteomes generating organelle-specific net surface charges. These properties have been exploited by the technique of free flow electrophoresis (FFE). FFE instruments consist of a separation chamber with a laminar buffer flow perpendicular to an electric field. Thus, individual particles are deflected from the linear flow in the separation chamber according to their electrophoretic mobility and isoelectric point (Krivankova and Bocek 1998). FFE has been successfully applied for the separation of a variety of organelles and like density gradient centrifugation, usually required a pre-purification of the initial homogenates which was generally accomplished by a series of differential purification steps (Islinger et al. 2010b).

Unfortunately, the isolation of PO is generally hampered by the similar electrophoretic mobility of mitochondria leading to insufficient separation of both organelles (Hannig and Heidrich 1974). To circumvent this problem, Völkl and colleagues invented the technique of immuno-FFE to increase the electrophoretic differences between both organelles (Völkl et al. 1997) Immunoglobulin molecules exhibit an isoelectric point close to a pH of 8.0, hence, binding of antibodies to PO surface membrane proteins will reduce the organelles' mobility in a buffer pH 8.0.

Incubation of a peroxisome-enriched light mitochondrial fraction with an antibody directed against the cytoplasmic C-terminus of the PO membrane protein PMP70 (ABCD3) significantly reduced the electrophoretic mobility of PO in the FFE thereby allowing a successful isolation from contaminating organelles, which was comparable to a PO fraction purified by density centrifugation using a sigmoidal Optiprep gradient (Völkl et al. 1997). Remarkably, immune-FFE could be successfully applied for the purification of PO subpopulations from the microsomal and heavy mitochondrial subcellular pre-fractions (Völkl et al. 1988; Mohr and Völkl 2002). Comparing the relative amounts for different PO proteins in the individual PO fractions showed that liver peroxisomes consist of heterogeneous peroxisomal populations exhibiting distinct protein patterns. Despite of these findings, the purification of PO from the heavy mitochondrial fractions did also show the limitations of the separation technique. As the peroxisomes found in this fraction possess relatively low amounts of PMP70, the PO isolated from this fraction are not of high purity which prevented a successful functional characterization (Mohr and Völkl 2002). Antibodies coupled to magnetic metal beads are routinely used for the purification of protein complexes from cellular lysates. Thus, it is not surprising that such immunoprecipitation (IP) approaches have been adapted for the purification of organelles. However, physiological buffer conditions have to be maintained during the purification process, thus precluding the use of high salt and detergent concentrations usually applied during washing steps for the purification of protein complexes. To prevent unspecific aggregation of contaminating membrane structures during the precipitation process, Lüers and colleagues avoided pelleting of the peroxisome bound magnetic particles using a continuous immunomagnetic sorter (CIMS) (Luers et al. 1998). In this device magnetic poles were arranged perpendicular to the buffer flow in a separation chamber, thus, deflecting PO-carrying magnetic beads from the main particle flow. To attach PO to the magnetic beads an antibody directed against the cytoplasmic C-terminus of the PO membrane transporter PMP70 was directly covalently coupled to commercially available Dynabeads (now Thermo Fisher Scientific). The antibody-coupled beads were subsequently incubated with light mitochondrial fractions from either liver or HepG2 cells. Enyzme assays and immunoblots for PO marker proteins revealed a strong PO enrichment if compared to the starting materials. During more recent years, immunoisolation of PO was performed directly from post nuclear supernatants applying batch purification techniques.

To purify PO from L6 rat myoblasts, PMP70-directed antibodies were bound to Protein A coated silanized magnetic iron oxide beads (1–4 μm in diameter) and incubated with the respective post nuclear fraction (Wang et al. 2012). Measurements of lysosomal and mitochondrial markers implied a >30-fold enrichment above the starting material. Moreover, assays for PO fatty acid β-oxidation and biotransformation of doxorubicin revealed that the PO were sufficiently preserved most of their functional capabilities. A similar approach was recently used to purify a specific PO subpopulation from the PNS of mouse adipose tissue (Liu et al. 2015). To this end antibodies against the PO ABC transporter ABCD2 were coupled to biotin and incubated with streptavidin magnetic beads.

Subsequently, the beads were incubated with the adipose PNS. After washing with homogenization buffers the beads were directly eluted with lysis buffer and analyzed by immunoblotting and mass spectrometry. As revealed by the protein identifications, the eluate contained a significant amount of PO but was still considerably contaminated with mitochondria and ER (identification of 9 PO, 22 mitochondrial and 14 ER proteins). In summary, antibody-based purification approaches permit a highly specific and efficient approach for the enrichment of PO from various sources; however, purification quality is strongly dependent on the availability of high affinity antibodies against PO membrane proteins, which have to be present at a considerable surface density. Moreover, the method requires antibodies in significant amounts and thus limits the quantity of the isolated analytes to a relatively small scale.

Flow field-flow fractionation (FlFFF) is a non-immunological technique which was recently used for the isolation from PO from HEK 293T cells (Yang et al. 2015). In FlFFF particles or molecules are separated according to differences in their hydrodynamic volumes. To this end a across flow is subjected against the main buffer flow pushing the analytes to the accumulation wall (Yohannes et al. 2011). In the normal mode Brownian motion of molecules opposes the cross flow against the accumulation wall and smaller analytes migrate closer to the middle of the channel than larger ones. Since the flow rate of the main buffer flow is higher in the center of separation channel than in proximity to the chamber wall, smaller compounds are eluted before larger ones from the separation chamber. For larger particles (approx. 1 μm), however, the Brownian motion becomes negligible and the particles are pressed firmly to the separation wall (Yohannes et al. 2011). Consequently, the separation process becomes inverted and larger particles emerge before smaller ones from the separation chamber (steric/hyperlayer mode). Fractionating a 15,000g supernatant from HEK 293 homogenates in an asymmetric FlFFF channel, allowed to enrich for lysosomes/endosomes, mitochondria and PO/microsomes in different peaks of the elution profile, however, subsequent proteome analysis reveals, that the different fractions are still significantly prone to cross contamination (Yang et al. 2015). Thus, even if the technique has obvious advantages in the time-scale of the separation process, significant improvements in the resolution of the separation are required to provide organelle fractions with a purity comparable to those from classic centrifugation-based separation techniques.

7 The Era of Proteomics—Opposing Approaches to Characterize Organelle Proteomes

During the earlier years of organelle proteome studies PO were usually purified by standard purification protocols isolating the organelles by first producing an PO enriched light mitochondrial fraction using differential centrifugation and a subsequent density gradient centrifugation step for final purification (see methods

described above). Since such purification schemes yield organelle purities of above 90%, subsequent MS analyses allowed to considerably expand the peroxisomal proteome from animal liver and kidney (Ofman et al. 2006; Islinger et al. 2006, 2007) plant tissue (Fukao et al. 2002; Reumann et al. 2007; Arai et al. 2008) and yeast species (Marelli et al. 2004; Thoms et al. 2008; Managadze et al. 2010; Yi et al. 2002). The ongoing improvements in MS sensitivity allow the detection of proteins with increasingly lower abundance, thus enabling to mine deeper into the organelle proteomes. However, in parallel more proteins from contaminating organelles are identified in the samples. Moreover, it is getting increasingly obvious, that a large number of proteins are shared by two or several subcellular compartments (Thul et al. 2017). Additionally, PO form contact sites with the ER, mitochondria and lysosomes (Costello et al. 2017; Mattiazzi Usaj et al. 2015; Chu et al. 2015), which will lead to a specific enrichment of proteins from other organelles in the purified fractions. Thus, distinguishing correctly allocated proteins of an organelle species from mere contaminants remains one of primary challenges in organelle proteomics. This problem is currently tackled by two alternative strategies. Most obviously a higher purity of the PO fractions will lead to fewer identifications of contaminating proteins. To this end multistep purification strategies, which combine different separation principals, have been used in current PO proteome studies. The first study with such a 2-dimensional purification strategy purified a liver PO fraction by linear Nycodenz-gradient centrifugation; subsequently the PO were further immunoisolated using a PMP70 antibody coupled to magnetic beads (Kikuchi et al. 2004). As judged (Mattiazzi Usaj et al. 2015) by the low number of proteins identified from other compartments, this strategy allowed producing highly pure PO fractions and led to the identification of several novel peroxisomal constituents such as Lon protease 2 and ACAD11. Since the separation principle of FFE relies primarily on the surface net charge of the organelle it is possible to combine the technique with the density-based gradient centrifugation in 2D-purification scheme. For the isolation of PO from Arabidopsis cell cultures light mitochondrial fractions were first purified on a discontinuous 25/50% Percoll gradient (Eubel et al. 2008). After centrifugation, the PO-enriched fraction at the interface of the two Percoll-solutions was separated by continuous zonal-FFE. In this FFE mode, the organelle mixture is continuously injected into the separation chamber of the instrument close its cathodic side, while an electric field is applied perpendicular to a permanent thin laminar buffer flow. As a consequence, individual organelle species migrate in different velocities to the anode according to their net surface charge. At the upper end of the separation chamber the analytes are collected via 96 outlets and sampled into 96-weel plates. Subsequently, the mitochondrial and PO peak fractions were located by immunoblotting of the fractions detecting marker proteins for mitochondria and PO. The purification success of the FFE was documented by 2D-gels of the mitochondrial and PO peaks, which were compared by DIGE. Overlay of the protein patterns allowed validate the successful separation of the two organelles. Isolated whole PO and membrane fractions were subsequently analyzed by MS. From the 89 proteins associated to PO, 35 novel candidates were not previously reported.

While PO enrich in highest concentrations in light mitochondrial fraction, which is thus usually used for the isolation of the organelles, significant PO amount can be also found in the heavy mitochondrial and microsomal fraction. Compared to PO from the light mitochondrial fraction, such "heavy PO" (HM-PO) are largely devoid of the PO membrane protein PMP70, which was successfully used for immunoisolation of the PO (Völkl et al. 1998; Mohr and Völkl 2002; Kikuchi et al. 2004). As an alternative, a purification strategy combining density gradient centrifugation and FFE was used for PO isolation (Islinger et al. 2010c). For PO isolation the heavy mitochondrial fraction was initially separated on an Optiprep step gradient; a PO-enriched fraction accumulating at a density of 1.18 g/ml was further separated via zonal FFE. Compared to the FFE conditions used for the isolation of Arabidopsis PO, the separation buffer consisted of a two component, low ionic strength buffer system, permitting high voltages at a low current (Islinger et al. 2010c). PO peaks fraction were localized by marker enzyme assays and immunoblotting and subsequently subjected to quantitative MS analysis using ITRAQ technology. Comparing HM-PO with PO isolated from the light mitochondrial fraction by standard methods (LM-PO) allowed to confirm the obvious lack of PMP70 in HM-PO but also to detect further metabolic differences between both PO fractions. These findings are in line with ultrastructural observations detecting heterogeneous subpopulations of PO in neighboring hepatocytes of even inside single cells (Islinger et al. 2010c).

The emergence of increasingly sophisticated quantitative MS techniques and analysis software allows following a very different strategy to assign organellar protein allocations. Protein correlation profiling of serially eluted fractions allows comparing abundance of individual proteins across a density gradient. Thus, this method does not require a maximal purity of the PO fraction since contaminating proteins can be identified according to their continuous enrichment in non-PO fractions. Such a strategy was originally invented in a proteome study for the characterization of the mouse kidney PO proteome (Wiese et al. 2007). Light mitochondrial fractions were separated in a linear 10–35% Nycodenz gradient with a 50% cushion. After centrifugation the gradient was eluted into fraction of equal volumes and 6 fractions covering PO and mitochondrial peaks were analyzed by MS. Peptide peak volumes with the same M/Z ratio were used to quantify the abundance of proteins in the different fractions. Profiles of selected reference PO and mitochondrial proteins were used to generate organelle characteristic distribution patterns across the gradient, which were subsequently used to validate the allocation of proteins with unknown or unsecure organelle localization (Wiese et al. 2007). The approach led to the identification of a significant number of novel PO proteins, which were partially further confirmed in gene expression experiments. Comparable protein correlation profiling strategies were in the meanwhile used for the characterization of PO from rat and human liver, documenting the experimental significance of the method (Schollenberger et al. 2010; Gronemeyer et al. 2013a, b). In an attempt to allocate the total liver proteome, Jadot and colleagues 2017 even reduced the resolution of subcellular fractionation applying protein correlation profiling already directly to the subcellular fraction gained from differential

centrifugation (Jadot et al. 2017). Nevertheless, to address PO and lysosomal localization, which cannot be separated by mere differential centrifugation, Nycodenz and sucrose density gradients were used to specifically enrich these organelles. By combining the power of sensitive modern MS and statistical analysis of peptide distributions, the publication allocates more than 6000 proteins to a single or several subcellular compartments. In this respect, the publication is an intriguing example, how innovations in analytical technology alter the needs for highly resolving subcellular fractionation techniques. Nevertheless, powerful fractionation technologies will still be necessary to define the proteomes of specific organelle subpopulations, which do not enrich in gross subcellular fractions or to validate the precise allocation of a protein with multiple subcellular locations.

8 Challenges for the Future

The different purification protocols developed for the isolation of PO have been successfully used to purify the organelles for proteomic studies thus allowing to define the principal protein inventory of PO from animals, plants and fungi. Modern methods of quantitative mass spectrometry additionally permit to compare different peak fractions from the same separation in order to assign protein distribution profiles to discriminate between true organelle constituents from contaminants or to analyze PO in adaptation to different physiological states of cells and tissues. Nevertheless, sophisticated validation experiments using e.g. immunolocalization by electron microscopy or co-localization by confocal immunofluorescence microscopy should be performed to corroborate the proteomics findings. Despite the valuable improvements in the field of mass spectrometry, the development of novel purification strategies will still be necessary to tackle some of the unsolved problems of PO biology. PO exhibit a remarkable heterogeneity with respect to tissue and cell function. Especially in heterogeneous organs like brain, sophisticated separation protocols are required to isolate the individual PO from different cell types in order to define their function-associated proteome. Organelle contacts sites are enriched in a specialized set of proteins, which facilitate the transmission of metabolites or signals between different cellular compartments. Preserving and in parallel separating such structures in the purification process will be demanding challenge for the future but would certainly allow to identify contact-specific proteins and their associated functional significance. Comparably, the isolation and proteomic characterization of pre-PO vesicles could give important novel insights into the pathways of PO biogenesis. As a consequence, even after decades of PO purification the development of novel separation technologies and the improvement of the existing method inventory could still help to unravel hitherto unanswered questions in PO biology.

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Part II
Predicting Peroxisomal Proteomes

Prediction of Peroxisomal Matrix Proteins in Plants

Sigrun Reumann and Gopal Chowdhary

Abstract Our knowledge of the proteome of plant peroxisomes is far from being complete, and the functional complexity and plasticity of this cell organelle are amazingly high particularly in plants, as exemplified by the model species *Arabidopsis thaliana*. Plant-specific peroxisome functions that have been uncovered only recently include, for instance, the participation of peroxisomes in phylloquinone and biotin biosynthesis. Experimental proteome studies have been proved very successful in defining the proteome of Arabidopsis peroxisomes but this approach also faces significant challenges and limitations. Complementary to experimental approaches, computational methods have emerged as important powerful tools to define the proteome of soluble matrix proteins of plant peroxisomes. Compared to other cell organelles such as mitochondria, plastids and the ER, the simultaneous operation of two major import pathways for soluble proteins in peroxisomes is rather atypical. Novel machine learning prediction approaches have been developed for peroxisome targeting signals type 1 (PTS1) and revealed high sensitivity and specificity, as validated by in vivo subcellular targeting analyses in diverse transient plant expression systems. Accordingly, the algorithms allow the correct prediction of many novel peroxisome-targeted proteins from plant genome sequences and the discovery of additional organelle functions. In contrast, the prediction of PTS2 proteins largely remains restricted to genome searches by conserved patterns contrary to more advanced machine learning methods. Here, we summarize and discuss the capabilities and accuracies of available prediction algorithms for PTS1 and PTS2 carrying proteins.

S. Reumann (✉)
Department of Plant Biochemistry and Infection Biology,
Institute of Plant Science and Microbiology, University of Hamburg,
Ohnhorststr. 18, 22609 Hamburg, Germany
e-mail: sigrun.reumann@uni-hamburg.de

G. Chowdhary
KIIT School of Biotechnology, Campus XI, KIIT University,
Bhubaneswar 751024, India
e-mail: gkchowdhary@kiitbiotech.ac.in

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Abbreviations

CML	Calmodulin-like protein
EYFP	Enhanced yellow fluorescent protein
PEX	Peroxin
PTS1/2	Peroxisomal targeting signal type 1/2
TPR	Tetratricopeptide (34-amino acid) repeat
PWM	Position weight matrix

1 Prediction of PTS1 Proteins

All soluble matrix proteins of peroxisomes are nuclear-encoded and synthesized on free cytosolic ribosomes with specific signals that direct them post-translationally into the peroxisomal matrix. The majority of matrix proteins possess a peroxisome targeting signal type 1 (PTS1), which consists of a C-terminal tripeptide such as SKL> (">" refers to the C-terminal protein end) and auxiliary residues located immediately upstream (Gould et al. 1987, 1989; Swinkels et al. 1992; Kragler et al. 1998; Lametschwandtner et al. 1998). Transport of PTS1 proteins into the peroxisomal matrix is mediated by a set of peroxins encoded by *PEX* genes that are required for peroxisome biogenesis (Distel et al. 1996; Hu et al. 2012; Theodoulou et al. 2013; Baker and Paudyal 2014). In brief, soluble proteins carrying a surface-exposed PTS1 are recognized by the conserved cytosolic receptor, PEX5 (the number reflects the chronology of identification, Hayashi et al. 2005; Kragler et al. 1998; Distel et al. 1996; Wimmer et al. 1998). Cargo-loaded PEX5 diffuses to the peroxisomal membrane and docks to the importomer, which is the central membrane-embedded protein import complex that also enables cargo translocation into the matrix (Rayapuram and Subramani 2006; Meinecke et al. 2010). Interestingly, a second homolog of the tetratricopeptide repeat (TPR) protein family was identified recently in *Saccharomyces cerevisiae*, named PEX9, and was characterized as a specific receptor for a subset of peroxisomal matrix proteins, such as an oleate-inducible malate synthase isoform (Effelsberg et al. 2016; Yifrach et al. 2016).

1.1 Canonical Versus Non-canonical PTS1s

PTS1 tripeptides can be classified into canonical and non-canonical sequences. Canonical plant PTS1 tripeptides confer strong peroxisome targeting efficiency to reporter proteins and match the consensus sequence [SA][RK][LMI] > at all three tripeptide positions (Mullen et al. 1997; Lametschwandtner et al. 1998;

Kragler et al. 1998; Reumann 2004; Lingner et al. 2011). These PTS1 tripeptides and their position-specific individual tripeptide residues occur frequently in higher plant PTS1 proteins and have been experimentally demonstrated to function as strong tripeptides and residues for peroxisome targeting, respectively. Canonical PTS1 tripeptides generally are sufficient for peroxisome targeting and mediate high-affinity binding to PEX5. Nevertheless, upstream amino acid residues have been shown to affect PEX5 affinity also for canonical PTS1 tripeptides (Mullen et al. 1997; Hayashi et al. 1997; Kragler et al. 1998; Reumann 2004; Lingner et al. 2011; Brocard and Hartig 2006; Neuberger et al. 2003a, b; Fodor et al. 2012; Lametschwandtner et al. 1998; Maynard et al. 2004).

Non-canonical PTS1 tripeptides generally carry one non-canonical amino acid residue at one tripeptide position (e.g., TRL>, SDL>, and SRV>, non-canonical residues underlined). Nearly all experimentally verified plant PTS1 tripeptides identified to date follow the pattern that one low-abundance PTS1 residue (denoted as x, y, or z) is combined with two high-abundance PTS1 tripeptide residues (x[KR][LMI]>, [SA]y[LMI]>, [SA][KR]z>). Importantly, this PTS1 classification into canonical and non-canonical tripeptides is simplified and reflects the present status of experimental results and predictions. For instance, SNV> was also validated as a functional plant PTS1 tripeptide, carrying Asn (pos. -2) and Val (pos. -3) and, hence, two low abundance residues (Skoulding et al. 2015).

Non-canonical PTS1 tripeptides alone generally represent weak signals and often require auxiliary targeting-enhancing patterns (e.g., basic residues) for functionality. These are located immediately upstream of the tripeptide and are often kingdom-specific (Neuberger et al. 2003b; Lametschwandtner et al. 1998; Kragler et al. 1998; Ma and Reumann 2008). According to present knowledge, 35 functional plant PTS1 tripeptide residues have been reported. The residues are distributed in the following manner: ([SAPCFVGTLKIQ] [RKNMSLHGETFPQCYDA] [LMIVYF]>), leading to twelve (pos. -3), 17 (pos. -2), and six (pos. -1) allowed amino acid residues in plant PTS1 tripeptides. The targeting strength of PTS1 tripeptides could be classified by in vivo subcellular targeting analyses into three categories: strong, moderate and weak. This classification is based on the time required for the PTS1 to target a reporter protein such as enhanced yellow fluorescent protein (EYFP) to peroxisomes. Further details on this topic are summarized below Sect. (1.3) and available in the authors' publication (Skoulding et al. 2015).

1.2 Prediction Algorithms for PTS1 Proteins

Similar to fungi and animal PTS1s, plant PTS1s exhibit a conserved pattern in the primary sequence level that can be utilized to predict novel peroxisomal proteins by computational approaches. The PTS1 pattern with characteristic features includes the PTS1 tripeptide and several amino acids immediately upstream of the tripeptide. Global biochemical properties and N-terminal targeting information of the protein

can sometimes be added to the prediction models. By utilizing a suitable PTS1 prediction approach in combination with genome information for a species of interest, peroxisomal proteomes of PTS1 proteins can now be predicted in a straightforward manner.

Prediction models are validated for their accuracy by calculation of their prediction sensitivity and specificity. The sensitivity is usually determined as the ratio between correctly predicted peroxisomal proteins (true positives) and the number of all known peroxisomal proteins. The specificity can be assessed by dividing the number of true positives by the number of all (true and falsely) predicted peroxisomal proteins (for more details, see Reumann et al. 2016). Prediction models are usually trained on the larger subset of "training" example sequences, while the accuracy (i.e. sensitivity and specificity) is estimated on a so-called test set of the remaining "unseen" example sequences. In general, the prediction accuracy strongly increases with the size and sequence diversity of the set of example sequences.

In the past decades, several approaches for sequence-based prediction of PTS1 proteins were presented. The first approach developed by Nakai and Kanehisa (1992) was based on overall characteristic amino acid content and a conserved motif ([SA][KRH]L as defined in Gould et al. 1989). Due to a limited set of positive example sequences, the prediction accuracy of the later developed webserver PSORT remained low. The prediction algorithms of PSORTII and WoLF PSORT were based on a larger set of training sequences but did not improve the accuracy significantly (Nakai and Kanehisa 1992; Horton et al. 2007). The PTS1predictor (http://mendel.imp.ac.at/pts1/) built in 2003 is still leading in the field and is based on characteristic structural and functional features of more than 300 PTS1 sequences from metazoa, fungi and plants (Neuberger et al. 2003a, b). The algorithm takes the twelve C-terminal amino acids into consideration and evaluates both sequence conservation and structural properties. For plants, however, only a general prediction model is available, contrary to taxa-specific algorithms for metazoa and fungi. Further PTS1 prediction approaches comprise the PeroxiP method (Emanuelsson et al. 2003; discontinued) and the PTS1Prowler algorithm (Hawkins et al. 2007), which was later integrated into the PProwler server (Boden and Hawkins 2005). For details, the reader is referred to our previous publication (Reumann et al. 2016).

The first plant-specific prediction approach for PTS1-containing proteins was published by our group (Lingner et al. 2011), followed by presentation of the public web server PredPlantPTS1 (http://ppp.gobics.de/, Reumann et al. 2012). For development of the prediction model, a large set of plant PTS1 sequences homologous to known *A. thaliana* PTS1 sequences was manually identified in protein and EST databases and was manually verified. Positive and negative example sequences were analyzed by a discriminative machine learning model without any restrictions on the tripeptide pattern. The 14 C-terminal amino acids were found to contain discriminative properties. We confirmed the high prediction accuracy of the algorithm by in vivo subcellular targeting analyses of PTS1 decapeptides and full-length proteins fused N-terminally to reporter proteins.

Most importantly (because most challenging in terms of PTS1 protein prediction), several novel peroxisomal proteins bearing non-canonical PTS1 tripeptides were newly identified since publication of the algorithm (Lingner et al. 2011; Kataya et al. 2015a, b, 2016; Kataya and Reumann 2010; Chowdhary et al. 2012; for review see Reumann and Bartel 2016). Notably, the use of a large number of positive and negative example sequences allowed the statistically founded deduction of so-called posterior probabilities (or balanced targeting probability) for peroxisomal targeting between 0 and 100%, which are easier to interpret. Moreover, these balanced posterior probabilities of PTS1 peptides were found to correlate well with experimentally measured binding affinities to Arabidopsis PEX5 (Skoulding et al. 2015).

Wang et al. (2017) recently presented another computational model for the prediction of plant PTS1 proteins. A major difference compared to the above-mentioned machine learning methods, is the authors' claim that also the residues located distantly of the PTS1 tripeptide (between pos. -30 and -15) contained discriminative features distinct from non-peroxisomal proteins (Wang et al. 2017). The prediction model called PPero is publicly available (https://biocomputer.bio.cuhk.edu.hk/PP/).

1.3 Prediction and Analysis of Peroxisome Targeting Efficiency

For several reasons, it is often desirable to predict the efficiency at which proteins are targeted to peroxisomes, as outlined previously (Reumann et al. 2016). In vivo subcellular targeting analyses are the gold standard for studying protein localization in peroxisomes to date, and several suitable transient expression systems have been established for in vivo subcellular targeting analyses (Reumann et al. 2016). Only very few studies, however, have also addressed targeting efficiency and were shown to be suited to yield semi-quantitative results. Onion epidermal cells, for instance, used for long-term expression studies over several days of cold incubation allowed the observation of weak peroxisome targeting (Lingner et al. 2011). In the same expression system, it was possible to even resolve significant differences in strong peroxisome targeting efficiency for two canonical PTS1 decapeptides terminating with either SRM> or SRI> after very short expression times (Skoulding et al. 2015).

Thermodynamic in vitro analyses of binding constants are a valuable complementary method to obtain quantitative data of cargo-PEX5 interactions. In fluorescence anisotropy-based assays the affinity of synthetic PTS1 peptides to recombinant PEX5 is determined in a competition experiment, in which a constant, fluorescently labelled peptide bound to PEX5 is replaced by diverse PTS1 peptides of interest (Gatto et al. 2000, 2003; Maynard and Berg 2007). We carried out a systematic comparative analysis of in silico predictions, in vivo subcellular localization data and in vitro thermodynamic binding constant analyses for one model

PTS1 decapeptide and its cytosolic receptor PEX5 and several amino acid residue point mutations of the PTS1 (Skoulding et al. 2015). A good correlation was found between the two experimental methods and the prediction scores. While in vivo subcellular localizations studies turned out to be more sensitive, thermodynamic binding assays yielded quantitative results and allowed a finer discrimination between similar PTS1 peptides (Skoulding et al. 2015). The finding that the position weight matrix (PWM) prediction scores and posterior probabilities also predict the efficiency of protein import into plant peroxisomes is valuable because both experimental methods are laborious and time-consuming compared to the application of prediction tools.

2 PTS2 Nonapeptide Definition and Prediction of PTS2 Proteins

The second targeting signal of peroxisomal matrix proteins is the so-called PTS2 (Swinkels et al. 1991; Osumi et al. 1992). The major targeting information of the PTS2 is included in a conserved nonapeptide of the prototype RLx_5HL located in the N-terminal domain. Four residues of the nonapeptide are highly conserved and spaced by five rather variable residues (Kato et al. 1996, 1998). Interestingly, the number of known PTS2 proteins is rather small in most organisms. In plants, however, as exemplified for Arabidopsis, the number of known PTS2 proteins is with approx. 20 matrix proteins relatively high.

The targeting pathway from the cytosol to the peroxisomal matrix uses pathway-specific PEX proteins in the beginning, before thought to merge with the PTS1 pathway at the peroxisomal membrane. The cytosolic receptor for PTS2 proteins is PEX7, a soluble protein with six WD40 domains. Contrary to PEX5, which is sufficient to target PTS1 containing proteins to the peroxisomal membrane, many organisms require one or two additional co-receptors for proper targeting of PTS2 containing proteins. For instance, *S. cerevisiae* needs PEX18 and PEX21 (Purdue et al. 1998), while in other fungi PTS2 protein import depends on PEX20 (an ortholog of PEX21). In plants and mammals co-receptors of the PEX18/20/21 family have not been reported yet, but PTS2 protein import by PEX7 requires the long version of PEX5 with its PEX7 interaction domain, implying that PEX5 takes over the function of the PEX7 co-receptor in these kingdoms (Dodt et al. 2001; Woodward and Bartel 2005; Ramon and Bartel 2010; Khan and Zolman 2010). The Erdmann group (Ruhr-University Bochum, Germany) recently characterized electrophysiologically a distinct PTS2-specific pore, which consisted of the PTS2 co-receptor PEX18 and the PEX14/Pex17-docking complex as major constituents and also allowed import of folded PTS2 proteins (Montilla-Martinez et al. 2015). Contrary to the PTS1 pore, the reconstituted PTS2 channel was constitutively present in an open state. The new results question the previous concept according to which both import pathways were thought to converge at the peroxisomal

membrane (Montilla-Martinez et al. 2015). Contrary to PTS1 proteins, which are not processed in the matrix, the PTS2 domain is cleaved upon import into peroxisomes by a trypsin-like endopeptidase, referred to as DEG15 in plants (TYSND1 in mammals, Helm et al. 2007; Schuhmann et al. 2008). Dimerization of AtDEG15 was shown to be mediated by the Calmodulin-like protein, AtCML3 (Dolze et al. 2013).

Initial PTS2 analyses by sequence conservation and site-directed mutagenesis revealed that the first two and the last two positions are most conserved in PTS2 nonapeptides in all organismal groups. According to present knowledge, pos. 1 and 8 of the PTS2 nonapeptide are nearly constant with Arg and His, respectively. Both residues are only rarely replaced each by single alternatives, namely Arg by Lys (pos. 1), thus showing a requirement for a positively charged residue, and His by Gln (pos. 8, Fig. 1). Four and three possible hydrophobic residues can occur at pos. 2 (L, I, Q or V) and pos. 9 (L, A or F), respectively (Fig. 1; Petriv et al. 2004).

Initially, the five middle residues were considered highly variable and flexible, any lacking sequence conservation in different orthologous groups. However, advanced computational analyses revealed a preference also at these positions for certain residues. A preference for hydrophobic residues was found also at pos. 5 (L, V, I, H or Q) and 6 (L, S, G, A or K, Petriv et al. 2004). Moreover, with increasing knowledge of the peroxisomal proteome additional PTS2 proteins were identified and an extended consensus PTS2 motif was deduced that included all known PTS2 nonapeptides ([RK][LVIQ]x_2[LVIHQ][LSGAK]x[HQ][LAF], Fig. 1; Petriv et al. 2004).

Kunze et al. (2011) added structural characteristics of the PTS2 receptor and the PTS2 proteins to prediction algorithms by performing mutational studies of PTS2. Using the PTS2 of human thiolase as model nonapeptide, the authors revealed that bulky aliphatic amino acids are essential at pos. 5 for a functional PTS2, while both positively and negatively charged residues at the same position rendered the signal non-functional (Kunze et al. 2011). At pos. 4 the amino acid preference and mutational effect was similar for negatively charged residues.

The similarity between peroxisomal PTS2 and mitochondrial presequences had been early noticed, and single amino acid mutations in the PTS2 domain, such as H-to-R/L (pos. 8), could redirect reporter genes to mitochondria (Osumi et al.

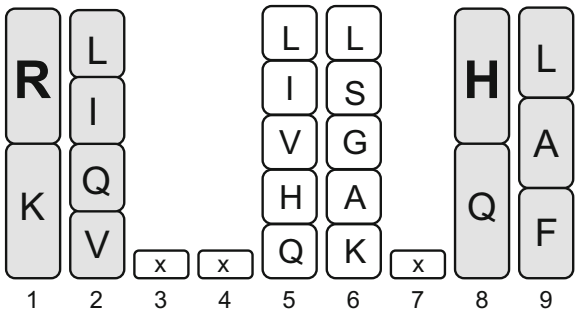

Fig. 1 Graphical presentation of the general PTS2 motif (Petriv et al. 2004). The four most conserved residues of the PTS2 nonapeptide are shaded gray

1992). Even single point mutations in the x_5 sequence, such as the introduction of a basic residue at pos. 4 or 5, directed the reporter protein partially to mitochondria (Kunze et al. 2011).

The secondary structure of PTS2 nonapeptides remained long unknown. The hypothesis that the PTS2 nonapeptide forms an α-helix (Reumann 2004; Fig. 2) was strongly supported by the fact that the mutation of a hydrophobic residue to the helix breaking residue, proline, at pos. 6 abolished peroxisome targeting (Kunze et al. 2011; Fig. 2). By generating a homology-based structural model of PEX7, Kunze et al. (2011) could show that human PEX7 formed a groove with an evolutionary conserved charge distribution complementary to the PTS2. The predicted PTS2-PEX7 interaction site was confirmed by mammalian two-hybrid studies. Based on all these PTS2 characteristics, the authors developed a computational screening method and identified a fourth PTS2 protein for mammals, namely potassium channel interacting protein 4 (RVx_5HL, Kunze et al. 2011).

Conclusive evidence for the PTS2 forming an amphipathic α-helix (Fig. 2), similar to mitochondrial presequences and plastidic transit peptides (Kunze and Berger 2015), was provided by structural analyses. Pan et al. (2013) determined the structure of the ternary complex of *S. cerevisiae* PEX7, the C-terminal domain of PEX21 and the PTS2 from thiolase at 1.8 Å resolution. Accordingly, PEX7 forms a ring structure with a seven-bladed propeller fold formed by the typical WD40 repeats and acts as a platform for binding of both PEX21 and PTS2 cargo. Both receptors form a binding groove in a cooperative manner for the amphipathic α-helix of the PTS2 (Pan et al. 2013).

Prediction methodologies for PTS2 proteins can be classified into simpler motif-based methods and more advanced machine learning methods. Motif-based methods are solely based on the detection of short peptides included in the applied motif, which can be relaxed or specific (stringent, see above). Bodén and Hawkins (2006) combined different motifs in a hierarchical manner from relaxed to stringent. Their PTS2 motif included both "positive" and "negative" properties. The authors claimed that their prediction method had a discriminative accuracy exceeding previously manually curated motifs and could be used to screen genomic data for putative peroxisomal proteins. Applied to the Arabidopsis genome, 76 putative PTS2 proteins were identified (Bodén and Hawkins 2006). Unfortunately, the Arabidopsis proteins were not published, and a public prediction webserver was not created.

Machine learning methods require a large and diverse dataset of positive example sequences to discriminate between PTS2-specific and other protein-specific conserved features. Due to the low number of PTS2 proteins in most organisms (except for plants) and due to the lack of a sufficiently large training data set of positive example sequences, true machine learning methods are not available yet for the prediction of PTS2 proteins. However, in due course of time, (i) as the peroxisomal proteome knowledge will get deeper and richer, (ii) as more peroxisomal PTS2 proteins will become known and (iii) as more genome sequence information will become available, the training data set of positive PTS2 protein example sequences will steadily increase. This development will facilitate the establishment of robust accurate PTS2 protein prediction algorithms for plants,

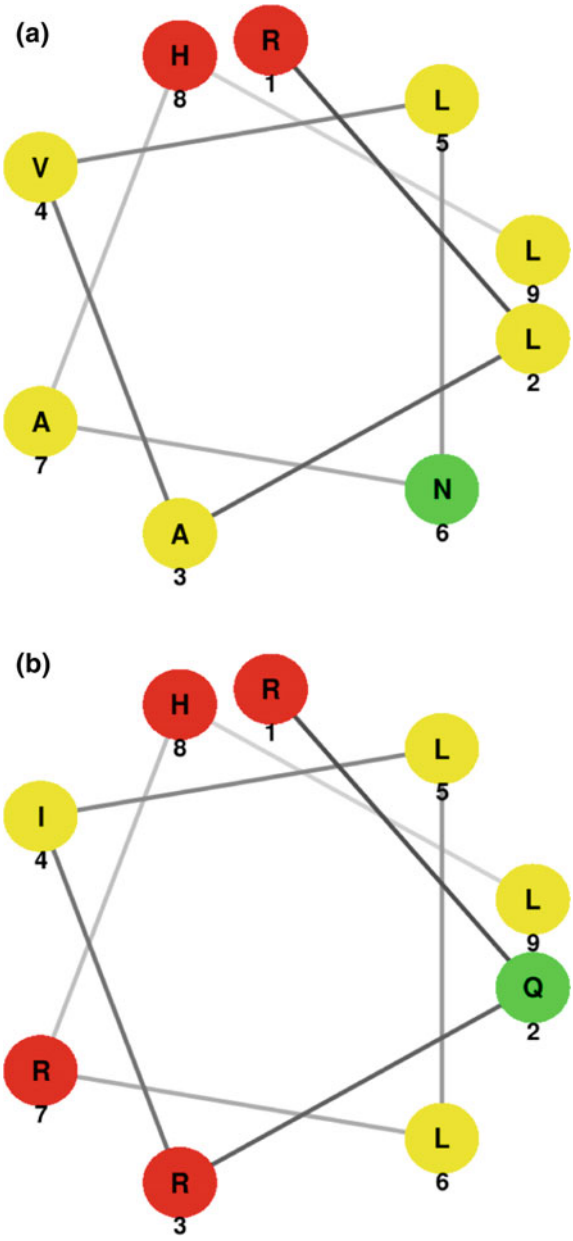

Fig. 2 Helical wheel presentation for two PTS2 of Arabidopsis proteins. NetWheels (http://lbqp.unb.br/NetWheels/) was applied to show the positioning of the nine residues of the PTS2 nonapeptide in the amphipathic α-helix. **a** Arabidopsis citrate synthase (At3g58740, CSY1, RLAVLNAHL) and **b** thiolase (At1g04710, KAT1/PKT4, RQRILLRHL) serve as examples of plant PTS2 proteins. The nonapeptide residues are numbered below the circles (from 1–9). Polar residues are colored red (basic residues), blue (acidic) and green (uncharged), and nonpolar residues are shown in yellow. To mimic a 3-dimensional view from the top into the helix, the lines indicating the peptide bonds are shown as a color gradient from black (beginning of the peptide) to light gray (end of the peptide)

which will most likely also be well applicable to all other eukaryotes that possess the import route of the PTS2 pathway into peroxisomes. Key to successful PTS2 protein prediction will also be the integration of quantitative affinity data between PEX7 and its PTS2 cargo as well as structural data into the prediction models.

3 Conclusions and Future Perspectives

It is well established that the PTS1 is the predominant targeting signal for peroxisome import of matrix proteins. The larger number of positive PTS1 example sequences and the signal's precise position at the C-terminus made it possible to develop successful prediction algorithms. Regarding the PTS2, the restriction to small data sets of positive example sequences, the signal's flexibility in primary structure and its positional flexibility in the N-terminal domain made it difficult to develop accurate prediction algorithms for PTS2 proteins up to now. However, in due course of time, as the peroxisome proteome resources will become richer, the number of known PTS2 proteins will increase. Even more rapidly, the number of fully sequenced eukaryotic genomes increases exponentially, leading to a significant increase of orthologous PTS2-containing sequences per newly identified PTS2 protein. These facts altogether will increase and improve the quantity and quality (e.g. diversity) of the dataset of positive PTS2 protein example sequences, which will facilitate the development of robust PTS2 protein prediction algorithms in the near future.

The peroxisome is the only organelle having two different types of targeting signals for soluble proteins of the organelle matrix, while mitochondria, plastids and the ER evolved only one type of targeting signal, namely a presequence, a transit peptide and a signal peptide, respectively. It is presently unknown why eukaryotes evolved and maintained two different import pathways for peroxisomal matrix proteins and whether one of them is superior, for instance, in terms of import efficiency or specificity, or substrate range and size. A model for the sequential evolution of the two import pathways for peroxisomal matrix proteins has been proposed, starting with the evolution of the PTS2 import pathway and being followed by the PTS1 import pathway for soluble proteins into peroxisomes. (Reumann et al. 2016). It will be interesting to validate this model, for instance by the detection of cargo intermediates of both pathway in ancient organisms.

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The Obvious and the Hidden: Prediction and Function of Fungal Peroxisomal Matrix Proteins

Johannes Freitag, Thorsten Stehlik, Alina C. Stiebler and Michael Bölker

Abstract Fungal peroxisomes are characterized by a number of specific biological functions. To understand the physiology and biochemistry of these organelles knowledge of the proteome content is crucial. Here, we address different strategies to predict peroxisomal proteins by bioinformatics approaches. These tools range from simple text searches to network based learning strategies. A complication of this analysis is the existence of cryptic peroxisomal proteins, which are overlooked in conventional bioinformatics queries. These include proteins where targeting information results from transcriptional and posttranscriptional alterations. But also proteins with low efficiency targeting motifs that are predominantly localized in the cytosol, and proteins lacking any canonical targeting information, can play important roles within peroxisomes. Many of these proteins are so far unpredictable. Detection and characterization of these cryptic peroxisomal proteins revealed the presence of novel peroxisomal enzymatic reaction networks in fungi.

Keywords Motif prediction · Dual targeting · Peroxisome · Translational readthrough · Alternative splicing · Glycolysis · Redox homeostasis

1 Peroxisomes in Fungi

Peroxisomes are ubiquitous organelles that are surrounded by a single membrane and play a major role in fatty acid degradation and hydrogen peroxide detoxification (for a general review about peroxisomes see Smith and Aitchison (2013)). Comprehensive understanding of peroxisomes in fungi is of special interest, since fungal species display a wide variety of specialized metabolic pathways and peroxisomal functions, that are not found in plants and animals (van der Klei and Veenhuis 2006a; Stehlik et al. 2014). Moreover, peroxisomes are required for

J. Freitag · T. Stehlik · A. C. Stiebler · M. Bölker (✉)
Department of Biology, Philipps-Universität Marburg, Marburg, Germany
e-mail: boelker@staff.uni-marburg.de

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sexual reproduction and fruiting body formation in different fungal species (Peraza-Reyes and Berteaux-Lecellier 2013). Many fungi produce and secret secondary metabolites (Keller et al. 2005). For some of these metabolites, e.g. penicillin or surface active glycolipids it was shown that part of their biosynthesis take place inside peroxisomes (Bartoszewska et al. 2011; Freitag et al. 2014). Functional peroxisomes are critical for virulence in many fungal plant pathogens that are responsible for severe agricultural damage (Dean et al. 2012). The ascomycetous fungi *Magnaporthe oryzae* and *Colletotrichum orbiculare* infect their hosts with specialized heavily melanized infection structures called appressoria (Wilson and Talbot 2009). Melanin biosynthesis requires acetyl-CoA derived from beta-oxidation. Thus, *M. oryzae* and *C. orbiculare* mutants with defective peroxisomal functions are less melanized and not virulent (Kimura et al. 2001; Bhambra et al. 2006; Wang et al. 2007; Fujihara et al. 2010). Remarkably, after penetration of the plant timely degradation of peroxisomes via Atg26 mediated autophagy is necessary for further pathogenic development of *C. orbiculare* (Asakura et al. 2009). In addition, filamentous ascomycetes contain highly specialized peroxisomes (Woronin bodies) to seal septal pores after wounding of hyphae to prevent leakage of the cytoplasm (Jedd and Chua 2000). In *M. oryzae,* woronin bodies are essential for the infection of rice plants (Soundararajan et al. 2004). Also in pathogenic basidiomycetes an important role for peroxisomes and peroxisomal beta-oxidation for virulence has been described (Klose and Kronstad 2006; Kretschmer et al. 2012b; Freitag et al. 2012). *Ustilago maydis* cells compromised for the import of peroxisomal matrix proteins (e.g. Δ*pex6* cells) are nonpathogenic on corn plants while mutants lacking only the multifunctional enzyme required for peroxisomal ß-oxidation are still virulent (Klose and Kronstad 2006; Freitag et al. 2012). This data indicate that *U. maydis* peroxisomes play an important role during plant infection beside breakdown of fatty acids.

Fungi have been widely used to study general aspects of peroxisome function and biogenesis. A large fraction of today's data and models about peroxisomal protein import are derived from studies in *Saccharomyces cerevisiae* or related yeasts such as *Pichia pastoris* and *Hansenula polymorpha* (van der Klei and Veenhuis 2006a; Pieuchot and Jedd 2012). Filamentous fungi are also interesting models to study long distance transport of peroxisomes via microtubules (Guimaraes et al. 2015; Salogiannis et al. 2016).

In the basidiomycetes *Cryptococcus neoformans* and *U. maydis* intact peroxisomes are important for sugar metabolism beside their classical function in fatty acid degradation (Idnurm et al. 2007; Freitag et al. 2012). In contrast to budding yeast, *U. maydis* cells contain a high number of peroxisomes already in the absence of external fatty acids. Analyzing the putative peroxisomal proteome of *U. maydis* revealed a number of novel PTS1 proteins and interesting parallels of human and fungal peroxisomes (Camões et al. 2015).

2 Predicting Peroxisomal Proteins

All peroxisomal matrix proteins are translated in the cytosol and have to be imported. Since peroxisomes are difficult to purify and are diverse depending on their biological function, their proteome is not easy to determine. Therefore bioinformatic prediction plays an important role in the elucidation of the peroxisomal proteome. Soluble proteins destined for import into the peroxisomal matrix are characterized by conserved signal sequences located either at the very carboxy-(C)-terminus or within the amino-(N)-terminus. The majority of peroxisomal matrix proteins contain a C-terminal targeting signal termed peroxisomal targeting signal type 1 (PTS1). The amount of proteins containing N-terminal peroxisomal targeting signal type 2 (PTS2) with the consensus motif (R/K)Lxxxxx(H/Q)L varies: while *Saccharomyces cerevisiae* only codes for a handful of proteins with PTS2 motif, approximately 25% of all peroxisomal matrix protein contain a PTS2 motif in the plant *Arabidopsis thaliana* (Reumann et al. 2004; Lazarow 2006). In some species PTS2 proteins are even absent (Motley et al. 2000; Gonzalez et al. 2011).

The minimal PTS1, originally identified in firefly luciferase, consists of a tripeptide motif with the prototype sequence SKL* located at the C-terminus (Gould et al. 1989). Later, it turned out that many variations of this motif exist. In addition, the wider context of the C-terminus also influences import efficiency (Brocard and Hartig 2006). PTS1 motifs of different species are recognized by a soluble receptor protein termed Pex5 that mediates import into peroxisomes (Rucktäschel et al. 2011). Structural studies revealed that the C-terminal tetratricopeptide repeats (TPR) of Pex5 recognize the C-terminal tripeptide of PTS1 proteins (Gatto et al. 2000). Their studies also suggested that amino acids upstream of the C-terminal tri-peptide modulates binding affinity of the TPR domain to the core tripeptide PTS1 (Gatto et al. 2000).

A straightforward approach to identify putative peroxisomal matrix proteins in databases is to perform simple text searches for a list of published variants of the prototype SKL motif. Depending on the number of variants included such queries either tend to fail to identify peroxisomal proteins (e.g. PSORT) or predict too many false positives (e.g. PROSITE) (Nakai and Horton 1999; Brocard and Hartig 2006; Sigrist et al. 2012). Emanuelsson et al. (2003) combined machine learning and cross species analysis to annotate peroxisomal proteins in plants, fungi and animals. The authors used a positive learning set containing known peroxisomal proteins with PTS1 motifs and a negative learning set of non-peroxisomal proteins with sequences similar to the C-terminal SKL motif (Emanuelsson et al. 2003). In addition, their data analysis revealed that the 12 C-terminal amino acids influence the efficiency of a given PTS1. Their algorithm was also able to identify many previously unannotated peroxisomal proteins. They detected the highest number of putative PTS1 proteins in plants, while fungi contained the lowest number (Emanuelsson et al. 2003). Another peroxisomal prediction tool was developed, that makes use of a recurrent network to improve prediction rates (Bodén and Hawkins 2005; Hawkins et al. 2007).

It has been shown that different organisms show some degree of variation in their recognition of PTS1 motifs and prediction tools need to be adapted to a specific organism to improve accuracy (Lametschwandtner et al. 1998; Neuberger et al. 2003b). Therefore, Neuberger et al. (2003a) used 355 proteins sequences from different species either known to interact with Pex5 or annotated as PTS1 proteins in public databases as learning set for their PTS1 predictor. This public web-based program allows for the prediction of PTS1 motifs in the proteomes of fungi, animals or other taxa. The algorithm is essentially based on two different scores that positively affect peroxisomal import: S_{score} (amino acid composition) and $S_{profile}$ (other physical properties of the C-terminal sequence). Proteins obtaining a score bigger than zero are considered to contain a functional PTS1 (Neuberger et al. 2003a). The authors declared all scores between 0 and −10 as twilight PTS1-motifs. C-terminal motifs with a score smaller than −10 are regarded as non-functional (Neuberger et al. 2003a).

In 2006, Schlüter et al. launched a peroxisomal proteome database, which integrates data from humans and *S. cerevisiae* (Schlüter et al. 2006). This database serves as a registry for both peroxisomal proteins and metabolic pathways. In addition, the authors also provide a predictor software for PTS1, PTS2 and for mPTS-Signals (targeting motifs found inside peroxisomal membrane proteins). Their prediction tool is based on the motif search programs MEME and BLOCKS (Henikoff and Henikoff 1992; Bailey et al. 2009). For PTS2 proteins an alternative online prediction tool has not been established yet. A combination of regular expression based data mining in combination with Hidden Markov Models revealed potential new candidates for PTS2 proteins in mice. However, all of them failed to localize a reporter to peroxisomes (Mizuno et al. 2008). A more reliable prediction platform for PTS2 proteins requires more experimental data; e.g. systematic and quantitative binding studies of the PTS2 receptor Pex7 to a variety of proteins and peptides. This data might help to create positive and negative learning sets (Mizuno et al. 2008; Hu et al. 2012).

In recent years several machine-learning approaches have been applied to build a more comprehensive view on PTS1-containing proteins (see also previous chapter in this book). In the plant model organism *A. thaliana* 23 novel functional PTS1 motifs and a number of so far undescribed peroxisomal proteins were identified by Lingner et al. (2011). The authors also provided a web based tool for prediction of plant peroxisomal proteins (Reumann et al. 2012). A similar machine learning strategy was recently used for *S. cerevisiae.* Two novel peroxisomal matrix proteins were identified and the consensus motif for PTS1 was expanded to ([S/A/H/C/E/P/Q/V] [K/R/H/Q] [L/F]) (Nötzel et al. 2016).

Most of the knowledge about fungal peroxisomes was deduced from work in *S. cerevisiae* and related ascomyetes. In addition, most prediction tools are mainly based on data from *S. cerevisiae.* However, more recent experimental evidence indicates that there is some degree of variability in the recognition of PTS1 in different fungal species. A C-terminal dodecamer derived from a putative glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the early-diverged fungus *Phycomyces blakesleeanus* mediates peroxisomal import of a fluorescent

protein in the basidiomycetous fungus *U. maydis* but fails to mediate import in budding yeast (Freitag et al. 2012). This dodecamer ends on the more unusual tripeptide GNL. Peroxisomal import in *U. maydis* shows an even higher degree of flexibility compared to *S. cerevisiae* (Julia Ast and Michael Bölker, unpublished data). One reason for this increased plasticity might be the occurrence of two Pex5 proteins with different TPR domains in *U. maydis* and several other basidiomycetes such as *C. neoformans* (Freitag et al. 2012).

A comprehensive knowledge of peroxisomal proteins in basidiomycetous fungi might reveal further peroxisomal metabolic pathways and even help to understand the general biology of these organelles. However, establishment of a highly sophisticated PTS1 prediction tool would require systemic analysis of PTS1 import in a basidiomycete (e.g. *U. maydis*) to generate sufficient information for a specific learning set of PTS1 motifs.

3 Cryptic Peroxisomal Proteins

In the next sections, we will discuss cases, where prediction of peroxisomal proteins solely based on sequence information can be extremely difficult. These include proteins with weak targeting signals, proteins where functional targeting signals result from posttranscriptional modification and proteins lacking any obvious PTS motif but nevertheless are targeted efficiently into peroxisomes via unconventional mechanisms.

3.1 Weak Peroxisomal Targeting Signals Promote Dual Targeting

Many enzymatic functions occur in more than one cellular compartment. If two different compartments are targeted this phenomenon is called “dual targeting” (Yogev and Pines 2011; Ast et al. 2013). A number of NADH-dependent dehydrogenases are prominent examples residing both inside peroxisomes and the cytosol. These enzymes drive a redox shuttle using small intermediates e.g. malate/oxaloacetate. This is required since the peroxisomal membrane is impermeable for NADH generated during ß-oxidation (Antonenkov and Hiltunen 2012). Peroxisomal malate dehydrogenase catalyzes NADH dependent reduction of oxaloacetate to malate inside peroxisomes resulting in regeneration of NAD^+ required for ß-oxidation. Organisms use different strategies to achieve both peroxisomal and cytosolic localization of malate dehydrogenase isoforms (for review see: Ast et al. 2013).

If dually targeted protein isoforms are derived from the same gene, several problems arise in regard to prediction and experimental validation of peroxisomal

localization. In *U. maydis* the central glycolytic enzyme fructose-bisphosphate aldolase (FBA) contains a quite unusual PTS1 at its C-terminus (GKL). Although bioinformatic analysis failed to predict peroxisomal localization the PTS1 motif was shown to be functional (Neuberger et al. 2003a; Freitag et al. 2012). The same seems to hold true for GAPDH2 from *P. blakesleeanus*, which was predicted to be non-targeted with a PTS1 predictor score of −35.3 (Neuberger et al. 2003a).

When *U. maydis* FBA was tagged with the green fluorescent protein (GFP) it was found to localize predominantly in the cytosol. Therefore it was not possible to decide whether the protein also resides inside of peroxisomes (Freitag et al. 2012). Similarly, peroxisomal targeting of *A. thaliana* glutathione reductase 1 containing the non-canonical tripeptide TNL was also difficult to detect due to its high cytosolic localization (Kataya and Reumann 2010). This suggests that more proteins may exist, that contain non-canonical PTS1 motifs and thus are imported only with limited efficiency (see also last section of this chapter). Such proteins are both difficult to predict by bioinformatics and difficult to confirm experimentally. Even in proteomic analysis it is not easy to identify these proteins with low-efficiency PTS since they can be considered as contamination (Managadze et al. 2010).

Several experimental approaches can be used to overcome problems associated with proteins containing low efficiency PTS1. To enhance fluorescence of peroxisomal GFP fusion proteins in cells with high cytosolic background photo-bleaching experiments can performed (Stiebler et al. 2014). Repeated illumination of a defined area in a cell result in predominant bleaching of cytosolic GFP fusion proteins, which diffuse freely. By this technique proteins inside of the less mobile peroxisomes become visible (Stiebler et al. 2014). Alternatively, self-assembly GFP could be used to visualize proteins with low-efficiency PTS1 motifs. This assay makes use of two GFP fragments, which only assemble into a fluorescent probe if both polypeptides reside inside the same compartment (Cabantous et al. 2004). Thus, localization of proteins fused to one GFP fragment can be studied in a series of strains expressing the other fragment exclusively in one specific compartment.

3.2 *Differential Splicing and More: Alternative Transcripts Encoding Peroxisomal Isoforms*

It is generally assumed that in contrast to metazoa fungi make much rarer use of alternative splicing to generate protein isoforms with altered cellular function or localization. *S. cerevisiae* and related fungi only contain a very limited number of genes with introns (for review see: Kempken 2013). However, recent papers indicated that in many fungal species differential splicing is a conserved mechanism to create peroxisomal isoforms of central metabolic enzymes (Freitag et al. 2012). In both ascomycota and basidiomycota C-terminally extended variants of the glycolytic enzymes phosphoglycerate kinase (PGK) and GAPDH are generated by alternative polyadenylation and splicing. It was demonstrated that dual localization

of GAPDH is important since this enzyme acts as part of a peroxisomal redox shuttle (Freitag et al. 2012). Alternative splicing as a means to generate peroxisomal proteins in fungi was also described for other central metabolic enzymes. In *Yarrowia lipolytica* peroxisomal targeting of NADH dependent malate dehydrogenase is controlled by differential splicing (Kabran et al. 2012). In contrast to dual localization of proteins via weak targeting signals, differential splicing has the advantage that the ratio between the two variants can be regulated by different RNA binding proteins and splice enhancers (Matlin et al. 2005).

Peroxisomal ß-oxidation of unsaturated fatty acids depends on NADPH acting as cofactor for peroxisomal 2,4-dienoyl-CoA reductase (Poirier et al. 2006). Different enzymes can participate in NADPH regeneration (Visser et al. 2007). The oxidative branch of the pentose phosphate pathway (PPP) is a major source for NADPH. 6-phosphogluconate dehydrogenase generates NADPH via oxidation of 6-phosphogluconate to 3-keto-6-phosphogluconate, which spontaneously decarboxylates to ribulose-5-phosphate (Stincone et al. 2015). Differential splicing gives rise to a PTS2 containing isoform of 6-phosphogluconate dehydrogenase in *Candida albicans* (Strijbis et al. 2012). Differential splicing as a means to produce peroxisomal isoforms of enzymes involved in the turnover of co-factors is not restricted to fungi but was also described in other eukaryotes. Inorganic pyrophosphatase is found at various cellular locations and hydrolyzes pyrophosphate to accelerate thermodynamically unfavorable biochemical reactions (Kornberg 1962). In peroxisomes activation of imported free fatty acids by ATP dependent acyl-CoA synthetases requires pyrophosphatase and its activity has been observed in purified rat liver peroxisomes already in the 1990s (Shimizu and Ohkuma 1993; Visser et al. 2007). In *U. maydis,* inorganic pyrophosphatase contains a PTS2, while in *Drosophila melanogaster* differential splicing triggers formation of a PTS1 (Stiebler et al. 2014).

Recently, a systematic approach identified a number of *A. thaliana* genes encoding PTS1 or PTS2 containing proteins that depend on alternative splicing (An et al. 2017). The authors identified a total of 66 PTS1 and five PTS2 in protein isoforms resulting from differential splicing. The isoforms identified in *A. thaliana* represent a variety of different biological functions, while in fungi and animals most characterized examples of PTS proteins derived from alternative splicing are enzymes involved in co-factor turnover (Ast et al. 2013; Stiebler et al. 2014). Mining of RNA-sequencing data of fungal and animal species for splice variants encoding potential peroxisomal proteins should tell us if the functional capacity of the peroxisomal proteome is considerably enlarged by differential splicing in all eukaryotic kingdoms.

Beside alternative splicing different transcriptional start sites can also trigger the generation of compartment-specific isoforms in fungi. $NADP^+$ dependent isocitrate dehydrogenase (IDP) is used to regenerate NADPH inside peroxisomes and catalyzes oxidation of isocitrate to alpha-ketoglutarate (Visser et al. 2007). In *Aspergillus nidulans* the intracellular distribution of IDP is controlled via generation of two *idpA* transcripts from distinct transcriptional start sites (Szewczyk et al. 2001). The longer transcript codes for an IDP variant containing both an N-terminal

mitochondrial targeting signal and a C-terminal PTS1. This protein was found to be predominantly localized inside mitochondria (Szewczyk et al. 2001). The shorter protein lacks the mitochondrial targeting signal and is found in the cytosol and inside peroxisomes. A similar example of different transcripts leading to differentially targeted protein isoforms was identified in *S. cerevisiae*. Carnitine acetyltransferase (Cat2) is used to shuttle fatty acids through membranes (Visser et al. 2007). *S. cerevisiae* Cat2 contains an N-terminal signal for mitochondrial import followed by a PTS. Oleic acid induces a shorter transcript encoding an N-terminally truncated protein, that lacks mitochondrial targeting information and thus localizes to peroxisomes (Elgersma et al. 1995).

3.3 Translational Stop Codon Readthrough: A Widely Conserved Mechanism to Create Peroxisomal Isoforms

In the last two sections we have described cryptic peroxisomal proteins, whose peroxisomal targeting requires generation of alternative transcripts or are targeted with low efficiency. Recently, several studies reported an alternative post-transcriptional mechanism leading to formation of C-terminally extended PTS1 containing proteins (Freitag et al. 2012; Schueren et al. 2014; Stiebler et al. 2014; Hofhuis et al. 2016). In fungi, several glycolytic enzymes are partially targeted to peroxisomes via programmed readthrough of stop codons (Freitag et al. 2012). Remarkably, the mechanism to generate C-terminally extended isoforms varies between different fungal species. While in *Ustilago maydi*s and other basidiomycetes alternative splicing is used to create a peroxisome targeted variant of GAPDH, in many ascomycetes this results from translational readthrough. In contrast, readthrough generates the peroxisomal isoform of phosphoglycerate kinase (PGK) in basidiomycetes, while differential splicing leads to the formation of a PTS1-containing PGK in ascomycetes (Freitag et al. 2012). Further analysis in *U. maydis* revealed that a UGA stop codon followed by the di-nucleotide motif CU constitutes a core element that triggers efficient stop codon readthrough (Stiebler et al. 2014). Genome mining using this core motif lead to the identification of additional metabolic enzymes, which are partially sorted to peroxisomes by this mechanism. Among these enzymes are members of different metabolic pathways, e.g. the pentose phosphate pathway (PPP) (Stiebler et al. 2014).

Comparative genomics and machine learning approaches uncovered that the short stop codon context UGA CU triggers readthrough also in animals (Loughran et al. 2014; Schueren et al. 2014; Stiebler et al. 2014; Hofhuis et al. 2016; see also next chapter). Interestingly, it was shown that in humans PTS1 containing extended variants of lactate dehydrogenase and malate dehydrogenase are produced by translational readthrough. Both enzymes participate in redox-homeostasis and act in peroxisomes most likely as redox shuttles to regenerate NAD^+ during ß-oxidation (Baumgart et al. 1996; Visser et al. 2007). In addition, hallmarks for translational

readthrough were discovered also for inorganic pyrophosphatases in mammals and nematodes indicating that this is a common mechanism to achieve dual peroxisomal localization of key metabolic enzymes (Stiebler et al. 2014).

Translational readthrough of stop codons is perfectly suited for the synthesis of C-terminally extended proteins such as PTS1 containing isoforms. Beside this obvious reason to use readthrough as mechanism to target proteins to peroxisomes recent studies indicated another layer of regulation that may explain the evolutionary conservation of readthrough derived peroxisomal proteins. In *S. cerevisiae*, it was shown that translational accuracy is affected by proline-hydroxylation of ribosomal protein Rps23 catalyzed by the oxygen dependent hydroxylase Tpa1 (Loenarz et al. 2014). The presence of hydroxylated Rps23 resulted in higher readthrough rates at stop codon with UGA CU context. A recent ribosomal profiling approach with human cell culture under conditions mimicking ischemia by depleting both oxygen and glucose also demonstrated that ribosomal readthrough rates of transcripts containing the stop codon context UGA CU are reduced within short time (Andreev et al. 2015). ß-oxidation requires molecular oxygen for the initial oxidation of acyl-CoA (Poirier et al. 2006). Thus, readthrough dependent modulation of peroxisomal targeting of metabolic enzymes may be an adaptation to cope with conditions of altered oxygen availability. While UGA CU triggered readthrough is less effective in cells with a *tpa1* deletion, readthrough rates increase if the UGA stop codon is followed by an adenosine residue. This data suggests differential regulation of stop codon readthrough by Tpa1 depending on the context of the stop codon.

Identification of the UGA CU readthrough core motif by different approaches will help to improve prediction of the peroxisomal proteome by including proteins with readthrough derived PTS1 containing C-terminal extensions (for review see: Schueren and Thoms 2016). Remarkably, ribosome profiling experiments with *D. melanogaster* revealed further readthrough derived protein isoforms with functional PTS1 that apparently do not depend on the UGA CU motif (Dunn et al. 2013). This suggests additional sequence motifs or structural determinants yet to be identified that trigger readthrough of stop codons.

3.4 Peroxisomal Matrix Proteins Without PTS: The Really Hidden Ones

Although most cryptic peroxisomal proteins have been difficult to identify, prediction of such proteins is in principle possible, since they contain genetically encoded PTS motifs. In these cases prediction of peroxisomal localization requires a significantly broader definition of PTS motifs, an accurate prediction of splice sites or identification of motifs that trigger efficient stop codon readthrough. But still, a number of proteins remain that are known to reside inside fungal peroxisomes without containing any obvious targeting signals. Such proteins are hardly to

predict but can be identified in proteomic approaches or by intracellular localization studies. Peroxisomal matrix proteins are imported in a folded state and can even be imported as oligomers or complexes with other proteins (Glover et al. 1994; McNew and Goodman 1994; Walton et al. 1995). This mode of translocation allows for peroxisomal import of proteins without any PTS via hitchhiking with proteins that harbor a canonical PTS. In plant cells enzymes of the glyoxlate pathway can be targeted to peroxisomes even if they lack the canonical PTS1 due to mutation. Peroxisomal targeting of these enzymes requires the wild-type variants of respective enzymes (Lee et al. 1997). A natural example of such a piggy-back mechanism was identified in mammalian cells. Cu/Zn superoxide dismutase does not contain an obvious PTS but is imported into peroxisomes via interaction with a PTS1 containing copper chaperone (Islinger et al. 2009). It was also shown that human LdhA is transported into peroxisomes via interaction with LdhB that contains a cryptic PTS1 (Schueren et al. 2014). Recently, in *S. cerevisiae* a piggy-back based import mechanism was described for Pnc1 (pyrazinamidase/nicotinamidase I) (Effelsberg et al. 2015; Kumar et al. 2016). Pnc1 is part of an NAD^+ salvage pathway and important for stress response and longevity (Anderson et al. 2003). Pnc1 is imported into peroxisomes in a complex together with glycerol-3-phosphate dehydrogenase (Gpd1), which harbors a PTS2 and needs to form a homodimer to co-import Pnc1 (Effelsberg et al. 2015; Kumar et al. 2016; Al Saryi et al. 2017). So far, only a relatively small number of examples for "piggy-backing" import into peroxisomes have been described. In theory, all PTS containing peroxisomal proteins might be able to carry other proteins into peroxisomes maybe only in small amounts. Systematic interaction analysis of PTS proteins with "*prima facie*" cytosolic proteins will be useful to characterize further instances of "piggy-backing". The maximum complex size, which can still be imported into peroxisomes, is still unclear. It was already shown that colloidal gold particles coated with PTS1 proteins in a size range from 4 nm to 9 nm enter mammalian peroxisomes (Walton et al. 1995), suggesting that very large complexes can be transported through the peroxisomal membrane. However, it will be also interesting to measure the force that can be generated by a single PTS to receptor interaction. A more detailed understanding of the physical properties and limitations of PTS1 and PTS2 import is an important prerequisite for a better understanding of peroxisomal import of oligomeric complexes.

Some peroxisomal matrix proteins do not harbor PTS motifs but are imported into peroxisomes without hitchhiking (for review see: van der Klei and Veenhuis 2006b). In *S. cerevisiae,* acyl-CoA oxidase (Pox2) and carnitine acetyltransferase (Cat2) are imported into peroxisomes in a Pex5 dependent manner (Klein et al. 2002; Schäfer et al. 2004). Cat2 contains a PTS but is imported even when the motif is deleted. It was demonstrated that peroxisomal import of both proteins relies on interaction with the N-terminus of Pex5 and does not depend on the TPR domains (Klein et al. 2002; Schäfer et al. 2004). Similarly, alcohol oxidase from *H. polymorpha* and the multifunctional enzyme for ß-oxidation from *U. maydis* can reach the peroxisomal matrix independent of PTS motifs (Gunkel et al. 2004; Klose and Kronstad 2006; Kretschmer et al. 2012a). Further instances of peroxisomal proteins

without PTS have been reported or PTS1 motifs were found to be redundant. In all these cases, other information encoded in the protein sequence or structure confer recognition by Pex5 (van der Klei and Veenhuis 2006b). It was speculated that additional targeting information beside classical PTS motifs requires proper cytosolic folding before import into peroxisomes thereby preventing accumulation of unfolded proteins inside peroxisomes (van der Klei and Veenhuis 2006b). The so far described examples of proteins exhibiting additional targeting information or unconventional Pex5 binding are all core enzymes of peroxisomal metabolism. It is possible that these proteins need to be transported with priority. It remains to be determined whether one Pex5 molecule is able to import simultaneously a normal PTS1 containing protein together with a protein with an unconventional Pex5 recognition site.

4 Dual Targeting Reveals a Broader Metabolic Capacity of Fungal Peroxisomes

In the following section we take the basidiomycete *U. maydis* as an example to describe how an important metabolic pathway can be partially compartmentalized inside fungal peroxisomes. This occurs by a combination of the mechanisms introduced in the previous sections. Moreover, we provide an example for a novel peroxisomal metabolic pathway, which was identified by predicting cryptic peroxisomal targeting signals.

Glycolysis was thought to occur only in the cytosol with the notable exception of trypanosomes. In their bloodstream phase these organisms contain highly specialized peroxisomes called glycosomes that harbor the majority of glycolytic enzymes (Szöör et al. 2014). Identification of alternative splicing and stop codon readthrough as widespread mechanisms to generate PTS1 motifs revealed that many fungi including *U. maydis* contain several glycolytic enzymes with a dual localization both in the cytosol and inside peroxisomes (Freitag et al. 2012; Stiebler et al. 2014). Interestingly, many of the different mechanisms discussed in the previous paragraphs contribute to partial peroxisomal targeting of glycolytic enzymes (Fig. 1). The peroxisomal isoform of GAPDH is generated by alternative splicing, while dual targeting of PGK results from stop codon readthrough. In contrast, fructosebisphosphate aldolase (FBA) contains a low efficiency PTS1. Glycolysis is interconnected with the pentose phosphate pathway. Remarkably, members of the PPP also show partial peroxisomal targeting in fungi including *U. maydis* (Fig. 1). It remains to be investigated whether also those enzymes involved in glycolysis or PPP that lack obvious targeting information for peroxisomes (e.g. fructose-1,6-bisphospatase) are found in peroxisomes. These enzymes could be transported into peroxisomes by means of piggybacking or yet undiscovered mechanisms leading to dual targeting. This might be possible, since glycolytic enzymes were suggested to occur in oligomeric complexes to enhance efficiency by

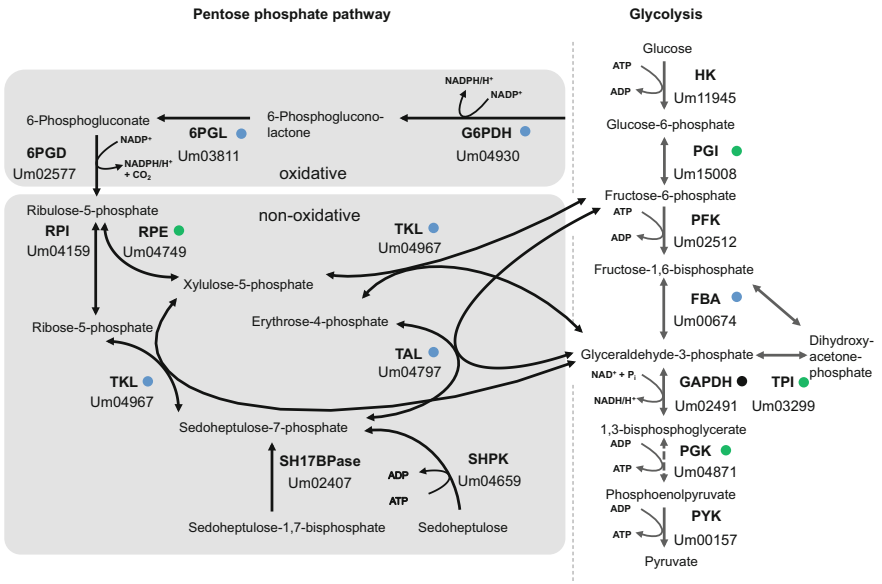

Fig. 1 Partial peroxisomal compartmentalization of sugar metabolism in *U. maydis*. The figure shows a schematic representation of glycolysis/gluconeogenesis and the PPP. Mechanisms leading to peroxisomal targeting are coded with different colors. Blue circles indicate enzymes harboring constitutive but sometimes non-canonical PTS1 motifs. Black circles specify peroxisomal targeting via alternative splicing. Enzymes containing extensions derived from stop codon readthrough are marked with a green circle. Abbreviations refer to the following enzymes: 6PGD: 6-phosphogluconate dehydrogenase, 6PGL: 6-phosphogluconolactonase, FBA: Fructose-1,6-bisphosphate aldolase, GAPDH: Glyceraldeyde-3-phosphate dehydrogenase, G6PDH: Glucose-6-phosphate dehydrogenase, HK: Hexokinase, PFK: phosphofructokinase, PGK: Phosphoglycerate kinase, PYK: Pyruvate kinase, RPE: ribulose-5-phosphate 3-epimerase, RPI: ribose-5-phosphate isomerase, SHPK: Sedoheptulose kinase, TAL: Transaldolase, TKL: Transketolase, TPI: triosephosphate isomerase

preventing diffusion of intermediates (Menard et al. 2014). Alternatively, intermediates have to pass the peroxisomal membrane. The peroxisomal membrane of rat liver peroxisomes has been shown to be permeable for solutes up to a size of 400 Da (Antonenkov et al. 2004). It is noticeable the many dually targeted enzymes have a role in redox shuttling (e.g. NADH). This may also help to connect peroxisomal metabolism to intermediates of major pathways in other compartments. GAPDH couples peroxisomal metabolism to cytosolic glycolysis and peroxisomal MDH to the mitochondrial citric acid cycle.

Identification of further enzymes targeted to peroxisomes via programmed translational readthrough revealed another metabolic pathway that partially resides inside *U. maydis* peroxisomes (A. Stiebler and M. Bölker, unpublished). Microorganisms can degrade phenolic compounds such as resorcinol and hydroquinone. The genome of *U. maydis* encodes a hydroxyquinol-1,2-dioxygenase, which contains a canonical PTS1 (Fig. 2). In addition, ribosomal readthrough at a UGA CU stop codon context

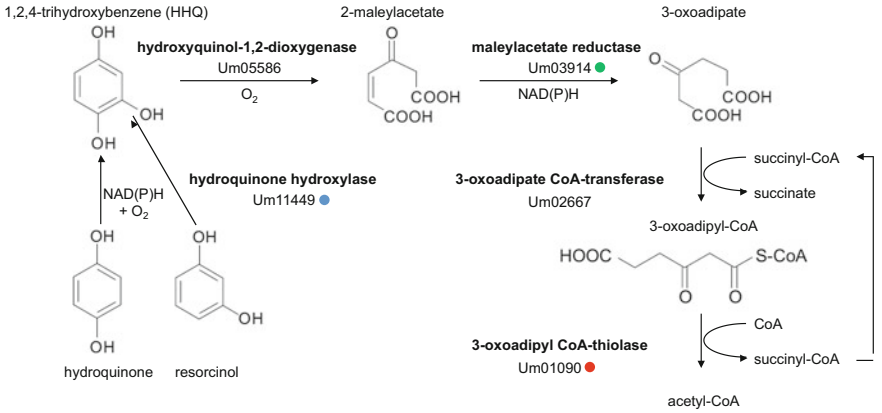

Fig. 2 Degradation of phenolic compounds inside fungal peroxisomes. Depicted is the metabolic pathway to catabolize hydroquinone or resorcinol. The blue circle refers to a protein with a canonical PTS1 motif. The green circle specifies peroxisomal targeting via stop codon readthrough. The red circle indicates a PTS2

results in the formation of a PTS1 containing variant of maleylacetate reductase (Stiebler et al. 2014). This enzyme catalyzes an intermediate step in the degradation of hydroquinone (Fig. 2). The last step of the degradation pathway is likely to be catalyzed by a peroxisomal thiolase containing a PTS2. Peroxisomal targeting of maleylacetate reductase via readthrough is restricted to *U. maydis* and close relatives. It will be interesting to determine whether the presence of this pathway is related to their phytopathogenic lifestyles. This finding suggests that fungal peroxisomes are likely to contain even more unexpected metabolic pathways, some of which might be specific for particular groups of fungi.

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Predicting Peroxisomal Targeting Signals to Elucidate the Peroxisomal Proteome of Mammals

Markus Kunze

Abstract Peroxisomes harbor a plethora of proteins, but the peroxisomal proteome as the entirety of all peroxisomal proteins is still unknown for mammalian species. Computational algorithms can be used to predict the subcellular localization of proteins based on their amino acid sequence and this method has been amply used to forecast the intracellular fate of individual proteins. However, when applying such algorithms systematically to all proteins of an organism the prediction of its peroxisomal proteome *in silico* should be possible. Therefore, a reliable detection of peroxisomal targeting signals (PTS) acting as postal codes for the intracellular distribution of the encoding protein is crucial. Peroxisomal proteins can utilize different routes to reach their destination depending on the type of PTS. Accordingly, independent prediction algorithms have been developed for each type of PTS, but only those for type-1 motifs (PTS1) have so far reached a satisfying predictive performance. This is partially due to the low number of peroxisomal proteins limiting the power of statistical analyses and partially due to specific properties of peroxisomal protein import, which render functional PTS motifs inactive in specific contexts. Moreover, the prediction of the peroxisomal proteome is limited by the high number of proteins encoded in mammalian genomes, which causes numerous false positive predictions even when using reliable algorithms and buries the few yet unidentified peroxisomal proteins. Thus, the application of prediction algorithms to identify all peroxisomal proteins is currently ineffective as stand-alone method, but can display its full potential when combined with other methods.

Keywords Peroxisomes · Targeting signal · Prediction algorithm
Proteome · PTS1 · PTS2

M. Kunze (✉)
Department of Pathobiology of the Nervous System,
Center for Brain Research, Medical University of Vienna,
Vienna, Austria
e-mail: markus.kunze@meduniwien.ac.at

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Abbreviations

ANN	Artificial neural network
HMM	Hidden Markov Model
mPTS	PTS for membrane proteins
PBD	Peroxisome biogenesis disorder
PEX	Peroxin
PSSM	Position specific scoring matrix
PTS	Peroxisomal targeting signal
SETD	Single enzyme and transporter deficiency
SVM	Support vector machines

1 Introduction

Eukaryotic cells are divided by a complex endomembrane system into the cytosol and various membrane bound compartments. Traditionally, endosymbiotic organelles such as mitochondria or chloroplasts and peroxisomes have been separated from the complex system of the secretory and endosomal compartments involving the endoplasmic reticulum (ER), Golgi apparatus, trans-Golgi network as well as endosomes and lysosomes. These organelles contribute to cellular and organismal physiology by housing a plethora of enzymatic activities partially cooperating as metabolic pathways, by sequestering and inactivating toxic compounds or by buffering ionic stores such as calcium. These capabilities depend on the proteinaceous equipment concentrated within these compartments rendering knowledge of total proteins, nowadays termed the proteome, an important question. Originally, such compilations were obtained secondarily by listing all published proteins attributed to an organelle, but the steady improvement of bioanalytical technologies facilitated the systematic identification of some and later most proteins of a biochemically isolated fraction enriched in the organelle of interest (Taylor et al. 2003; Ho et al. 2006). However, the deciphering of whole genome sequences from many organisms up to humans (International Human Genome Sequencing Consortium 2004; Venter et al. 2001), which are available in large databases such as Ensembl (https://www.ensembl.org/index.html) or others (Wang et al. 2013), facilitates another approach. Given that nearly all proteins compartmentalized in an organelle (or parts of the endomembrane system) reach their destination by active transport processes, each of these proteins needs a postal code, which can be interpreted by the cellular transport machineries to initiate the transport of this protein into a specific compartment. Therefore, a detailed knowledge of these postal code(s), which determine(s) the intracellular destination of a protein, usually known as targeting signals, allows the forecast of the subcellular localization of a protein based solely on its amino acid sequence (Emanuelsson and von Heijne 2001). Generating algorithms to effectively identify such postal codes within protein

sequences and applying them to a compilation of all proteins of an organism also allows the prediction of the proteinaceous equipment of an organelle, which is the proteome.

Among the different organelles peroxisomes are special due to their limitation by a single membrane and the complete absence of DNA and the corresponding transcriptional and translational machineries (Subramani 1998). Thus, all proteins are imported into peroxisomes in a posttranslational manner, but the transport of soluble matrix proteins across the peroxisomal membrane occurs in a folded state, which is a characteristic difference compared to the other organelles (Dias et al. 2016). In contrast, peroxisomal membrane proteins (PMPs) can reach peroxisomes either by direct import from the cytosol or by vesicular transport from the ER (Kim and Hettema 2015).

The importance of peroxisomes for mammalian physiology is highlighted by a variety of severe inherited human disorders caused by mutations in genes coding either for an individual peroxisomal enzyme or transporter protein or for proteins involved in the formation or propagation of peroxisomes (Waterham et al. 2016). Accordingly, these disorders have been categorized as single enzyme and transporter deficiencies (SETD) (Kunze and Berger 2014) and peroxisome biogenesis disorders (PBD) (Steinberg et al. 2006; Fujiki et al. 2012). The genes causing PBD have been summarized as peroxins, which are encoded by PEX-genes (Waterham et al. 2016). Peroxisomes can exert a plethora of metabolic reactions, but many of these are restricted to individual branches of the tree of life, whereas ubiquitous enzymatic activities such as the degradation of hydrogen peroxide (H_2O_2) by catalase, the degradation of fatty acids by β-oxidation or the oxidation of D-amino acids are relatively scarce. In contrast, the oxidation of branched chain fatty acids by α-oxidation, the shortening of the side chain of bile acid precursors or the first steps in the biosynthesis of ether-phospholipids are considered animal-specific peroxisomal activities (Wanders and Waterham 2006). Conversely, typical examples for taxon-specific metabolic pathways, which do not occur in mammals, involve the glyoxylate cycle of plants and fungi (Kunze et al. 2006), parts of photorespiration in plants (Bauwe et al. 2010), glycolysis in protozoa (Allmann and Bringaud 2017), biosynthesis of penicillin in some fungi (Martin et al. 2010) or that of biotin in plants (Tanabe et al. 2011). This highlights a surprising variability of peroxisomes, which can also be observed at the level of an individual organism, where the protein composition of peroxisomes (Mi et al. 2007) or their size and number can differ remarkably between tissues, differentiation states or environmental states (Kunze et al. 2006).

Nonetheless, our understanding of the contribution of peroxisomes to the organismal physiology is still rather incomplete. However, a complete list of proteins, which can be found in peroxisomes, would be an important next step. Listing all peroxisomal proteins may also be of medical importance as the classification of an inherited human disorder as a single enzyme and transporter deficiency would place it in the context of peroxisomes and might reveal surprising interconnections with other peroxisomal functions such as the recent identification of a susceptibility gene for hearing deficits upon exposure to loud noise (Delmaghani et al. 2015).

This chapter is focused on the prediction of peroxisomal proteomes from mammalian organisms based on the identification of peroxisomal targeting signals (PTS) and the evaluation of whole protein sequences. Other taxa such as plants are covered in another chapter of this book (cf. chapter by S. Reumann et al.).

2 Elucidation of the Peroxisomal Proteome

In contrast to the proteome of an organism, which can be defined as the sum of all proteins generated by this organism, the compilation of a fraction of these proteins as peroxisomal proteome requires a selection criterion, which at first glance is the localization of the protein to peroxisomes. However, such an attribution mostly depends on experimental results such as the identification of an endogenous protein in a peroxisome-enriched fraction obtained by biochemical methods from a specific tissue (e.g. the liver) or by microscopic colocalization of an ectopically expressed tagged protein with a peroxisomal marker protein in a specific cell type or cell line.

Accordingly, the peroxisomal proteome of an organism is a theoretical construct, which summarizes all proteins ever detected in peroxisomes, but this compilation of proteins does not necessarily exist in any cell. Moreover, many proteins resist a simple attribution to peroxisomes, because the protein is prominently found in other subcellular locations such as mitochondria, chloroplasts or even the cytosol or because only one of the protein variants derived from a gene locus is found in peroxisomes. In the context of this article the *peroxisomal proteome* is considered as the compilation of all proteins occurring in peroxisomes limited by the peroxisomal membrane, which includes matrix and membrane embedded proteins, but also proteins attached by posttranslational modifications such as a prenyl-chain (farnesyl- or geranyl-geranyl-) to the peroxisomal membrane. In contrast, proteins which are solely attached to the outer face of the peroxisomal membrane via an interaction with a PMP are not included, because they never enter the organelle and remain in the cytosol although they may become co-isolated with peroxisomes by biochemical approaches (e.g. cytosolic chaperones, parts of the ribosome or peroxisome attached proteasomes). This criterion might appear unintuitive for some proteins exerting important functions for peroxisomes like those mediating motility (motor proteins) (Neuhaus et al. 2016) or those performing the last step of fission (dynein-like proteins, DLP1) (Schrader et al. 2016). However these proteins should rather be considered as part of cytosolic structures, which are not actively transported to peroxisomes, but are recruited to the peroxisomal surface to perform a specific task there. Finally, the assignment of a protein to the peroxisomal proteome demands that a considerable fraction of the protein or of the individual protein isoform is found in peroxisomes. This definition includes proteins also found in other compartments next to peroxisomes (bi- or even tri-localized proteins), and proteins hardly expressed in the tissue of interest, but excludes minor fractions of abundant proteins, of which only traces appear in peroxisomes due to mistargeting.

Early descriptions of the peroxisomal proteome—even without coining this particular term—listed all proteins and enzyme activities, which had been attributed to peroxisomes at that time, and often added orthologous enzymes from other species (Tolbert 1981). The identification of the genes encoding these proteins allowed a more detailed compilation of protein sequences (Wanders and Waterham 2006; Hayashi and Nishimura 2006). Moreover, the investigation of the subcellular distribution of systematically generated fusion proteins with fluorescent proteins provided large data sets to pick peroxisomal proteins (Huh et al. 2003; Marion et al. 2008). Later on, the advance of mass spectrometric techniques facilitated the systematic identification of most proteins within a biochemically isolated fraction highly enriched in peroxisomes derived from liver or kidney of mice, rats and humans (Fig. 1, left side) (Wiese et al. 2007; Kikuchi et al. 2004; Gronemeyer et al. 2013b; Islinger et al. 2006). Whereas in the beginning only the major components of a protein band (Kikuchi et al. 2004) or of a protein dot from a 2-dimensional gel (Mi et al. 2007) could be identified, later experiments identified nearly all proteinaceous components of a complete peroxisomal fraction, as described for example in Wiese et al. (2007). However, in such approaches, the discrimination of real peroxisomal proteins and proteins, which were only co-isolated with peroxisomes, became a major challenge. Accordingly, the ever increasing sensitivity of mass spectrometry not only allowed the identification of rare peroxisomal proteins, but these new candidates were buried more and more beneath a pile of contaminants. This problem could be addressed by improved experimental procedures such as organellar profiling (Wiese et al. 2007; Andersen and Mann 2006), but also by the use of orthogonal methods to rank and prioritize the most promising candidates from long lists of identified proteins, which were then confirmed to be located in peroxisomes by alternative techniques (e.g. the intracellular distribution of EGFP-fusion proteins). Among these methods, the prediction of PTS has been

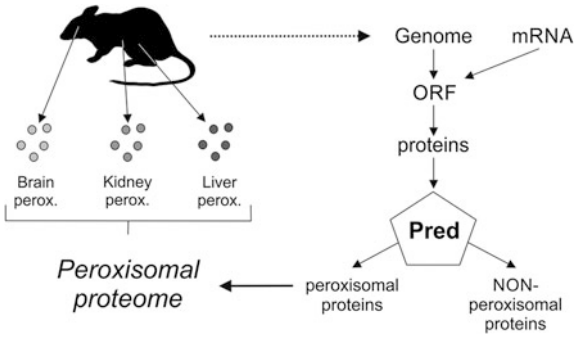


Fig. 1 Extracting the peroxisomal proteome: The peroxisomal proteome of an organism (e.g. mouse) can be obtained by summing up all peroxisomal proteins from diverse tissues identified experimentally (left side) or by compiling all proteins, which have been tagged as peroxisomal by a prediction algorithm (*Pred*) that analyses all possible protein sequences delineated from the whole genome sequence of the organism (right side)

frequently used, because the presence of peptide sequences satisfying the minimal consensus sequence of PTS was easy to verify and the first predictor for type-1 PTS (PTS1) motifs was available online in 2003 (Neuberger et al. 2003b).

Although the proteomic approaches described above have been rather successful in identifying novel peroxisomal proteins in humans and mice, this approach has systematic limitations as well. On the one hand, the isolation of peroxisomes of individual tissues cannot identify peroxisomal proteins, which are not expressed there. On the other hand some proteins might be peroxisomal only under specific living conditions of the organism. Unfortunately, some tissues of exceptional interest for the scientific community such as the brain proved rather refractory to an isolation of peroxisomal fractions with sufficient purity to render mass spectrometric analyses informative. Therefore, the development of a complementary approach was desirable, which specifically circumvents the limitations of the above mentioned experimental techniques. The computational analysis of the amino acid sequence of proteins aiming at forecasting their subcellular localization has been such an approach. Therefore, algorithms have been generated, which use characteristic sequence elements of a protein, such as PTS, to generate an output indicating, whether a protein should be found in peroxisomes or not. When applying such algorithms to all proteins of an organism (proteome), a subset of proteins will be identified, which are predicted to be in peroxisomes and together these proteins comprise the *predicted peroxisomal proteome* of the organism.

3 Predicting the Peroxisomal Proteome

3.1 Concept

The *in silico* prediction of the peroxisomal proteome should be able to identify all proteins or protein variants with the potential to become part of this organelle in any tissue or any living condition, because this approach accesses all protein variants, which can be derived from the whole genome sequence of the organism. Moreover, this approach necessitates a computational algorithm, which analyzes the amino acid sequence of all these protein variants and reliably identifies and tags peroxisomal proteins. This tagging depends on the preceding classification of proteins based on properties that can be linked to their primary sequence. By that, proteins can either be categorized into groups of peroxisomal and non-peroxisomal proteins or classified into several groups of proteins sharing the same subcellular compartmentations among which peroxisomal proteins are clustered into one group. Classification algorithms can utilize many different properties of proteins, which are either known to correlate with the subcellular distribution of proteins or which are identified as useful indicators during the development of the algorithm. The subcellular compartmentation of proteins depends on specific transport processes, which distribute proteins according to a postal code embedded in their

primary sequence. Accordingly, these postal codes are highly informative for the prediction of the subcellular location of a protein. In the traditional terminology of cell biology, the term "targeting signal" has been coined for such postal codes (Blobel et al. 1979). Such targeting signals have been described as short peptide sequences, which are *necessary* and *sufficient* for the transport of the encoding protein into the organelle of interest, such as the ER (Blobel et al. 1979; Blobel and Dobberstein 1975). Functionally, targeting signals mediate the interaction between the encoding protein and a specific receptor protein, which is the connection element between its cargo proteins and the import machinery of the organelle. Accordingly, the term targeting signal always implicates the ability to interact with a receptor protein, because this interaction is a prerequisite for its functionality and can be considered as extension of the definition of targeting signal. Conversely, a peptide sequence, which has the ability to interact with a receptor protein, is not a functional targeting signal, whenever the interaction with the receptor protein cannot occur within the context of the protein. Targeting signals have been characterized in the N-terminal part of the primary sequence of proteins transported into mitochondria, chloroplasts, or the secretory pathway including the ER as entrance point, but also in proteins imported into peroxisomes (Schatz and Dobberstein 1996). The latter are equipped with PTS, which interact with specific receptor proteins that initiate their transport to the peroxisomal membrane (Kim and Hettema 2015). However, peroxisomal matrix and membrane proteins are imported by different machineries and even matrix proteins can utilize different types of PTS. Thus, the primary interaction between any PTS and its receptors can only be understood within the framework of all the diverse transport routes into peroxisomes. Accordingly, these routes are shortly described before the details of each PTS motif are presented.

3.2 Peroxisomal Protein Transport

The vast majority of peroxisomal proteins can be categorized into soluble matrix proteins and PMPs, which reach their destination by separated transport routes, whereas a few proteins are attached to peroxisomes via a lipid anchor. Fully folded *soluble proteins* including their cofactors and even oligomerized protein complexes are transferred across the peroxisomal membrane via a protein machinery with pore-like properties (Liu et al. 2012; Francisco et al. 2017). This transport process can be initiated by two types of PTS, which are recognized by soluble receptor proteins. Type-1 PTS (PTS1) is located at the extreme C-terminus of the protein and interacts with the receptor PEX5, which is sufficient to translocate its cargo to the docking complex embedded in the peroxisomal membrane (Fig. 2a) (Brocard and Hartig 2006; Erdmann and Schliebs 2005). In contrast, the majority of type-2 PTS (PTS2) are found in proximity to the N-terminus of the encoding protein and are bound by a bipartite receptor complex consisting of the PTS2-receptor PEX7 and a co-receptor (Fig. 2b) (Kunze et al. 2015; Lazarow 2006). In animals and plants, the

co-receptor is a specific isoform of the PTS1-receptor PEX5 (PEX5-long; PEX5L) harboring an additional exon for the interaction with PEX7, whereas in fungi independent proteins exert this function (Schliebs and Kunau 2006). These co-receptors stabilize the interaction between PTS2 and PEX7 and mediate the transfer of the complex to the docking site (Kunze et al. 2015). The peroxisomal import machinery facilitates the transfer of receptor-bound folded proteins across the membrane, and a dynamic assembly with several copies of the membrane protein PEX14 is crucial in this process (Francisco et al. 2017). Accordingly, all cargo-bound receptor proteins reach into the peroxisomal lumen to release their passengers, but only PTS2 carrying proteins are processed inside peroxisomes to release the N-terminal sequence including the PTS2. Subsequently, the receptor proteins are recycled to the cytosol by a complex mechanism involving mono-ubiquitination of the (co-)receptor and their ATPase-mediated extraction from the membrane (Platta et al. 2016). When a protein harbors an N-terminal targeting signal for mitochondria or the ER next to its PTS1, it is hardly found in peroxisomes, because the late exposure of the PTS1 during translation renders peroxisomal import subordinate within the hierarchy of targeting signals (Neuberger et al. 2004; Kunze and Berger 2015).

In contrast, two different routes have been described by which PMPs can reach peroxisomes (Kim and Hettema 2015). On the one hand, PMPs encoding a "type-1 PTS for membrane proteins" (mPTS-I) interact with the soluble receptor protein PEX19 in the cytosol, and this complex translocates to the integral membrane protein PEX3, where the integration of polytopic PMPs into the membrane is initiated (Fig. 2c). In this process the farnesylation of the C-terminus of PEX19 is crucial and has been linked to a conformational change (Rucktaschel et al. 2009; Emmanouilidis et al. 2017). Moreover, some PMPs such as PEX26 or FIS1 share the combination of a PEX19 binding domain and a transmembrane domain close to the C-terminus. This unit facilitates a special type of integration, by which only the C-terminus of the protein is inserted into the peroxisomal membrane, whereas the soluble domain reaches into the cytosol (Halbach et al. 2006; Yagita et al. 2013; Costello et al. 2017). These proteins might be integrated into peroxisomes by a special mechanism, but their structure resembles proteins, which have been found in the outer mitochondrial and the ER membrane, and have been termed tail-anchored (TA) proteins (Yagita et al. 2013; Chen et al. 2014).

On the other hand, some PMPs reach peroxisomes after passing the ER, which implicates that these PMPs require targeting information for the ER and for the transfer from the ER to peroxisomes (Fig. 2d) (Mayerhofer 2016; Kim and Hettema 2015). However, the relation between these two transport routes is not completely clear, because they could either be totally separated or present as alternative routes, which are used under different conditions (e.g. peroxisomal proliferation upon stimulation or peroxisomal re-establishment in peroxisome-free cells) (Agrawal and Subramani 2016; Kim and Hettema 2015).

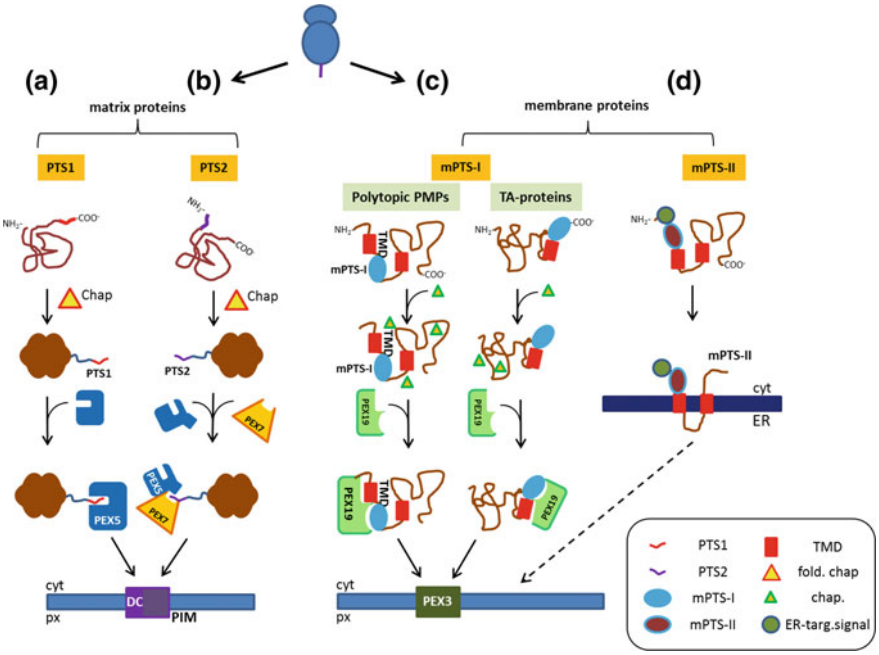


Fig. 2 Protein transport routes to peroxisomes: The transport routes of peroxisomal matrix proteins (**a**, **b**) and peroxisomal membrane proteins (PMPs) (**c**, **d**) are depicted schematically to distinguish different pathways in animal cells. **a** PTS1 motifs are encoded at the extreme C-terminus of proteins, which are produced by free ribosomes and folded in the cytosol with the help of chaperones. These PTS1 motifs are specifically bound by the PTS1 receptor PEX5, which transports its cargo to the peroxisomal surface, where it binds to the docking complex (DC) as entrance point to the peroxisomal import machinery (PIM). **b** Similarly, PTS2 motifs are found in proximity to the N-terminus of proteins and are sequentially bound by the PTS2 receptor PEX7 and the co-receptor (e.g. PEX5). This trimeric complex translocates to the DC in the peroxisomal membrane and becomes transported across the membrane. **c** Some PMPs are produced in the cytosol and their mPTS-I motifs are recognized by the receptor protein PEX19, which transfers its cargo to the peroxisomal membrane by attaching to the integral membrane protein PEX3. This mechanism can act on polytopic membrane proteins and tail-anchored (TA) proteins. **d** Other PMPs are first integrated into the membrane of the ER, and are subsequently transferred to peroxisomes; the dashed line indicates that the mechanism of transfer is not yet understood

3.3 Peroxisomal Targeting Signals

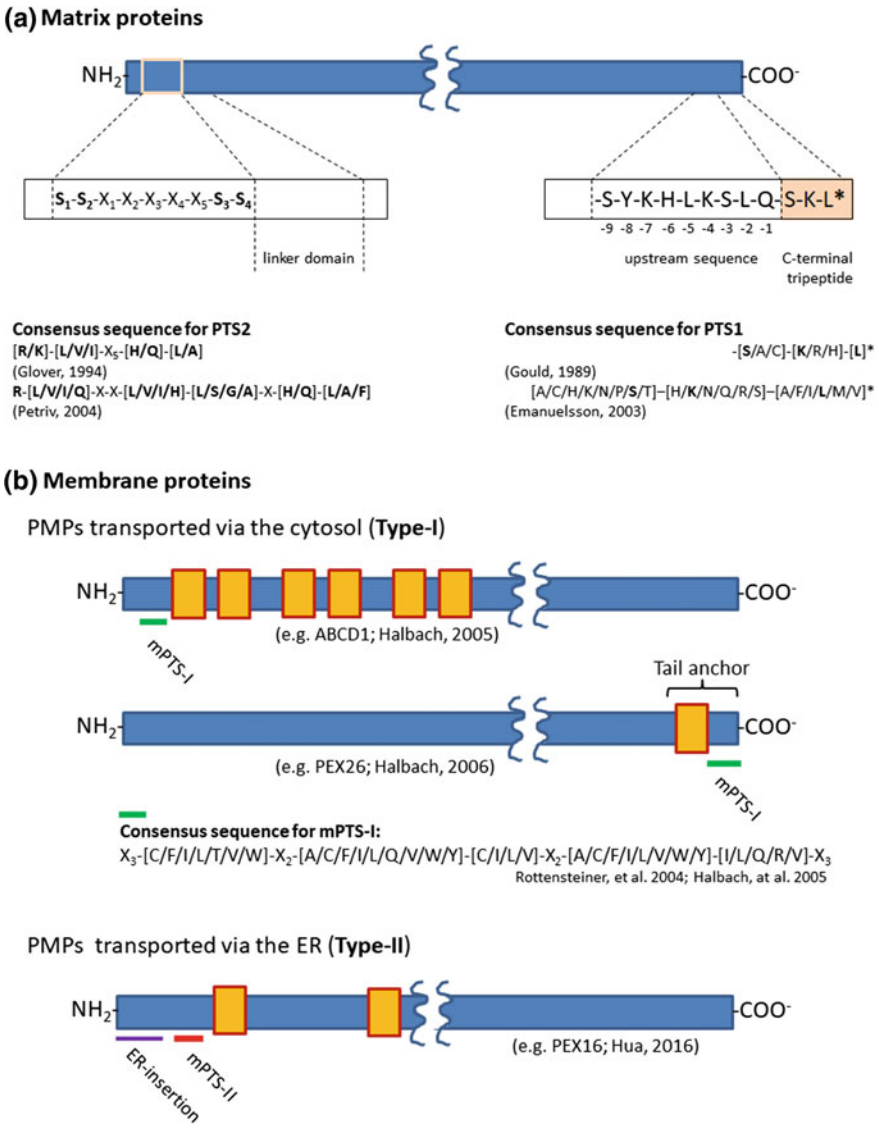
Targeting signals have been found in all peroxisomal proteins residing either in the lumen or in the limiting membrane of peroxisomes. These motifs have been described as PTS (Subramani 1998). Soluble matrix proteins can use one of two types of targeting signals, PTS1 or PTS2. Whereas the targeting signal initiating the transport of PMPs from the cytosol to peroxisomes, termed the targeting signal for

membrane proteins (mPTS), has been characterized to a reasonable extent (Van Ael and Fransen 2006), the signal mediating the transport of individual membrane proteins from the ER to peroxisomes is poorly understood.

3.3.1 Targeting Signals for Matrix Proteins

PTS1: PTS1 reside at the extreme C-terminus of the encoding protein (Fig. 3a) and the motif has originally been defined as the tripeptide serine-lysine-leucine (-SKL) mediating the transport of firefly luciferase into mammalian peroxisomes (Gould et al. 1988). Later, the PTS1 was described to be compatible with conservative variations within the tripeptide resulting in the first suggestion of a consensus sequence as [S/A/C]-[K/R/H]-[L] (Gould et al. 1989). This tripeptide motif is evolutionary conserved as demonstrated by similar analyses in yeast, plants and insects (Gould et al. 1990), but PTS1 motifs in endogenous proteins of these species showed marked deviations from the prototypical [–SKL] such as [–ARM] in isocitrate lyase from *Gossypium sp.* (Trelease et al. 1994) or [–AKI] in *Candida tropicalis* (Aitchison et al. 1991). Mutational analyses of PTS1 variants revealed that individual deviations from the consensus sequence are compatible with functional PTS1 motifs (Elgersma et al. 1996). However, only when using a yeast two-hybrid-assay to systematically screen a peptide library for possible binding partners of the PTS1-receptor PEX5, the group of Andreas Hartig was able to demonstrate that a broad variety of tripeptides can function as PTS1 motif, which includes marked deviations from conserved physico-chemical properties observed at the intermediate position of the tripeptide (-SLL instead of -SKL) (Lametschwandtner et al. 1998). Furthermore, a relaxed PTS1 consensus sequence ([A/C/H/K/N/P/**S**/T]–[H/**K**/N/Q/R/S]–[A/F/I/**L**/M/V]) has been suggested based on a broad range comparison of PTS1 motifs from diverse species (Emanuelsson et al. 2003). A similar variability in the amino acid composition has been found in PTS1 motifs of peroxisomal proteins from diverse plant species (Lingner et al. 2011).

Fig. 3 Peroxisomal targeting signals (PTS): **a** Targeting signals for matrix proteins: the PTS1 ▶ resides at the extreme C-terminus and is comprised of the C-terminal tripeptide as key determinant and an upstream sequence modulating the quality of the PTS1; the PTS2 has been described as nona-peptide close to the N-terminus with four highly conserved positions (S_1–S_4) and positions without recognizable conservation (X_1–X_5), which have been originally used to define a loose consensus sequence (69) that has been specified later on (73). **b** Targeting signals for membrane proteins: the mPTS-I (B1) has been found in all proteins transported from the cytosol into peroxisomes and mediates the interaction with the cytosolic receptor PEX19; such motifs can occur within polytopic membrane proteins and close to the C-terminus of tail-anchored proteins; the mPTS-II is crucial for the peroxisomal transport of PMPs, which are translocated to peroxisomes even without a PEX19 binding domain, which is supposedly linked to the transfer of the proteins from the ER to peroxisomes (B2); accordingly, these proteins contain a sequence element necessary to mediate the import into the ER. As the relative position of the mPTS within the protein varies, prototypic proteins are depicted such as ABCD1 (B1, upper) (Halbach et al. 2005), PEX26 (B1, lower) (Halbach et al. 2006) or PEX16 (B2) (Hua et al. 2015)



However, this seemingly low specificity of PTS1 receptors was contrasted by the finding that the preferences of PEX5 from *Homo sapiens*, *Saccharomyces cerevisiae* and tobacco (*Nicotiana tabacum*) differ markedly (Kragler et al. 1998; Lametschwandtner et al. 1998). Furthermore, a detailed study of the identified peptides and the determination of their relative binding strength to PEX5 proteins from different species revealed that the sequence preceding the tripeptide is important for the quality of the PTS1, which was later confirmed by computational

analysis of the PTS1 motifs in naturally occurring proteins (Lametschwandtner et al. 1998; Neuberger et al. 2003a). These upstream sequences were found enriched in amino acids conferring structural flexibility, which has been associated with the proper exposition of the C-terminal tripeptides (Neuberger et al. 2003a, b). Recently, the isolation and characterization of the proteome from *Arabidopsis thaliana* leaves provided an unprecedented number of proteins harboring a PTS1-like sequence at their C-terminus and most of them are able to mediate the transport of EGFP into plant peroxisomes (Reumann et al. 2007, 2009).

PTS2: PTS2 have been characterized as a conserved nona-peptide sequence, which is usually located in proximity to the N-terminus, although a few examples of internal PTS2 motifs have been described (Fig. 3a) (Osumi et al. 1991; Swinkels et al. 1991). The consensus sequence [R/K]-[L/V/I]-X_5-[Q/H]-[L/A] was delineated from sequence alignments and confirmed by a mutational analysis in yeast (Glover et al. 1994b), but very similar amino acid preferences have also been found in rat (Tsukamoto et al. 1994) and tobacco (Flynn et al. 1998). This consensus sequence was further defined by systematic computational analyses of the increasing number of PTS2 carrying proteins from diverse species (Reumann 2004; Petriv et al. 2004) resulting in the suggestion of a more specified consensus sequence for the most common PTS2 motifs R-[L/V/I/Q]-X-X-[L/V/I/H]-[L/S/G/A]-X-[H/Q]-[L/A/F]), which disregards some very rare nona-peptide motifs (Petriv et al. 2004). Later on, we reanalyzed the available PTS2 motifs systematically and experimentally verified in a mammalian system that the observed preference for amino acids with large hydrophobic side chains at the central position (X_3) is functionally relevant, because charged residues at this position inactivate the PTS2 (Kunze et al. 2011). Furthermore, we could demonstrate that PTS2 motifs have to form an α-helical structure orienting the side chain of all critical residues towards one side, which acts as binding domain for the receptor PEX7. The proper exposure of the PTS2-helix from the core protein is mediated by a stretch of flexible amino acids, which probably acts as an unstructured linker domain (Kunze et al. 2011). The final sequence element found in most PTS2-carrying proteins is a processing site for the peroxisomal processing peptidase, TYSND1 in animals and DEG15 in plants, which releases the N-terminal PTS2-carrying peptide from the core protein (Schuhmann et al. 2008; Kurochkin et al. 2007).

PTS3: The observation that in some peroxisomal matrix proteins no typical targeting signal could be found (Klein et al. 2002) or that the import of yeast catalase was affected by an internal amino acid sequence (Kragler et al. 1993) led to the suggestion of a third uncharacterized PTS, PTS3. However, the ability of peroxisomes to import PTS-less protein variants embedded within homo- or hetero-oligomeric complexes, which has been described as a piggy-back like mechanism (McNew and Goodman 1994; Islinger et al. 2009; Titorenko et al. 2002), provides an explanatory framework to resolve the above mentioned puzzles and abolishes the need to assume an independent PTS3.

3.3.2 Targeting Signals for Membrane Proteins

mPTS: The mPTS is encoded in PMPs and requires the presence of at least one transmembrane domain for its functionality (Halbach et al. 2005). However, at least two different types of mPTS motifs have been discriminated: type-1 mPTS (mPTS-I), which has been linked to the transport of proteins from the cytosol to peroxisomes (Fig. 3b) and type-2 mPTS (mPTS-II), which has been suggested for proteins that do not require a PEX19-binding domain for their transport to peroxisomes (Van Ael and Fransen 2006; Subramani 1998), and thus, might correspond to the mPTS for the alternative route via the ER.

Mutational analyses in different PMPs revealed characteristic sequences acting as mPTS-I, but these motifs differed markedly when comparing different PMPs or orthologous proteins, and sometimes more than one sequence element acted as mPTS (Brosius et al. 2002) (for review see Van Ael and Fransen 2006). This rendered the delineation of a consensus sequence rather difficult and frustrated all attempts to identify a common motif. As most PMPs share the ability to interact with PEX19, this interaction has been investigated in detail, although in some cases the sequence element acting as mPTS and the most effective PEX19-binding domain were found on separate parts of the protein (Fransen et al. 2001; Snyder et al. 2000). The characterization of the PEX19-binding domain has been approached by deletion analyzes and peptide binding assays, and by systematically mutating several positions of identified PEX19 binding domains to extrapolate a consensus sequence also applicable to mPTS-I motifs (Rottensteiner et al. 2004; Fransen et al. 2001; Halbach et al. 2005). Thereby, this motif could be narrowed down to the amino acid level in various PMPs resulting in the suggestion of the consensus sequence X_3-[C/F/I/L/T/V/W]-X_2-[A/C/F/I/L/Q/V/W/Y]-[C/I/L/V]-X_2-[A/C/F/I/L/V/W/Y]-[I/L/Q/R/V]-X_3 (Fig. 3b), which proved sufficient for the identification of other PEX19 binding motifs in different PMPs (Halbach et al. 2005). An overview of the data including the position of the transmembrane domains and the targeting signals has been compiled in an independent review (Van Ael and Fransen 2006). The conservation pattern of PEX19-binding elements was in agreement with the formation of an α-helical domain, which was confirmed by the elucidation of the 3D-structure of a complex consisting of an mPTS-I/PEX19-binding domain interacting with PEX19 (Schueller et al. 2010).

The targeting of TA-type proteins to peroxisomes requires a PEX19 binding domain and some specific properties of the amino acid composition of the transmembrane domain close to the C-terminus (Costello et al. 2017), which also conveys organellar specificity, because TA-anchored proteins have been found in various membranes (Borgese et al. 2007) (compare chapter in this book by M. Schrader).

However, it has been reported that a subclass of PMPs such as PEX3 and PEX16 reach peroxisomes via the ER (Kim et al. 2006; Lam et al. 2010) suggesting a completely different transport route. Therefore, these proteins need to encode at least two types of targeting information to initiate first the insertion into the ER and then the transfer from the ER to peroxisomes. This route has been suggested to

occur either constitutively (Aranovich et al. 2014) or during a restoration phase to generate novel peroxisomes upon complementation of cells lacking even content-free peroxisomal remnants (ghosts) in animals (Kim et al. 2006) and yeast (Hoepfner et al. 2005). The insertion of PEX3 into the ER has been reported to occur via a signal peptide-like mechanism (Mayerhofer et al. 2016), but also via the specific interaction with PEX16 (Aranovich et al. 2014). However, the specific transfer of such proteins from the ER to peroxisomes should require an additional targeting signal, but up to now only one report investigated a domain of PEX16 required for this transport in mammalian cells (Hua et al. 2015).

3.4 Computational Algorithms to Predict the Subcellular Localization of Proteins

In this chapter, we summarize prediction algorithms, which have been used to forecast the peroxisomal localization of proteins. These algorithms have been designed to either exclusively evaluate the quality of isolated sequence elements, such as PTS, or to predict the peroxisomal compartmentation of a protein based on its overall similarity to other peroxisomal proteins. Conceptually, the first type can be used to identify all proteins encoded in a genome and harboring any kind of PTS, assuming that such a motif is a reliable indicator for the peroxisomal compartmentation of the protein. In contrast, the second type parameterizes different properties of each protein to characterize it by a large set of properties, among which the presence of a PTS can be listed. However, both approaches are based on the analysis of data sets comprised of proteins with known compartmentation, termed the learning set, which can be subdivided according to the subcellular localization of the proteins either into two (peroxisomal/non-peroxisomal) or more (diverse subcellular localizations including peroxisomes) groups. Next, these proteins are analyzed to attribute a panel of values to each amino acid sequence, which describes different properties of this protein or of well-defined parts of the protein. A subclass of these properties can then be used to describe either a PTS (in the first type), or to define a kind of barcode, which is sufficiently characteristic to predict the localization of the protein. Therefore, these barcode-like compilations of properties are compared among the members of each group to identify similarities and between the groups to identify characteristic differences, which are sufficient to reclassify all proteins into the originally defined categories of the learning set. As the majority of these barcode-elements (properties) is rather independent and expressed in different units or dimensions, their analysis and comparison is a highly complex task, which can only be performed with specialized algorithms. Finally, an additional algorithm is generated, which determines the same set of parameters for any input protein and then uses the values of these parameters to generate a barcode allowing the classification of the novel protein either into the group of peroxisomal proteins or the group of non-peroxisomal ones.

Altogether, prediction algorithms to identify peroxisomal proteins are either based exclusively on the identification of PTS motifs, or they combine the latter with the determination of other properties, such as alternative targeting signals, to improve their performance by a preselection (e.g. excluding proteins harboring a mitochondrial leader sequence or a signal peptide from being a candidate for a peroxisomal protein). Alternatively, they use a broad variety of properties to classify proteins into different groups sharing the same compartmentation, but in these cases the prediction of PTS plays an ancillary role.

3.4.1 Prediction Algorithms to Identify Targeting Signals

As targeting signals consist of core elements embedded within a loosely defined context, any prediction algorithm needs to grasp the difference between essential elements, which are often reflected by a strict consensus sequence and modulatory elements, which improve or deteriorate the quality of the targeting signal, but are not essential. Furthermore, the relative abundance of residues with specific physico-chemical properties within a stretch of amino acids (e.g. a certain number and ratio of positive, hydrophobic and hydroxylated residues within the N-terminal sequence of mitochondrial or chloroplast proteins) (Nakai and Kanehisa 1992; Imai and Nakai 2010) can be critical, but also the combination of two or even more independent elements has been used to describe a targeting signal [e.g. in the prediction of signal peptides for the ER (von Heijne 1986)]. However, the degree of conservation within a targeting signal varies remarkably as PTS2-motifs contain five highly conserved positions within a nona-peptide, whereas mitochondrial or chloroplast targeting signals just share amphiphilic properties together with some loosely defined properties (Kunze and Berger 2015). To identify targeting signals, prediction algorithms scan either the whole amino acid sequence of a protein of interest or just a critical domain (e.g. the N- or the C-terminal part) to identify peptide sequences with reasonable similarity to known targeting signals. Alternatively, a theoretical representation of a targeting signal can be used, which has been delineated by statistical means from a large set of proteins sharing that particular type of targeting signal, and which displays the variability of amino acid residues at each position by the frequency distribution or other probabilistic terms (Emanuelsson and von Heijne 2001; Imai and Nakai 2010).

Prediction algorithms for targeting signals can be classified according to the methodical approach to delineate characteristic properties of the targeting signals. On the one hand, targeting signals can be effectively analyzed by multiple sequence alignments based on the assumption that a large number of naturally occurring motifs reflects the majority of possible combinations of residues within the targeting signal. On the other hand, machine learning approaches try to extract the crucial properties of targeting signals by collecting a broad variety of properties, which characterize these motifs and compare the values reflecting these properties with those of other amino acids sequences, which do not function as PTS.

Prediction Algorithms Based on the Conservation Pattern at Individual Positions of the Targeting Signal

This approach is based on multiple sequence alignments of targeting signals derived from many different proteins and aims at the identification of crucial positions within ungapped motifs, which can be analyzed by probabilistic algorithms for each position individually. Crucial positions present with low variability, usually expressed as high evolutionary conservation, and reflect high information content. The relevance of such crucial positions can be verified experimentally by exploring the functional consequences of property-changing substitutions at these positions of the targeting signal (for PTS2 motifs see Kunze et al. 2011). A traditional depiction of a consensus sequence, in which only the variability at the most conserved positions of a motif is depicted, can be considered as a simplified model of such an approach. Furthermore, at less conserved positions the preference for residues with specific properties or the contribution of specific residues to the formation of secondary structures can be analyzed. However, all these results are based on the comparison of the frequency distribution of different amino acids at individual positions relative to the expected amino acid frequency in arbitrarily chosen protein sequences. Thereby, for each position of such a motif, either the information content (e.g. represented by the relative Shannon entropy), the property distribution of the amino acids (e.g. a preference for positively charged residues or an underrepresentation of negatively charged ones) or the preference for certain amino acids (e.g. a preference for arginine, but not for lysine, although both residues are positively charged) can be depicted. However, also structural properties of targeting signals can be delineated from the distribution of amino acids at individual positions (Lund et al. 2005). Using the frequency of different amino acids in well-defined secondary structures as an external reference, the observed amino acid distribution within a targeting signal can be used to predict structural properties of the motif. Accordingly, a high α-helical content can be delineated from the high relative abundance of amino acids frequently found in α-helical domains, whereas amino acids characteristic for unstructured domains should be underrepresented (Kunze et al. 2011).

The generation of a prediction algorithm for a targeting signal implements all information about each position of the motif into a matrix, in which the positions of the targeting signal are outlined in one dimension and the measures for different properties delineated from the amino acid distribution at each position are outlined in the other dimension. Subsequently, the relevance of each property at each position for a successful prediction is estimated by comparing amino acid frequencies and assigning weights to each property at each position. Thereby, the learning set can be used to generate a matrix with maximal predictive performance when classifying amino acid sequences into those harboring a targeting signal and those without targeting information (Schneider and Fechner 2004). This matrix is known as position specific scoring matrix (PSSM), which can be used to estimate the similarity of a given amino acid sequence identified within a protein and the targeting signals observed in other peroxisomal proteins. Consequently, the quality

of such an amino acid sequences as functional targeting signal can be predicted and presented by assigning a score. A typical depiction of the multitude of implemented parameters obtained by such analyses and their implementation into a prediction algorithm can be studied in papers describing the investigation of the PTS1 motif using a PSSM (Neuberger et al. 2003a, b).

Generating Prediction Algorithms for Targeting Signals by Combining Various Properties and Considering the Context

In contrast to sequence alignment-based methods, machine learning approaches such as support vector machines (SVM), artificial neural network approaches (ANN), Hidden Markov Models (HMMs) and Bayesian network analyses, can treat different amino acid sequence elements or even the full-length protein as independent units, thereby allowing the implementation of properties, which depend on the utilization of external information (e.g. the comparison with databases for conserved structural domains or the utilization of information stored in the gene ontology (GO) database).

For machine learning approaches, the different properties, which can be delineated from amino acid sequences encoding targeting signals or even subdomains thereof, are compiled as a set of properties, which can be depicted as vector with each of n properties representing one dimension of an n-dimensional space. Thereby, each protein of the learning set can be depicted as a vector in the n-dimensional space and by consideration of a subset of parameters, the group of vectors representing peptide sequences harboring a PTS (or just peroxisomal proteins) can be spatially separated from the residual vectors corresponding to arbitrary amino acid sequences of the learning set not containing a PTS (or simply all other proteins). Next, an algorithm is generated, which allows the assignment of a novel amino acid sequence either to the group of sequences encoding a targeting signal or to the control group. Therefore, the novel amino acid sequence is analyzed to delineate values for the same properties previously used to establish the n-dimensional space before, which allows the estimation of a vector representing the new amino acid sequence and the determination of its spatial proximity to one of the other groups of vectors, representing either sequences encoding the targeting signals or control sequences.

SVM: This assignment can be based on the subdivision of the n-dimensional space into sections representing the two groups of vectors, by means of an $(n - 1)$-dimensional hyperplane acting as decision boundary (e.g. a 2-D plane subdividing a 3-D space). However, the computation of the position of the hyperplane within the n-dimensional space can follow different criteria in regard of the optimal course of the plane (e.g. aiming at optimally separating the centers of gravity between the groups of vectors or optimizing the margin for vectors in spatial proximity to the border between the groups). Also, the algorithms to combine different properties (vector dimensions) may use either linear combinations or implement an additional mathematical function, the so-called kernel function, to transform the data set and

capture non-linearity rendering the groups better separable. An SVM based algorithm has been used to predict the targeting signals governing the transport of tail-anchored proteins in mammalian cells (Costello et al. 2017).

ANN: Alternative approaches try to relate the amino acid sequence of each individual protein of the learning set (used as input) to a classifying evaluation, which indicates the presence or absence of a targeting signal as output. Therefore, the learning set can be depicted as multilayer arrangement, in which the amino acid sequence (input layer) and the classifying evaluation (output layer) are linked by a layer of connecting elements ("neurons"), each representing the combination of variables of the different properties that can be delineated from the primary sequence or from the output of a more complex evaluation. The relative contribution of each of these connecting elements to the output is adjusted by the training of this system using the proteins of the learning set. Additional layers can specifically combine the results of a subclass of connected elements and can convert the output into an understandable format (e.g. the classification of the amino acid sequence as a PTS). Such an arrangement as a neural network has been used to generate the prediction algorithm TargetP, which evaluates amino acid sequences for the presence of N-terminal targeting signals (Emanuelsson et al. 2000).

HMM: Another approach to generate a prediction algorithm is based on the analysis of the relation between input (amino acid sequence) and output (ability to function as PTS) supposing the existence of a variety of internal (=unknown) states within the algorithm, each of which can adopt different values. When the system is in one state, it can transit exclusively to several other states, and thus, each state of the system can be retraced to a limited number of preceding states. Within the system, a change between states occurs with a certain probability (*important: probability of the conversion and not of the state*) and each of these states can give rise to different outputs, but the relation between an internal state and the output can be described by a probability as well (Eddy 1998). Utilizing the learning set to train this arrangement of states, the probability of transitions is set appropriately to optimize the relation between input and output. A plethora of properties, which can be derived from the amino acid sequence, can be linked to the probability of reaching a certain internal state and may also affect the probability of the transition to another state. A well-known prediction algorithm of signal peptides using this method has been described and commented previously (Nielsen and Krogh 1998; Emanuelsson and von Heijne 2001).

BN: Finally, a prediction algorithm can use the subdivision of the learning set into positive and negative samples to determine for both samples the probability of occurrence for each of the many properties delineated from the amino acid sequence (e.g. the presence of a characteristic domain within the primary sequence). When focusing on properties, for which the probability of occurrence differs between the positive and negative data set, the classification of a protein can be based on the probability to find a protein with a given combination of values for a subset of properties (barcode) within an organelle, which contains other proteins that have compilations of property values (barcodes) in a certain range. Accordingly, the elucidation of the probability to find proteins with a specific value for a certain

property (e.g. an isoelectric point within a given range) is used to classify a novel protein either with the positive sample (e.g. harboring a PTS) or the control group of the learning set. The prediction is based on the product of individual evaluations for the protein´s probability to be peroxisomal, before and after considering each of the analyzed properties of the protein. According to Bayes´ equation for conditional probability, the probability of being peroxisomal (PX) given property X is $P(PX|X) = \frac{P(X|PX)*P(PX)}{P(X)}$. This could be applied to a hypothetical probability of an amino acid sequence to act as PTS (P^{PTS}) when the property X has a value within a certain range (e.g. X = "the number of positive charges within the last 12 amino acids is larger 4") ($P^{PTS}_{\text{given X}}$). This P^{PTS} would then correspond to the probability that an amino acid sequence containing a PTS has property X (can be extracted from the positive examples of the learning set) ($P^{X}_{\text{given PTS}}$) multiplied by the probability that an amino acid sequence contains a PTS (can be calculated from a consensus sequence) (P^{PTS}) divided by the probability for any protein to have the property X (P^{X}). Using such an approach, the probability to encode certain types of conserved domains has been used as a tool to predict the subcellular distribution of proteins by the Bayesian network predictor PSLT (Scott et al. 2004) and was able to reach a satisfying performance.

As all these approaches have been successfully used to generate prediction algorithms, their utilization for the generation of a prediction algorithm for PTS appears reasonable, but more recently, even a combination of these approaches with homology based methods has been reported (Lingner et al. 2011).

If the learning set consists of more categories (e.g. by subdividing the learning set into groups of proteins sharing the same transport route or the same subcellular compartment), the n-dimensional space should be populated with a corresponding number of groups of vectors representing these assemblies of proteins. By this, the group of non-peroxisomal proteins can be better characterized, because characteristic properties of the subgroups can be revealed more easily when the proteins of this group are not intermixed within a large set of arbitrarily composed proteins. This might reveal suitable separation criteria not only among the groups of non-peroxisomal proteins, but allows a better distinction of peroxisomal from non-peroxisomal proteins. However, the multiplicity of separation criteria might also generate the problem of misclassification simply for statistical reasons, because more separation processes have to be performed.

3.4.2 Prediction Algorithms to Classify Proteins into Groups Sharing the Same Subcellular Compartment

The other type of prediction algorithms tries to forecast the subcellular localization of whole proteins using classifier algorithms to categorize a novel protein with pre-defined groups of proteins, which are confined to the same compartment based on all properties of these proteins. Such algorithms can classify either into two

groups [e.g. peroxisomal and non-peroxisomal) or into several groups [e.g. up to 22 subcellular locations and the cytosol (Chou and Shen 2007)] according to a weighted combination of properties, which are sufficient to describe groups of proteins that share the same location. Usually, these properties involve the presence of targeting signals, but also other properties, which can only be extracted from full length proteins, but correlate with the distribution of proteins (e.g. pI, similarity score to other proteins, the presence of characteristic motifs, GO-annotations…). This allows the algorithm to predict the subcellular localization of a protein based on the probability to classify into one of the groups, which is reflected by the assignment of a probability to be part of one each cluster [e.g. Wolf Psort predictor, https://www.genscript.com/wolf-psort.html, scoring % probability to find the protein in a specific location (Horton et al. 2007)]. Several of such classifying prediction algorithms have been described, but the relatively low number of peroxisomal proteins renders this group of proteins rather problematic for the use in systematic classifications. Thus, PTS1 prediction algorithms have been often implemented into these classifiers, but these do not handle PTS2 and mPTS motifs.

3.5 Available Prediction Algorithms for the Identification of Peroxisomal Proteins

The increasing understanding of PTS facilitated the development of prediction algorithms, which evaluated the quality of PTS using diverse approaches such as similarity measures (position specific scoring matrix; PSSM) or machine learning (SMV). Moreover, classifiers have been developed to assign individual proteins to different subcellular compartments, which categorize proteins according to a variety of different criteria. Several of these algorithms are listed below concentrating on tools, which can be applied to animal proteins.

3.5.1 Prediction Algorithms for Targeting Signals

PTS1 motifs

The prediction of peroxisomal proteins based on the similarity of the actual C-terminal sequence with the PTS1 consensus sequence started early (Gurvitz et al. 2000; Kal et al. 2000). However, in 2003 two prediction algorithms for PTS1 motifs have been published, namely the **PTS1-predictor** (Neuberger et al. 2003b) and the **PeroxiP** predictor (Emanuelsson et al. 2003), which was later on modified to generate the **PTS1Prowler** (Hawkins et al. 2007). Furthermore, in an independent approach the prediction of PTS1 motifs was used to identify novel peroxisomal proteins among compilations of human and rodent proteins (Kurochkin et al. 2005). In the context of the generation of a peroxisome database (http://216.92.14.62/home.php) another prediction algorithm for PTS1 motifs was provided

(Schluter et al. 2007). Special prediction algorithms for PTS1 motifs from plants have been developed such as **PPero** (http://biocomputer.bio.cuhk.edu.hk/PP/) (Wang et al. 2017) as well as an untitled predictor (Lingner et al. 2011), which has recently been implemented into a plant specific prediction server (**PredPlantPTS1**), which is available at http://ppp.gobics.de/ (Reumann et al. 2012).

The **PTS1-predictor** has been developed using a PSSM approach for a broad data set, which does not only include naturally occurring peroxisomal proteins known to harbor a PTS1 (positive data set), but also all peptide sequences obtained by two-hybrid screens using PEX5 proteins from diverse phylogenetic origin (yeast, human and *A. thaliana*) as bait and a library of arbitrary peptide sequences as prey (Neuberger et al. 2003b). Therefore, the prediction covers a broad variety of PTS1 tripeptides, some of which had not been observed in naturally occurring proteins at the time, but can mediate an interaction with a PEX5 protein in the two-hybrid assay. Moreover, the evaluation of the quality of the upstream sequence, also described as flexible linker domain, is based on many sequences with diverse properties as well (Neuberger et al. 2003a, b). The output of the predictor is an evaluation score to rank C-terminal peptides, but two thresholds are specified, which categorize peptides into highly probable PTS1 motifs ("targeted"), intermediately probable PTS1 motifs ("twilight") or peptides, which probably do not function as PTS1 ("non-targeted") (Neuberger et al. 2003b). The prediction algorithm has been provided online (http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp) and allows the selection of a phylogenetic class, from which the protein of interest originates, thereby taking into consideration taxon-specific differences. Furthermore, the qualification of the C-terminal tripeptide and that of the upstream sequence are displayed separately. Accordingly, this predictor has been amply used to evaluate PTS1 motifs of individual candidate proteins, but also to rank proteins obtained by proteomic approaches (Wiese et al. 2007; Gronemeyer et al. 2013b).

PeroxiP consists of two major elements evaluating the 12 C-terminal residues of a protein, but separates the information of the tripeptide and that of the upstream sequence, which is especially considered from the perspective of its amino acid composition (Emanuelsson et al. 2003). PeroxiP has also been provided online (http://bioinfo.se/PeroxiP/).

PTS1Prowler is a derivative of the more general predictor for targeting signals called Prowler (http://pprowler.imb.uq.edu.au) and was generated using data sets used by PeroxiP (Emanuelsson et al. 2003) with adaptations (Hawkins et al. 2007; Boden and Hawkins 2005). However, the algorithm was modified to use a three-step procedure consisting of (i) a basic motif check accepting a broad variety of PTS1 like motifs, (ii) an SVM-classifier evaluating the last 12 amino acids based on amino acid composition and an evaluation of the whole sequence, and (iii) the exclusion of proteins, which are predicted to be secreted based on the assumption that such proteins cannot reach peroxisomes.

Altogether, the available PTS1 predictors use very different approaches: *PTS1-predictor* is based on PSSM (Neuberger et al. 2003b), *PTS1Prowler* is founded on a sequence-biased recurrent network mode (Boden and Hawkins 2005; Hawkins et al. 2007), the predictor provided by Lingner and Reumann uses position-specific

weight matrices (PWM/PSSM) and a residue interdependence (RI) model-based prediction, and *PPero* uses a Bayesian network based algorithm (Wang et al. 2017). Some of these prediction algorithms have also been included into more complex prediction algorithms uniting various independent algorithms aiming at a better overall prediction quality such as PROlocalizer (Laurila and Vihinen 2011).

PTS2 motifs

The prediction of PTS2-motifs has been hampered by the very low number of independent proteins harboring this type of targeting signal even when considering all branches of the tree of life (Kunze et al. 2011). In an early approach, the search for putative PTS2 motifs in proteins from protozoa (*Leishmania* and *Trypanosoma*) was guided by a loose consensus sequence that was transformed into {[M]-x{0,20}-[RK]-[LVI]-x_5-[HQ]-[ILAF]} (Opperdoes and Szikora 2006). However, neither of the predicted PTS2 motifs has been tested rendering the quality of this prediction algorithm unclear, because the number of peptides conforming with the suggested consensus sequence is as high as 1.5×10^8 or about 0.3 per million of all possible nona-peptides. In another approach, a prediction algorithm for the PTS2 motif was generated by combining the definition of a consensus sequence obtained by sequence alignments of known PTS2 motifs, an independent HMM profile and the expression profile of the predicted proteins to identify murine proteins encoding a PTS2. However, none of the five tested PTS2 motifs was functional within the full-length protein and this disappointing result was traced back to the low number of positive samples in the learning set (Mizuno et al. 2008). Finally, our group approached this problem by combining statistical analysis of naturally occurring PTS2 motifs using a PSSM, the knowledge on previously published mutations affecting the functionality of the PTS2, and our own mutational analysis of the central, less conserved positions of the PTS2. The PSSM was generated by selecting three orthologues of each PTS2-carrying protein derived from evolutionary distant species and comparing them to the N-terminal regions of 1000 arbitrarily chosen proteins (Kunze et al. 2011). Moreover, we considered the α-helical property of the PTS2 core domain and the unstructured domain connecting the helix with the core domain. When the N-terminal sequences of naturally occurring human proteins were scanned for the presence of PTS2 nona-peptides and several high-ranked peptides were tested in a reporter protein context, we found that about 25% (4/15) of the tested peptides were able to act as functional PTS2, but only one of the encoding proteins was actually found in peroxisomes (Kunze et al. 2011). Moreover, minimal prediction tools for PTS2 motifs have been generated based on the MEME system (http://meme-suite.org/) and processed by the Blocks-Server (http://blocks.fhcrc.org/blocks/). This "Do-It-YourSelf Block Search" has been implemented into the peroxisome database platform (http://www.peroxisomedb.org/home.jsp) (Schluter et al. 2010) and the prediction algorithm for PTS2 motifs is accessible at http://216.92.14.62/diy_PTS2.html.

mPTS motifs

The analysis of motifs effectively interacting with the receptor PEX19 has been a key step in the characterization of mPTS and the results of these investigations together with extensive mutational analyses of PMP sequences facilitated the generation of a prediction algorithm for mPTS motifs (Rottensteiner et al. 2004; Halbach et al. 2005). This mPTS predictor is based on the MEME system as well and is provided by the Blocks-Server, which can be accessed at the *Peroxisome-DB* homepage (http://www.peroxisomedb.org/home.jsp).

3.5.2 Prediction Algorithms Based on Classifiers Using General Properties of Peroxisomal Proteins

In contrast to prediction algorithms, which are restricted to the recognition of targeting signals, other classifiers predict the subcellular location of proteins based on their similarity to other proteins of the same subcellular compartment. This similarity can be based on diverse properties, which are known to correlate with the subcellular distribution, such as amino acid composition (Cedano et al. 1997), general physical properties of proteins (Sarda et al. 2005), homology to other proteins with known location (Mott et al. 2002) or gene ontology (Wan et al. 2014). However, these classifiers also include elements to identify specific targeting signals, which are necessary to reach a certain cellular location. These different parameters can be combined either by successive application of different prediction algorithms, exemplarily demonstrated for manual execution in a tutorial for the sequential utilization of different available prediction algorithms (Emanuelsson et al. 2007), which has later been implemented into the PRO-localizer algorithm (http://bioinf.uta.fi/PRObalizer/) acting as sequence of binary classifiers (Laurila and Vihinen 2011). Other successful prediction algorithms have been developed including peroxisomes as individual category such as PSORT-II (https://psort.hgc.jp/form2.html) and WolfPSort (https://www.genscript.com/wolf-psort.html) (Horton et al. 2007), MultiLoc (http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc/) (Hoglund et al. 2006) and pTarget (http://bioinformatics.albany.edu/~ptarget) (Guda and Subramaniam 2005; Guda 2006). Later, various other prediction algorithms were introduced, in which the analysis of alternative specific properties of proteins had been implemented as additional classification criteria, such as protein homology and GO-annotation in the CELLO2GO predictor (http://cello.life.nctu.edu.tw/cello2go/) (Yu et al. 2014), the consideration of taxon-specific differences within targeting signals and among protein properties in the Cell-PLoc 2.0 predictor (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/). Special predictor variants for human proteins are available at Hum-mPLOC 2.0 (Shen and Chou 2009) or SCLpredT (http://distillf.ucd.ie/sclpredt/). Another predictor has been designed to enable the classification to many different subcellular compartments and to provide a platform for the analysis of large data sets such as Euk-mPLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/) (Chou and Shen 2010). However,

the relatively low number of independent peroxisomal proteins within the learning sets utilized for the generation of diverse algorithms might limit the statistical power of these predictors, when classifying peroxisomal proteins, which coincides with a relatively low performance in the identification of peroxisomal proteins (Sprenger et al. 2006). Other prediction algorithms just concentrate on compartments enclosing many proteins and do not even evaluate peroxisomes independently but only as part of the class "organelles", e.g. SCLpredT (http://distillf.ucd.ie/sclpredt/) (Adelfio et al. 2013). Moreover, in several classifiers, the prediction of a signal peptide or a mitochondrial targeting signal precedes the prediction of other destinations, because an early sequestration of a protein from the cytosol renders other destinations implausible, which has been supported by the observation that mimicking the cellular transport process improves the quality of some prediction algorithms (Nair and Rost 2005). For the predictive performance of proteins harboring a PTS, this decision might be beneficial, because it reflects the hierarchy of targeting signals suggesting that proteins harboring such signals at their N-terminus are sequestered from the cytosol before the PTS1 can become functional (Kunze and Berger 2015; Neuberger et al. 2004). However, such decisions might preclude the identification of PTS within bi-localized proteins, when two targeting signals act concomitantly.

Altogether, algorithms for the reliable detection of PTS1 motifs considering taxon-specific differences are available, but prediction algorithms for PTS2 and mPTS have been less successful. Moreover, many general classifiers involve peroxisomes as individual category and some of them even incorporate a PTS1 predictor.

4 Strengths and Weaknesses of Prediction Algorithms

4.1 General Considerations

Similarly to other methods prediction algorithms for targeting signals or for a certain subcellular location are no perfect tools, which can be applied without considering the limitations of the method. Obviously, such limitations are highlighted by the observation of an incorrect prediction of a targeting signal, but a certain error rate might be acceptable, when forecasting the distribution of an individual protein or when ranking a group of candidate proteins. However, a critical evaluation of this tool is crucially important when applying it to large data sets aiming at the prediction of the peroxisomal proteome. Therefore, we will first discuss internal reasons for an incorrect or biased prediction (Sect. 4.2) and then list intracellular mechanisms, which might explain a seemingly incorrect prediction in spite of the correct evaluation of the targeting signal (Sect. 4.3).

4.2 Incorrect Predictions Based on the Limitations of the Algorithm

Certainly, a large number of false positive or false negative classifications of targeting signals and of incorrect predictions of the subcellular compartmentation of proteins are caused by limitations inherent to the prediction algorithms, which can be either *too restrictive* (many peroxisomal proteins are not detected) or *too loose* (many non-peroxisomal proteins are suggested), or might suffer from an internal distortion producing a bias within the results.

In such cases, the composition of the learning set is often problematic, because the selection of the subset of proteins either representing the positive (PTS, peroxisomal) or the negative (no PTS, non-peroxisomal) examples is not perfect. Such deviations from optimal conditions might be unavoidable in some cases (e.g. the low number of evolutionary independent proteins found in peroxisomes), whereas others are caused by a false estimation of the importance of different criteria. However, as the learning set is the fundament of any prediction algorithm, its compilation is highly critical.

A major problem is the mis-classification of individual proteins into the positive (e.g. cytosolic proteins as peroxisomal) or the negative data set (e.g. a peroxisomal protein as non-peroxisomal), resulting in a distortion of the data set, which is highly relevant for data sets consisting of a low number of proteins such as proteins harboring a PTS2. Moreover, the composition of the data set should be balanced across the phylogenetic tree or across a specific branch of the tree of life, if the prediction algorithm should be specialized for such a subdomain (e.g. the data set for a global prediction algorithm should involve proteins from many evolutionary distant species, whereas a prediction algorithm for plants can be restricted to plant proteins), because otherwise the results of the prediction algorithm might be biased and reflect the composition of the learning set. For instance, PEX5 proteins from yeast, human and tobacco show a remarkable difference in their preference for PTS1 peptides with different amino acid composition, which results in different orders when peptides are ranked according to their affinity to these PEX5 proteins (Kragler et al. 1998; Lametschwandtner et al. 1998). Accordingly, prediction algorithms for PTS1 motifs need to consider these taxon-specific differences, which can be implemented by providing different learning sets to generate predictors for each branch of the tree of life, which can be chosen depending on the phylogenetic origin of the protein of interest (Neuberger et al. 2003a, b). However, this restriction of the data set reduces the number of independent peroxisomal proteins within the learning set, and thereby decreases the statistical power of the analysis. Alternatively, a learning set including peroxisomal proteins from all taxa needs to be well-balanced to reflect all branches of the tree of life, although even then disregarding the phylogenetic origin of the proteins of interest might bias the ranking of functional PTS1 motifs. In contrast, a plant specific prediction algorithm for PTS1 motifs does not need to account for the specificities of the other branches of the tree of life (Lingner et al. 2011; Reumann et al. 2016).

Furthermore, the composition of the control data set, representing the non-peroxisomal proteins of the learning set, is rather important as well, because it serves as a reference for all statistical comparisons. Therefore, a biased data set might cause a disguise of important differences or cause an overrating of a comparatively irrelevant property. Exemplarily, the relative amino acid frequency at different positions of the consensus sequence of a targeting signal is crucial for the evaluation of the conformity of a novel peptide sequence with a given targeting signal (e.g. the PTS1). In this case, the amino acid frequency of the background (control data set) could be defined either by the analysis of the amino acid frequency within naturally occurring proteins or by the amino acid frequency within N- and C-terminal domains. The latter sequences are more often exposed to the aqueous environment, whereas the interior of most proteins is dominated by hydrophobic residues (Sweet and Eisenberg 1983) rendering the decision of the appropriate reference data set a tricky task.

Finally, the number of independent samples within each class of the learning set is crucial, because a low number of examples drastically reduces the power of any statistical evaluation and increases the chance to implement an arbitrary property not related to the quality of the targeting signal.

4.3 Biological Background of the Misevaluation

Discordance between the prediction of a targeting signal and the actual localization of the encoding protein is not necessarily caused by deficits in the prediction algorithms. Several intracellular mechanisms disconnect the link between the presence of a targeting signal and the justified assumption of the according subcellular location of a protein, which is discussed below for the prediction of PTS.

A *false positive prediction* of a PTS is the inappropriate positive evaluation of a peptide as functional targeting signal and therefore, the suggestion of a peroxisomal localization of the encoding protein, although it is found elsewhere in the cell. Next to a simple mis-attribution of a targeting signal to a peptide sequence by the prediction algorithm, the observation would also be in line with a functional targeting signal, which cannot exert its function in the context of the full length protein. Several mechanisms have been described explaining such effects, namely (A) steric hindrance preventing the binding of the receptor to the targeting signal, (B) the presence of an alternative targeting signal sequestering the protein into another organelle before it can fold and become imported into peroxisomes, and (C) inactivation of a targeting signal by secondary modifications.

(A) Steric hindrance: The finding that PTS1 and PTS2 motifs are separated from the core domain of the encoding proteins by linker domains (Kunze et al. 2011; Neuberger et al. 2003a) suggests that the PTS need to be properly exposed to facilitate an interaction with the cognate receptor proteins. Conversely, the lack of exposure or the embedding of the targeting signal within the folded core of

the protein should prevent the peroxisomal transport of the encoding protein. This mechanism can serve as an explanation for the observation that the tripeptide –SKL cannot act as a PTS1 when directly fused to the C-terminus of murine dihydrofolate reductase (DHFR), but efficiently mediates transport into peroxisomes upon insertion of a short peptide sequence (6–7 amino acids) between the tripeptide and the core protein (Kragler et al. 1993). This finding retraced the functionality of the tripeptide to its proper exposure from DHFR and served as a test system for the evaluation of the predictive power of the PTS1-predictor (Neuberger et al. 2003b).

(B) Hierarchy of targeting signals: Peroxisomal matrix proteins are imported in a folded state, but the transport of proteins into mitochondria, chloroplasts or the ER occurs in a linear and unfolded state. Therefore, these proteins are either inserted co-translationally into the ER or are retained partially unfolded by cytosolic chaperones (Kunze and Berger 2015). Moreover, these N-terminal targeting signals can direct proteins to other organelles before the C-terminal PTS1 is produced. Accordingly, a C-terminal PTS1 is not active in proteins with a mitochondrial targeting signal (Mukhopadhyay et al. 2004). Moreover, PTS1 motifs have been found at the C-terminus of mitochondrial or secreted proteins, which had never been observed in peroxisomes, but these proteins can become peroxisomal when blocking the N-terminal targeting signal by fusion to a fluorescent protein (Neuberger et al. 2004). The occasional occurrence of PTS1 motifs in mitochondrial or secreted proteins has been interpreted as indicative of the lack of evolutionary counter selection, due to the constant inactivity of the PTS1. However, the dominance of a mitochondrial targeting signal over a PTS1 motif can be overcome by actively supporting the complete folding of the protein. When murine DHFR is equipped with a mitochondrial targeting signal and a PTS1, the protein can be redirected to peroxisomes by treatment with methotrexate, which acts as DHFR antagonist, but also supports the folding of DHFR.

(C) As PTS are peptide motifs, which can become targets for secondary modifications such as phosphorylation, the quality of a targeting signal can be affected by such events. In the yeast *S.cerevisiae* the peroxisomal import of the cytosolic protein NAD^+-dependent glycerol 3-phosphate dehydrogenase (Gpd1p) is regulated by phosphorylation occurring in the linker sequence between the PTS2 motif and the core enzyme (Jung et al. 2010). However, until now no example of such modulatory effects of phosphorylation have been described for proteins harboring a PTS1, although many PTS1 motifs contain a serine residue (e.g. in -SKL).

These examples illustrate that a targeting signal, which can interact with its receptor under specific conditions, is not necessarily active under all conditions. Therefore, a study of the subcellular distribution of an ectopically expressed tagged version of a full-length protein needs to be discriminated from the analysis of an isolated targeting signal in the context of a minimal reporter protein. Moreover, when trying to confirm the prediction of a targeting signal by investigating the

intracellular distribution of the encoding protein in a tagged form, the used protein isoform should be carefully selected, because an alternative targeting signal might repress the peroxisomal transport of the tagged protein. However, it needs to be stressed that many prediction algorithms already try to evaluate other targeting signals to properly take such possibilities into account.

In contrast, the term *false negative prediction* indicates that based on the protein sequence, no PTS has been suggested by the prediction algorithms, although the protein is found in peroxisomes (and not only in the peroxisomal fraction in a mass spectrometric analysis). Next to the inability of the prediction algorithm to recognize a targeting signal within a given protein sequence, such observations might be explained by cellular mechanisms, which allow the transport of individual proteins into peroxisomes although they do not encode an independent targeting signal. The most obvious mechanism is protein transport by a piggy-back mechanism, by which oligomerized proteins can become co-imported into peroxisomes. The effectivity of this mechanism has been conceptually demonstrated for an ectopically expressed PTS-less isoform of thiolase, which can become co-imported into peroxisomes (Glover et al. 1994a), but has recently been demonstrated as naturally occurring import mechanism for copper-zinc dependent superoxide dismutase (SOD1) (Islinger et al. 2009). SOD1 itself does not encode a recognizable PTS, but can reach the peroxisomal interior by interactions with its chaperone CCS1 harboring a PTS1. Similarly, a protein could interact with one of the receptor proteins via an atypical interaction mediated by another peptide sequence, which cannot be predicted by traditional algorithms. Another mechanism, which is expected to increase the number of false negatives in approaches to predict peroxisomal proteins is the read-through of stop-codons. This recently described cellular mechanism generates alternative C-termini in proteins by disregarding the natural stop codon. Surprisingly, in different taxa several cases of proteins with alternative C-terminal ends have been described, which are generated by read-through of a stop codon and some of them terminate with a functional PTS1 (Ast et al. 2013; Freitag et al. 2012; Schueren et al. 2014). However, as these protein variants cannot be delineated directly from genomic or mRNA sequences, they will not be predicted as peroxisomal proteins, because the relevant protein sequence is not annotated.

Furthermore, it should be stressed that prediction algorithms for PTS are not suitable to predict proteins that are attached to the outer side of the peroxisomal membrane via a prenyl-anchor, such as members of the RAB-family of small GTPases (Gronemeyer et al. 2013a). Finally, additional routes for peroxisomal protein import could exist, which either have not yet been sufficiently investigated to reveal details of the targeting signal or of the receptor protein [e.g. the transport of proteins from the ER to peroxisomes, which has been described for human PEX16 (Hua et al. 2015; Kim et al. 2006)] or which have not been described at all [e.g. the transport route of peptide hormones derived from proopiomelanocortin (POMC) to peroxisomes (Hoftberger et al. 2010)].

Last but not least, paralogous receptors might exist, which mediate the import of peroxisomal proteins independently of PTS1 or PTS2. Recently, in yeast, a homologue of the PTS1 receptor Pex5p has been identified, which has been termed

Pex9p and acts as independent import receptor for a subgroup of peroxisomal proteins under specific conditions, because Pex9p is only produced in the presence of oleic acid as carbon source (Yifrach et al. 2016; Effelsberg et al. 2016). In this case, the substrate specificity of Pex9p partially overlaps with that of Pex5p, but provided that Pex9p might recognize some unidentified proteins, which are not bound by Pex5p, these proteins would be prototypic for proteins translocated independently of the PTS1 or PTS2 motif. Similarly, a structural homologue of PEX5 was found in humans (PEX5-like; PEX5-L), but this protein cannot mediate translocation of its cargo to peroxisomes (Fransen et al. 2008). Altogether, false negative predictions do not necessarily indicate a problem of the prediction algorithm but might highlight interesting aspects of further research.

4.4 Evaluation of the Quality of Prediction Algorithms

As the above mentioned reasons for the suboptimal performance of prediction algorithms might raise general reservations against their broad application, the evaluation of the performance of individual prediction algorithms appears desirable and can be accomplished by different methods. These methods either use computational methods as well or rely on an experimental verification of predicted targeting signals, but the parameters to describe the performance are the same.

Similar to definitions in medical diagnostics the *quality of a prediction algorithm* can be characterized by the specificity and sensitivity or the positive predictive value. The *sensitivity* is defined as the number of true positives (TP) answering the question "*how many % of the positive samples are found again*" ($Sn = \frac{TP}{TP+FN}$), whereas the specificity is the number of true negatives (TN) answering the question "*how many of the excluded samples are correctly excluded*" ($Spc = \frac{TN}{TN+FP}$), but this value can also be considered as a measure for the number of false positive values as $FP = (1 - Spc)$. These parameter can be combined to the *accuracy* (Acc) answering the question "*how many % of the predictions are actually correct*" ($Acc = \frac{(TP+TN)}{(TP+TN+FP+FN)}$) or to Matthew's correlation coefficient (MCC), which is represented by $MCC = \frac{TP.TN-FP.FN}{\sqrt{(TP+FN)(TP+FP)(TN+FP)(TN+FN)}}$ representing a normalized value for the difference between correct and incorrect predictions that lies in the range between −1 and +1 (Matthews 1975). In contrast, the *positive predictive value* (PPV) determines "*how many % of the samples evaluated as positive are correct*" $PPV = \frac{TP}{TP+FP}$ (Altman and Bland 1994).

For the internal validation of the prediction algorithm, the elucidation of the above-mentioned parameter, should be performed with an independent data set generated at the beginning of the investigation by dividing the original data set into a learning set used to generate the prediction algorithm and a test set used to evaluate it. However, in case of a low number of available examples for the learning set (e.g. the number of evolutionary independent peroxisomal proteins of each

taxon) any further reduction of the learning set is highly undesirable. Therefore, a characterization of the algorithm can also be performed by re-utilizing the data from the learning set. For this, the learning set is subdivided into several groups (e.g. five groups containing 20% of the proteins each) and the predictive performance of an algorithm, which has been based on only 80% of the learning set, is determined when acting on the residual 20% of excluded proteins. When systematically generating such estimates for all subgroups of the data set, the performance of the predictor can be estimated by statistical means (Schneider and Fechner 2004).

Alternatively, the performance of such algorithms can and should be tested experimentally by demonstrating that it can correctly predict targeting information within naturally occurring proteins, which have not yet been linked to the organelle of interest. Therefore, the prediction algorithm is applied to a large set of protein sequences (e.g. the entirety of human protein variants known at the time) to obtain a list of high-scoring PTS motifs which are then tested experimentally. This can be done by ectopically expressing tagged variants of the suggested candidate proteins and determining their subcellular distribution by microscopic techniques or by testing the isolated sequence elements within well-described reporter proteins. However, even this approach may not be flawless, because weak targeting signals might be overrated, as high overexpression can induce residual import that is overestimated due to the concentrating effect of the small volume of mammalian peroxisomes. Although such trace amounts are probably biologically irrelevant, the suggested targeting signal will be evaluated "functional". Conversely, it should be noted that a reasonable number of false positive suggestions can be expected even when using valuable predictors. Considering the high number of independent proteins encoded in the genome—more than 23.000 genes in the human genome not considering the different protein isoforms—even low false positive rates can pose a severe challenge for prediction algorithms. Exemplarily even with a adequate specificity of 95% (5% false positives), about 1150 false positive predictions are to be expected when applying such a predictor to a compilation of human proteins, in which each gene is represented by a single protein.

5 Prediction of the Peroxisomal Proteome

The elucidation of the peroxisomal proteome by prediction algorithms depends on the classification of all proteins encoded in the genome of the organism of interest either into the category *peroxisomal* or the category *non-peroxisomal*. The computational algorithms listed above are either able to identify PTS motifs within the amino acid sequence of a given protein or to calculate a measure for the ability of an unknown protein to preferentially cluster with peroxisomal proteins or not. However, the limitations of these algorithms might prevent a successful prediction of the peroxisomal proteome, because the number of false positive or false negative predictions is unacceptably high when applied to a proteome. Therefore, the ability

of these predictions to correctly identify peroxisomal proteins has to be the gold standard for their evaluation.

The verification of the predictive performance of diverse prediction algorithms is rather difficult, because the low number of peroxisomal proteins renders the differentiation between a learning set and a test set not feasible without dropping the number of samples for the learning set below a critical threshold. Moreover, the reliability of the internal verification of targeting signals has recently been questioned especially for small data sets (Imai and Nakai 2010). However, the performance of several prediction algorithms for PTS has been tested by experimental verification of the functionality of PTS motifs, which obtained a high score when evaluating all human proteins (at the time).

The prediction of PTS1 motifs by the *PTS1 predictor* was first verified only internally, but later analyses demonstrated that PTS1 motifs could be successfully predicted in human proteins and that the identified C-terminal peptides interacted specifically with the human PTS1 receptor PEX5 (Neuberger et al. 2004). In contrast, *PeroxiP* was used to screen diverse genomes and used the ability to identify PTS1 motifs in several orthologues as a criterion for the reliability of the prediction algorithm (Emanuelsson et al. 2003). *PTS1Prowler* has been used to successfully predict PTS1 motifs, as three out of five proteins harboring a high-ranked PTS1 were found in peroxisomes, when expressed as myc-tagged full-length proteins (Hawkins et al. 2007). Furthermore, it should be noted that among the PTS1 predictors specifically developed for plant proteins *PPero* has not been tested experimentally, whereas the PTS1 predictor implemented into the *PredPlantPTS1* server has been tested extensively and proved to be reliable (Chowdhary et al. 2012; Lingner et al. 2011).

In contrast, the attempt to experimentally verify the quality of prediction algorithms for PTS2 motifs revealed a low success rate, as five murine proteins harboring a highly rated PTS2 were not found in peroxisomes upon ectopic expression in mammalian cells (Mizuno et al. 2008). Furthermore, among fifteen highly ranked PTS2 motifs identified in naturally occurring human proteins only four proved to be functional in a reporter protein context and only one of them was functional in the context of the full-length protein (Kunze et al. 2011). This inability to correctly predict PTS2 motifs has been retraced to the very low number of independent proteins using the PTS2-mediated transport route in animals (Mizuno et al. 2008).

The prediction algorithm for mPTS motifs has not been verified experimentally for its ability to predict novel animal PMPs, but it was able to predict the PEX19 binding site for several known PMPs (Halbach et al. 2005; Rottensteiner et al. 2004).

These results suggest that the reliability of the currently available prediction algorithms will not suffice for a prediction of the peroxisomal proteome, which exceeds the information already provided by experimental proteomic approaches.

6 Summary and Outlook

The *peroxisomal proteome* is the compilation of all proteins or protein variants, which can be found in peroxisomes, covering all tissues of an organism under different environmental conditions. This compilation can serve as a framework to elucidate all physiological contributions of peroxisomes, but also to suggest functional links between individual proteins (e.g. suggest novel metabolic pathways) or to classify an inherited human disease as peroxisomal single enzyme and transporter deficiency. The identification of the constituents of the peroxisomal proteome can be addressed by diverse experimental approaches to identify more and more peroxisomal proteins, but also by using prediction algorithms to tag the sequences of peroxisomal proteins among the entirety of protein sequences of an organism (proteome).

Various prediction algorithms are available to forecast the subcellular localization of a protein, which use different conceptual approaches and computational realizations. The identification of targeting signals within the primary sequence of proteins is a long-standing and effective tool to reliably predict the intracellular fate of a protein, because the interaction of these targeting signals with corresponding receptor proteins initiates transport processes ending at well-defined destinations. This approach has been extended by various methods taking into account many additional properties of proteins, which facilitate the proper classification of proteins according to their subcellular location.

Various prediction algorithms to identify and evaluate PTS have been developed, using homology based methods (PSSM), machine learning (SVM, NN, HMM) and combinations thereof. Moreover, these algorithms have been occasionally combined with external prediction algorithms for specific properties of proteins (e.g. prediction algorithms for targeting signals to other intracellular compartments) and have been embedded into complex classifiers forecasting the intracellular fate of proteins. The prediction algorithms for PTS1 motifs are rather successful, especially when considering taxon-specific peculiarities of this type of PTS (Neuberger et al. 2003a, b) or when generated exclusively for one branch of the phylogenetic tree (Lingner et al. 2011). In contrast, PTS2 and mPTS motifs are far less effectively predicted, although the identification of the most promising PTS2 or mPTS within the amino acid sequence of a peroxisomal protein is quite successful. However, an application of these prediction algorithms for the identification of all proteins from an animal genome (e.g. the human one), which harbor a functional PTS is not very promising. On the one hand, several approaches to experimentally verify the peroxisomal compartmentation of proteins that had obtained a high score by prediction algorithms for PTS revealed an ambiguous predictive performance as neither PTS1 nor PTS2 motifs were predicted with high specificity, although for different reasons. Whereas minimal PTS1 motifs were often correctly evaluated, some of the encoding proteins were not found in peroxisomes, because the context of the protein was not properly evaluated (e.g. in the case of the existence of a dominant alternative targeting signal). In contrast,

the prediction of PTS2 motifs was not reliable, because many predicted motifs were non-functional. Moreover, from a conceptual perspective a reasonable specificity for prediction algorithms (e.g. 95% true negative predictions) becomes problematic for large protein compilations such as the human proteome, due to the large number of false positive predictions.

A major barrier for a drastic improvement of prediction algorithms is the low number of peroxisomal proteins, e.g. slightly more than 50 proteins in mouse kidney (Wiese et al. 2007). This restricts the size of the learning set, which cannot be arbitrarily increased by other peroxisomal proteins from distantly related species, because of taxon-specific differences in the PTS. For human PTS1 motifs, the low number of proteins has been partially compensated for by mutational analyses and the consideration of two-hybrid assays picking peptides, which can interact with the PTS1 receptor protein PEX5. The prediction algorithm for PTS2 motifs benefited from the implementation of evaluations of the secondary structure (α-helix and linker domain), but the performance is still not satisfying. Finally, the data set for the prediction algorithm of mPTS-I motifs was supplemented by the results of binding assays with PEX19. However, the predictive performance should also benefit from a combination with algorithms analyzing additional properties of proteins and classifying proteins into groups sharing the same subcellular localization. However, these approaches also increase the complexity of the algorithm and thereby the uncertainty about the contribution of individual components. Thus, such approaches do not necessarily increase the performance of the algorithm for groups of proteins with a low number of members, but a systematic comparison of the results obtained with different prediction algorithms should increase the reliability of the prediction due to mutual compensation of the limitations.

Finally, it should be stressed that the compilation of the peroxisomal proteome from diverse tissues using different experimental approaches on the one hand and by prediction algorithms to identify PTS on the other hand are interconnected processes. The identification of novel peroxisomal proteins increases the number of independent proteins utilizing a certain type of PTS and thereby helps to improve prediction algorithms due to the enlargement of the learning set. A typical example is the improved performance of prediction algorithms for plant PTS1 motifs (Lingner et al. 2011; Ma and Reumann 2008; Reumann et al. 2012), which were based on the detailed resolution of the plant peroxisomal proteome enlarging the learning set (Reumann et al. 2007, 2009). Similarly, the elucidation of the mitochondrial proteome provided many proteins specifically processed by the mitochondrial processing peptidase and this improved the prediction of the processing site, which could then be implemented into novel prediction algorithms (Burkhart et al. 2015; Vogtle et al. 2009). Conversely, several experiments performed to verify the reliability of targeting signal predictions also revealed novel peroxisomal proteins, which had not been found by proteomic approaches to that date. Moreover, prediction algorithms are valuable tools to separate true peroxisomal proteins from contaminants.

Altogether, the observation that the performance of diverse prediction algorithms for peroxisomal proteins certainly discourages their exclusive utilization for the

elucidation of the peroxisomal proteome, but this does not implicate that they cannot provide a valuable contribution to reach this goal. These prediction algorithms are methodically orthogonal to different experimental approaches, which maximizes the expected benefit of a combined application. Sources of errors and systematic biases of these methods are unlikely to affect the same proteins, and their detection limits and separation capacities affect different types of properties of the investigated proteins and should counterbalance the limitations. Thus, the well-informed application of prediction algorithms and an awareness of strengths and limitations of this method will doubtlessly improve the effectivity, by which the aim of elucidating the complete peroxisomal proteome of humans and other animals can be achieved.

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Multiple Localization by Functional Translational Readthrough

Kristina Bersch, Ignacio Lobos Matthei and Sven Thoms

Abstract In a compartmentalized cell, correct protein localization is crucial for function of virtually all cellular processes. From the cytoplasm as a starting point, proteins are imported into organelles by specific targeting signals. Many proteins, however, act in more than one cellular compartment. In this chapter, we discuss mechanisms by which proteins can be targeted to multiple organelles with a focus on a novel gene regulatory mechanism, functional translational readthrough, that permits multiple targeting of proteins to the peroxisome and other organelles. In mammals, lactate and malate dehydrogenase are the best-characterized enzymes whose targeting is controlled by functional translational readthrough.

Keywords Peroxisome · Functional translational readthrough · Lactate dehydrogenase · Malate dehydrogenase · Dual targeting · Redox shuttle

Abbreviations

AQP4	Aquaporin 4
cAMP	Cyclic adenosine monophosphate
Drp1	Dynamin-related protein 1
eRF1	Eukaryotic release factor 1
EST	Expressed sequence tag
Fis1	Mitochondrial fission 1 protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gpd1	Glycerol-3-phosphate dehydrogenase (in yeast)
KANL	Lysine—alanine—asparagine—leucine
LDH	Lactate dehydrogenase
LDHBx	Readthrough-extended form of the B subunit of LDH
MAS	Malate-aspartate shuttle
MDH	Malate dehydrogenase

K. Bersch · I. Lobos Matthei · S. Thoms (✉)
Department of Child and Adolescent Health, University Medical Center Göttingen, University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany
e-mail: sven.thoms@med.uni-goettingen.de

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MDH1x	Readthrough-extended form of MDH1
Mff	Mitochondrial fission factor
Mo-MuLV	Moloney murine leukemia virus
NAD	Nicotinamide adenine dinucleotide (NAD^+ and NADH)
ORF	Open reading frame
PDE2	Phosphodiesterase 2
PGK	3-Phosphoglycerate kinase
PTS1	Peroxisome targeting signal type 1
SCC	Stop codon context
SKL	Serine—lysine—leucine
TMV	Tobacco mosaic virus
UTR	Untranslated region

Compartmentalization is the defining feature of the eukaryotic cell. As protein biosynthesis takes place in the cytosol, the cytosol is the default localization of a newly synthesized protein. In order to reach other places within the cell, a protein has to bear targeting information or needs to interact with a protein possessing targeting information. Frequently, proteins are present in several compartments in the cell. In this case, we speak of dual or multiple localization, the topic of this chapter.

- *Dual localization*: A protein is or can be present in two different cellular compartments.
- *Multiple localization*: A protein is or can be present in multiple compartments in the cell.

While the term *multiple localization* includes dual localization, this distinction is not always kept very strictly, so it is common to speak of dual localization even if a protein has been found in more than two cellular sites. The definitions of dual/multiple localization also include proteins that are normally present in one compartment, but can be transported to another one upon certain stimuli. Thus, cytosol-located transcription factors that are imported into the nucleus upon activation also fall under the definition of dually localized proteins.

How can proteins acquire multiple localizations? In order to answer this question we have to distinguish two cases: (A) The proteins present in multiple localizations are identical. (B) The proteins present in multiple localizations are isoforms of each other, that is, they are largely identical but differ in a targeting signal that gives them access to the secondary localization. This distinction is important with respect to the mechanistic details of how multiple localizations can be reached. In the first case, different receptor affinities may be the cause for multimodal distribution, while in the second case, localization is expected to be determined by the presence or absence of targeting signals in the respective isoforms.

In this chapter, we will discuss a novel and rather unusual mechanism of how proteins can acquire dual or multiple localization. Translational readthrough, the translation of a stop codon as a sense codon, can extend proteins, and the extensions

can contain functional peroxisomal targeting signals (Schueren and Thoms 2016). While this new class of proteins is exciting on its own, they are also associated with interesting methodological advances in the prediction of peroxisomal targeting signals and a novel method for the prediction of the 'leakiness' of stop codons and their associated nucleotide contexts.

We will start off the discussion of multiple localization by functional translational readthrough by a short summary on dual targeting, then turn to peroxisomal targeting signals and their in silico prediction, and the ways peroxisomal targeting signals affect multiple localizations. We will discuss translational readthrough, a new bioinformatics approach to translational readthrough, and lastly, the mammalian readthrough-proteins with dual localizations, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH), and their likely cellular functions.

1 Dual and Multiple Targeting of Proteins

Targeting of a protein to different cellular compartments enables the spatial separation of similar biological processes and creates additional regulatory options by which cells can adapt to physiological and pathological demands. Distribution of proteins to cellular organelles is mediated by organelle-specific targeting signals which are recognized by their respective receptors, leading finally to the translocation of the protein across an organelle membrane or through the pores of the cell nucleus.

There are a number of mechanisms by which proteins can acquire multiple localization (Karniely and Pines 2005; Ast et al. 2013). The minority of proteins with multiple subcellular localization are completely identical. These proteins contain two targeting signals with distinct affinity or accessibility to their organelle targeting receptors. In *S. cerevisiae*, catalase A contains both, a mitochondrial and a peroxisomal targeting signal. The distribution of the enzyme across the organelles is influenced by the nutrient status of the cell, but the mechanistic basis for the preference of one organelle over the other is unclear (Petrova et al. 2004).

The accessibility of a targeting signal can also be influenced by posttranslational modifications, binding to other proteins or the tertiary protein structure (Robin et al. 2002; Bandlow et al. 1998; Strobel et al. 2002; Anandatheerthavarada et al. 1999). The neuronal soluble epoxide hydrolase contains a peroxisomal targeting signal but is found in both, peroxisome and cytoplasm. The complete import into peroxisomes is hindered by dimerization of the enzyme and masking of the targeting signal (Nelson et al. 2016). In contrast, the targeting signal-bearing C-terminus of the peroxisomal lipolytic enzyme Lpx1 is not involved in the dimerization of the protein and dimerization does not block import of the dimeric protein into the peroxisome (Thoms et al. 2011a).

A protein with only one targeting signal can be transported into two organelles, e.g. upon recognition of this signal by import machineries of different organelles (Silva-Filho 2003; Peeters and Small 2001). Alternatively, incomplete translocation into an organelle with retention inside the cytoplasm or the active or passive transfer

out of an organelle of a fraction of the protein may lead to differential localization of proteins with only one targeting signal (Williams et al. 2012; Arand et al. 1991; Luo et al. 2008).

Most of the proteins with multiple cellular localization are not completely identical, as they normally differ by the existence of one or several targeting signals. In *S. cerevisiae*, gene duplication is a common cause of multiple targeting of proteins and functional diversity (Karniely and Pines 2005; Ast et al. 2013). The isoenzymes of malate dehydrogenase, Mdh1, Mdh2 and Mdh3 with mitochondrial, cytoplasmic and peroxisomal localization respectively, are encoded by different but paralogous genes and their expression is regulated at the transcriptional level (McAlister-Henn and Thompson 1987; Minard and McAlister-Henn 1991; Steffan and McAlister-Henn 1992).

Variable transcriptional initiation on a single gene or alternative RNA splicing leads to the generation of mRNA variants with distinct targeting information (Karniely and Pines 2005; Ast et al. 2013). In *C. albicans*, only one of the isoenzymes of 6-phosphogluconate dehydrogenase, generated by alternative splicing, carries a peroxisomal targeting signal, leading to cytoplasmic and peroxisomal localization of the isoenzymes (Strijbis et al. 2012).

Dually localized proteins resulting from several mRNAs are more common than those encoded by a single mRNA. The latter are generated on the translational level, either through multiple initiation sites or by readthrough of a stop codon leading to analogous proteins in- or excluding one or several targeting signals (Williams et al. 2014; Schueren et al. 2014; Hofhuis et al. 2016). As the difference of these isoforms arises only during translation, transcriptome analysis cannot be used for their identification (Ast et al. 2013).

2 Dual and Multiple Targeting to the Peroxisome and Other Organelles

Several peroxisomal enzymes have multiple localizations. They might be present in the cytoplasm, the endoplasmic reticulum, the nucleus and/or mitochondria where they catalyse similar or distinct metabolic reactions as in peroxisomes. For example, all the three malate dehydrogenases in *S. cerevisiae* are important for the redox balance in the cytoplasm, peroxisomes and mitochondria, but they also gained additional functions which are specific for the isoenzymes' location. In human cells, malate dehydrogenase has also been found inside the nucleus where it acts as a transcriptional co-activator of p53 in a glucose-dependent manner (Cho et al. 2009).

Mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff), and the GTPase dynamin-related protein1 (Drp1) have been found to be essential for division not only of mitochondria, but also of peroxisomes; the fission machinery is shared between these organelles (Koch et al. 2005; Motley et al. 2008). Fis1 is inserted into the peroxisomes through its C-terminal transmembrane domain and it

is one of the Drp1-recruiting proteins (Koch et al. 2005). For both organelles, fission is ultimately driven by Drp1 (Smirnova et al. 2001; Ingerman et al. 2005).

3 Identifying the PTS1ome by Bioinformatics

Peroxisomal matrix proteins are imported by a highly conserved machinery that is dependent on peroxisomal targeting signals (PTS), located either at the very C-terminus (PTS1) or within the N-terminal domain (PTS2) of the matrix protein. The PTS1 is present in the majority of all peroxisomal luminal proteins. In some species, it is the only targeting signal for soluble peroxisomal proteins, because the PTS2 has been completely lost in their evolution. The most common manifestation of PTS1 comprises the terminal tripeptide SKL (serine—lysine—leucine). A definition of PTS1, comprising most but not all PTS1 proteins, is [S/A/C] [K/R/H] L (Brocard and Hartig 2006). As the amino acids upstream of the tripeptide also contribute to targeting, more complete PTS1 definitions include the last ten or so amino acids (Brocard and Hartig 2006; Lametschwandtner et al. 1998). The important contribution of these upstream amino acids is illustrated by an SKL protein in yeast that had long been suspected to be peroxisomal, but it could be shown to target to lipid droplets instead—with no evidence for dual localization in the peroxisome (Thoms et al. 2011b). In other words, the prototypical SKL, albeit it is the consensus terminus, is neither necessary (there are other PTS1) nor sufficient (upstream sequences are important) for targeting into peroxisomes.

It is thus not surprising that in parallel to the investigation of the peroxisomal proteome by classical proteomics and high-throughput experimentation, computer algorithms have been developed to uncover the peroxisomal proteome, in particular the full set of PTS1 proteins—here referred to as the PTS1ome.

Such sequence-based prediction of PTS1 must take into account information from the region upstream of the SKL tripeptide. For example, the PTS1 predictor (Neuberger et al. 2003) outputs a composite score that derives from an independent evaluation of the tripeptide and the nine upstream residues. It offers three different models for metazoa, fungi and for species-independent predictions. PeroxiP and also the PTS1 Prowler (part of PProwler) evaluate the C-terminal tripeptide of a sequence according to known or canonical PTS1 tripeptides and then refine the result by machine learning algorithms that analyze the upstream region in order to assess whether they enhance or reduce the targeting propensity of the tripeptide (Emanuelsson et al. 2003; Bodén and Hawkins 2005).

For analysis of yeast and human PTS1, we have employed an integrative machine learning approach that has no pre-determined knowledge of the PTS1 tripeptide and treats all 15 terminal nucleotides in the same manner. The algorithm, originally developed for analysis of the plant PTS1ome (Lingner et al. 2011), uses a set of known PTS1 proteins as well as a large number of negative background sequences of proteins without known peroxisomal association as the learning sets of positive and negative examples, respectively (Nötzel et al. 2016). One of the critical

difficulties with such an approach is the low number of known positive examples (approximately 20 in the case of *S. cerevisiae*) that stands against a large number of negative examples which is virtually the remainder of the whole proteome. To overcome the problems associated with a low number of known PTS1 proteins, the set of positive examples was expanded by putative orthologous sequences from protein and EST databases (Nötzel et al. 2016). This step was based on the assumption that orthologues of peroxisomal proteins are also peroxisomal. The positive and the negative learning sets were then used to train a discriminative machine learning model. We then undertook genome-wide searches for new PTS1 and new peroxisomal proteins in *S. cerevisiae*. This search identified a number of new PTS candidates, of which we tested eleven. Ultimately, the analysis led to the identification of two new PTS1 proteins in *S. cerevisiae*, Yel020c and Yjr111c, which were termed Pxp1 and Pxp2, respectively (Nötzel et al. 2016). Interestingly, the PTS1 of both proteins are non-canonical meaning they contain new amino acids in the terminal tripeptide. The identification of essentially new PTS1 motifs underscores the power of the bioinformatics approach.

4 The Hierarchy of Targeting Signals

Nine of the eleven yeast PTS1-candidate decapeptides that we tested as GFP fusions for peroxisomal localization were able to target GFP to peroxisomes (Nötzel et al. 2016). Of these nine potentially new peroxisomal proteins, we tested five by labelling the full-length protein by introducing a promoter-GFP fusion in the genome. Surprisingly, only two of them proved to localize to the peroxisome (Nötzel et al. 2016). The other candidates were shown not to be peroxisomal in a genome-wide study of N-terminally GFP-labelled proteins (Yofe et al. 2016).

The finding that nine out of eleven PTS1 decapeptides do not target their parent protein to the peroxisome is remarkable in several ways. First, this introduces a methodological caveat: The ability of a protein terminus to target another protein to the peroxisome is not sufficient to prove that the protein is actually peroxisomal (Neuberger et al. 2004). Second, finding functional PTS1 (decapeptides) derived from natural proteins that are themselves not peroxisomal proteins is at odds with the definition of a targeting signal in general, because “transplantability” is one of the defining criteria of a targeting signal. Why, however, do not all proteins containing a functional PTS1 domain actually localize to peroxisomes in the living cell?

As we do not know the answer for all of the nine proteins in question, we can only suggest general mechanisms that may lead to the disregard of the PTS: It may be that targeting signals found more N-terminal in the protein have priority, being recognized and used for targeting before the C-terminus with the would-be PTS1 is even fully synthesized. Of the proteins studied by us, the nuclear-encoded mitochondrial protein Ptc5 may be an example for this case. The mitochondrial localization is acquired through a more N-terminal mitochondrial targeting signal.

The C-terminal SKL is only expressed once the protein has already reached the mitochondrion. A more extreme example is the ribosomal protein L2 in *S. cerevisiae*, which also contains a C-terminal SKL but it is never exposed to the peroxisomal receptor Pex5, because it spends its whole life in the mitochondrion (Neuberger et al. 2004).

Another conceivable explanation for the finding that not all PTS1 decapeptides are associated with peroxisomal proteins may be burying of the PTS1 within the protein structure, so that the PTS1 is not accessible for binding of Pex5 and thus for peroxisomal import (Thoms 2015). One interesting possibility is that a given PTS1 protein is peroxisomal only under certain environmental conditions. The PTS1 would have to be shielded until peroxisomal import is required.

An especially intriguing case of controlled dual localization is catalase, the hydrogen peroxide-degrading enzyme that is closely linked to the function of peroxisomes in redox metabolism. Human catalase has the rather weak PTS1 terminal tetrapeptide KANL (lysine—alanine—asparagine—leucine). 'Weak' in this context means that the affinity of the PTS1 to Pex5 is low and that import efficiency is so low that equilibrium of cytosolic and peroxisomal forms can easily be shifted towards the cytosol. Indeed, in aged cells bearing an increased oxidation level, the localization equilibrium is shifted towards the cytosol (Legakis et al. 2002). Interestingly, the catalase equilibrium controlled by the cellular redox state is dependent on the redox-sensitive import receptor Pex5 which through the redox-sensitive ubiquitylation site Cys-11 can act as a redox switch (Apanasets et al. 2014; Walton et al. 2017).

Before we discuss readthrough-dependent multiple localization, we have to take a brief look to translational termination and how it is related to translational readthrough.

5 Translational Readthrough

When in the course of protein synthesis and mRNA translation at the ribosome a stop codon appears in the A-site of the ribosome, translation is usually terminated. This is accomplished by the binding of a release factor in place of the incoming tRNA in the A-site, followed by hydrolysis of the completed polypeptide from the last tRNA and finally, the dissociation of the ribosome. In higher eukaryotes, eukaryotic release factor 1 (eRF1) recognizes all three stop codons UAA, UAG and UGA (Jackson et al. 2012).

Like all biological processes, translational termination is prone to errors. It is estimated that a stop codon terminates ribosomal translation with an error rate of 0.1% or lower (Namy et al. 2001; Harrell et al. 2002). If such an error occurs, a near-cognate tRNA binds instead of the release factor and translation ensues until the next in-frame stop codon reaches the ribosomal A-site. The actual degree of misinterpretation of a stop codon varies between the three stop codons in most organisms and can additionally be modulated by the nucleotides in the vicinity of

the stop codon. Especially the nucleotide immediately following the stop codon has a strong impact on the erroneous decoding (McCaughan et al. 1995).

Translational readthrough can be induced by the application of aminoglycosides, their derivatives or other small molecules. Induced readthrough is used as a therapeutic approach for the treatment of genetic diseases caused by premature stop codons and aims the generation of the full-length protein (Yang et al. 2016).

In some rare cases, the endogenous, non-induced translational readthrough is far higher than the error rate of about 0.1% (Williams et al. 2014; Harrell et al. 2002; McCaughan et al. 1995; Yang et al. 2016; Dunn et al. 2013; Yamaguchi et al. 2012). In these cases of interpretation of a stop codon as a sense codon, the C-terminal extension that is inevitably added to the parent protein by translation up to the next stop codon may exert a defined cellular function. If the readthrough extension contains a functional domain like, for example, a targeting signal, we speak of *functional translational readthrough* (Schueren and Thoms 2016).

6 Functional Translational Readthrough Diversifies Protein Function

Examples of translational readthrough leading to targeted localization and increased functionality of proteins are observed all over nature. The tobacco mosaic virus (TMV) produces two long proteins (of around 100 and 160 kDa) after infecting a host. Translational readthrough serves as a way to increase the encoded information in this limited genome (Brown et al. 1996). The Moloney murine leukemia virus (Mo-MuLV), in addition to its group-specific antigen (gag) and polymerase (pol), translates a gag-pol polyprotein by translational readthrough in amounts of 4–10% in relation to gag. Surprisingly, this fusion protein participates in the cleavage of gag protein, a prerequisite for its function (Yoshinaka et al. 1985; Felsenstein and Goff 1988). Translational readthrough can also be globally affected in nature. For example, the release factor Sup35 (eRF3) in *S. cerevisiae* and some other fungi has prion properties which upon activation forms amyloids. By aggregation, Sup35 gets inactivated and translational readthrough is globally increased. This is known as the [PSI^+] nonsense-suppressor phenotype (Wickner 2016). Also in *S. cerevisiae*, phosphodiesterase 2 (PDE2) undergoes functional translational readthrough, affecting its localization and reducing its enzymatic activity and consequently, increasing the intracellular cAMP concentration. Interestingly, PDE2 is also affected by the [PSI^+] phenotype, increasing its expression by over 20 fold (Namy et al. 2002).

In astrocytes, the readthrough-extended form of the water channel aquaporin 4 (AQP4) forms smaller and more homogeneous supramolecular structures in the plasma membrane than the non-readthrough AQP4 isoform and additionally, presents domains that could be associated with post-translational regulation (De Bellis et al. 2017).

7 Functional Translational Readthrough Diversifies Protein Targeting

As mentioned before, translational readthrough gives organisms the chance to attach a localization signal at the C-terminus outside of the boundaries of the first stop codon. This is the case for the enzymes 3-phosphoglycerate kinase (PGK), D-ribulose-5-phosphate-3-epimerase, and the NADH-dependent aldehyde reductase of *U. maydis*, for which dual localization to the cytosol and peroxisomes is achieved by the addition of PTS1 (Freitag et al. 2012). Similarly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *A. nidulans* is imported into the peroxisome as a secondary localization upon functional translational readthrough (Freitag et al. 2012). Surprisingly, translational readthrough can be evolutionary favoured over alternative splicing, or translational readthrough may be replaced by alternative splicing as a mechanism for protein targeting diversification. Both instances can be seen in *A. nidulans* where translational readthrough of GAPDH was evolutionary preferred over alternative splicing, while for PGK, alternative splicing causes the attachement of the targeting signal (Ast et al. 2013; Freitag et al. 2012; Stiebler et al. 2014).

LDH exists as a tetramer formed by a variable ratio of subunits A and B (heterotetramers), or by only one of the subunits (homotetramers). Readthrough of the transcript encoding for the B subunit of LDH gives rise to a C-terminally extended protein with the extension harbouring the peroxisomal targeting motif PTS1 (LDHBx) (Schueren et al. 2014; Stiebler et al. 2014). Tetramerization with LDHBx gives rise to new isoforms of LDH that are being transported into the peroxisome by virtue of an otherwise hidden PTS1 (Schueren et al. 2014). For stoichiometric reasons—the ratio of LDHBx/LDHB is about 1/50—the new isoforms contain one subunit of LDHBx together with conventional, non-extended subunits of LDHA and/or LDHB. The readthrough-subunit LDHBx thus provides a handle for a piggyback transport of the tetramer into peroxisomes, so that LDH tetramers are present inside peroxisomes (Schueren et al. 2014; Thoms 2015) (Fig. 1).

8 Omics Approaches for the Identification of Readthrough Proteins

How can one identify translational readthrough in an ‘omics’ systems biology context? Currently, four approaches are available for the detection and analysis of translational readthrough (Schueren and Thoms 2016). Mass spectrometry appears to be an ideal way of identifying readthrough proteins, and it is only a matter of time before this approach can yield new translation products with readthrough extensions. Ribosome profiling involves pull down and next-generation sequencing of the footprints of bound mRNA in actively translating ribosomes (Ingolia et al. 2009;

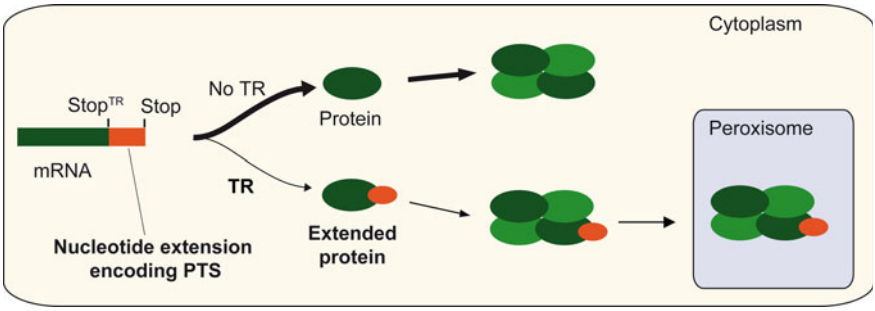

Fig. 1 LDHBx, the extended form of LDHB, is generated by translational readthrough (TR) of the first stop codon during translation of the normal mRNA into protein.The interpretation of the stop codon as a sense codon leads to the prolongation of translation until the next in-frame stop codon is reached. This leads to the C-terminal addition of seven amino acids to the normal LDHB. The extension (between $Stop^{TR}$ and Stop) harbors a peroxisomal targeting signal (PTS). Tetramers, which comprise the conventional subunits LDHB and/or LDHA together with a readthrough subunit LDHBx, can be transported into peroxisomes by the peroxisomal import machinery (piggyback import). The same mechanism combining readthrough and a hidden PTS1 after the first stop codon leads to the peroxisomal import of dimeric MDH1

Ingolia et al. 2011). By ribosome profiling, readthrough can be identified when regions downstream of ORFs are found to be occupied by ribosomes. Ribosome profiling has been employed to identify more than 350 potential translational readthrough genes in *D. melanogaster* (Dunn et al. 2013). Fifteen of these candidates were experimentally analyzed and in eight of these translational readthrough could be confirmed. Ribosome profiling has also been used to identify mRNAs undergoing translational readthrough in human skin fibroblasts (Dunn et al. 2013). While mass spectrometry and ribosome profiling are experimental methods, phylogenetic approaches and regression models involve dedicated bioinformatics for the identification of readthrough. Phylogenetic approaches analyze sequence alignments of homologous genes (Lin et al. 2011). This method is sensitive to unexpected sequence conservation in the regions annotated as 3'UTRs and to the coding potential of these regions by analyzing codon usage. The method can thus be used for the analysis of whole genomes of related species. Analysis of related *D. melanogaster* genomes identified more than 280 possible translational readthrough genes, some of which could be confirmed experimentally (Lin et al. 2007; Jungreis et al. 2011; Lin et al. 2011).

9 Readthrough-Omics in Silico

We have developed a fourth method for the identification of readthrough in the human genome. The method is essentially a regression model that combines bioinformatics and experimentation in an iterative manner. These bioinformatics

analyses require the exact and sensitive measurement of stop codon readthrough by reporter systems (Hofhuis et al. 2017). Most of these reporter systems are conceptually simple single- and dual-reporter systems. Single reporters either interrupt a reporter gene by a stop codon or are expressed directly behind the stop codon. Readthrough is then indicated by the re-expression of the functional reporter (Eswarappa et al. 2014; Reil and Hauser 1990; Cassan et al. 1990). Dual-reporter systems, on the other hand, express reporters before and after the stop codon. The advantage of dual-reporter systems is that they allow internal calibration by the N-terminal reporter gene. The reporters may either be enzymes like luciferase or fluorescent proteins. Fluorescent signals can be measured by flow cytometry, a method that does not require lysis of the cells. We employed two dual-reporter assays based on combined fluorescence/luminescence measurement of Venus and luciferase, and on flow cytometric fluorescence measurement of TagRFP and EGFP. Both dual reporters consist of an N-terminal tag as calibration control and a C-terminal tag as readthrough indicator. The reporter tags are flanking the stop codon context (SCC; stop codon and 10 nucleotides up- and downstream) which was introduced by simple primer annealing cloning in between the tags (Fig. 2).

As mentioned above, it is known for many decades that the three stop codons have different propensities for readthrough and that the nucleotides in the vicinity of the stop codon, especially the nucleotide immediately following the stop codon, influence its termination capacity (McCaughan et al. 1995). In this respect it is somewhat surprising that the termination efficiency of stop codon contexts had never been studied systematically. In order to identify readthrough-prone SCCs, we developed a genome-wide in silico readthrough analysis (Schueren et al. 2014). The algorithm is based on a set of experimental data of readthrough levels of random SCCs. These were formalized in a multidimensional binary vector that grants one dimension for every nucleotide at every position of the SCC. In our first model, 51 dimensions are used for six positions each preceding and following the stop codon, the stop codon itself is regarded as one position and therefore uses three dimensions. The algorithm calculates a linear regression between experimental

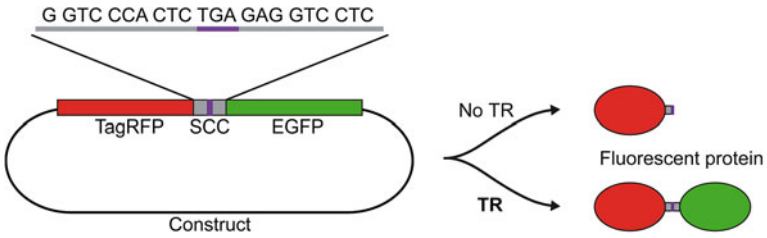

Fig. 2 Dual fluorescence reporter used for readthrough measurements.For the assay, mammalian cells are transfected with a DNA construct containing the stop codon context (SCC) of interest flanked by genes encoding the fluorescent proteins TagRFP and EGFP. Fluorescence is detected by flow cytometry and readthrough is calculated as the ratio of EGFP (only expressed upon translational readthrough [TR]) over TagRFP (internal control)

readthrough values and their respective sequences represented in the multidimensional vector space. The resulting regression coefficients describe the nucleotides' influence on translational readthrough of all four nucleotides in all positions of the SCC. The web-logo representation of the regression coefficient together with a feature selection to identify the positions that contribute most to the prediction allowed the identification of the high-readthrough context (Fig. 3).

We could show that the high-readthrough motif is associated with a readthrough level of at least one order of magnitude above the background (Schueren et al. 2014). More than 50 human genes/transcripts are known to carry this SCC nucleotide motif. So far, five have been confirmed experimentally (Schueren et al. 2014; Hofhuis et al. 2016; De Bellis et al. 2017; Stiebler et al. 2014; Loughran et al. 2014). Subsequently, we focused our analysis on those two genes that carry a functional peroxisomal targeting signal in the extension: *LDHB* and *MDH1*, giving rise to LDHBx and MDH1x, respectively (Schueren et al. 2014; Hofhuis et al. 2016). In these cases, about 1.6% of the transcripts encoding for LDHB and 4% of MDH1 are expanded during translation. We noticed that the quantitative readthrough levels were virtually identical for the SCC and for the full length cDNA including the full extension (Schueren et al. 2014; Hofhuis et al. 2016). This suggested that the SCC is the main contributor to stop codon readthrough, a suggestion that is very plausible in the case of LDHBx, where the conserved readthrough nucleotide extension is only 18 nucleotides long, thus not giving much room for RNA elements that may modulate readthrough. In the case of MDH1x, however, where the full extension comprises 57 nucleotides, the hypothesis that the SCC is the main contributor of readthrough had to be tested. To analyse the possible influence of the nucleotides following the MDH1 SCC or mRNA secondary structures that may be formed by this sequence, we expressed the MDH1 SCC

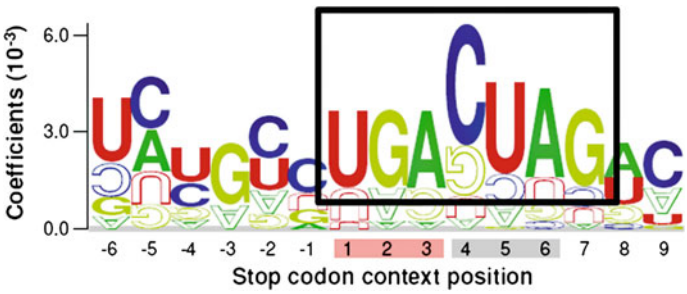

Fig. 3 The high-readthrough stop codon context (SCC) UGA CUAG meditates endogenous stop codon readthrough.Readthrough levels are at least one order of magnitude greater than background readthrough of a 'normal' stop codon. The high-readthrough motif was identified using a bioinformatics algorithm that was based on modelling the probability of SCC readthrough and verified experimentally. There are more than 50 genes in the human genome with this SCC motif. Of these, two have a PTS in the readthrough extension: LDHB and MDH1. Figure modified from Schueren et al. (2014)

together with either the full downstream stretch including the PTS1, the SCC with only 18 nucleotides following the stop codon, or the SCC alone in a dual reporter experiment. In addition, for a more direct control of the full-length downstream sequence, we expressed the MDH1 SCC together with a scrambled sequence of all the 57 nucleotides following the SCC. None of the possible extensions after the SCC significantly modulated translational readthrough (Hofhuis et al. 2016). While this experiment does not completely rule out contributions from other parts of the mRNA outside of the SCC, this is strong evidence that the main contribution of endogenous readthrough comes from the SCC, defining a process that takes place within or in close proximity to the ribosome.

10 Translational Readthrough as a Modification of the Genetic Code

The natural stop codon recoding of MDH1x and LDHBx constitutes a modification of the genetic code in humans. Mass spectrometric analysis of human MDH1x revealed that the amino acids tryptophan (normal codon UGG) and arginine (codons CGX, AGR; X = A, G, C or U; R = A or G) and, at lower levels, cysteine (UGY, Y = C or U) are integrated at the position of the stop codon UGA. This result supports the hypothesis that the second position of the triplet determines the tRNAs that binds to the stop codon instead of the release factor eRF1. Incorporation of tryptophan, arginine and cysteine at the stop codon UGA was also found by mass spectrometric analysis of a high-readthrough *S. cerevisiae* strain (Blanchet et al. 2014; Roy et al. 2015). Still, the mechanism of stop signal recoding is not entirely understood. A possible impact of the SCC on the type of near-cognate tRNA that couples to the stop codon is being discussed (Blanchet et al. 2014; Beznosková et al. 2016).

11 The Physiology of Peroxisomal Malate and Lactate Dehydrogenases

In contrast to vertebrates, *S. cerevisiae* has a dedicated peroxisomal *MDH* gene (MDH2) that arose in an early genome duplication. In the yeast *Y. lipolytica*, a peroxisome-specific MDH isoform results from alternative splicing, creating a mRNA containing a PTS1 (Kabran et al. 2012). The plant genome harbors two MDH1 genes, both encoding PTS2 motifs (Pracharoenwattana et al. 2007; Fukao et al. 2002). These findings indicate that the MDH requirement in peroxisomes has been conserved in evolution, and moreover, that convergent evolution ensured the presence of MDH in peroxisomes.

Peroxisomal β-oxidation of fatty acids and other β-oxidation substrates is dependent on the availability of NAD^+ in the peroxisome. NAD^+ is reduced to NADH during substrate oxidation. It is known, however, that nicotinamide adenine dinucleotides are not able to directly cross biological membranes (Antonenkov et al. 2004; van Roermund et al. 1995; Visser et al. 2007). Instead, reduction equivalents have to be transported in the form of small metabolites like lactate or pyruvate across the membrane to maintain redox homeostasis.

In yeast, it was hypothesized that the peroxisomal MDH, Mdh3, is involved in this process (van Roermund et al. 1995): Mdh3 catalyzes the conversion of oxaloacetate and NADH to malate and NAD^+. Thus NAD^+ is generated within the peroxisome, supporting β-oxidation, while smaller components are transported through the membrane, as in the mitochondrial malate-aspartate shuttle (MAS) (van Roermund et al. 1995). Interestingly, in yeast, also the cytoplasmic MDH isoform, Mdh2, is required for peroxisome function (Wolinski et al. 2009). This finding supports the hypothesis of a peroxisomal malate-shuttle required for peroxisome function.

In all eukaryotes, but mostly described in yeast, many shuttles seem to be responsible for maintaining the NAD^+ levels in the peroxisome. A glycerol-3-phosphate dehydrogenase (Gpd1)-dependent shuttle was recently described in yeast in addition to MAS. Both, Gpd1- and Mdh3-dependent shuttles are essential to maintain the intraperoxisomal redox balance by the delivery of NAD^+ that is required for β-oxidation, but also for L-lysine biosynthesis (Al-Saryi et al. 2017).

The LDHBx extension including the hidden targeting signal is conserved in mammals, but it is absent in non-mammalian species. In contrast, the MDH1x extension, including the hidden PTS1, is highly conserved not only in mammals but in all vertebrates (Fig. 4). Birds are an example of species containing a peroxisomal MDH1x, but not the peroxisomal LDHBx. This discrepancy has been dubbed "bird gap" (Hofhuis et al. 2016). It is currently not known if in non-mammalian vertebrates LDH has another means of entering the peroxisome, or if the MDH1x is the only NAD^+-recycling dehydrogenase in the peroxisomes of these species. Interestingly, the peroxisomal targeting of MDH1x-PTS1 from non-vertebrates, having no LDHBx, has been shown to be more efficient compared to mammalian MDH1x targeting into peroxisomes (Hofhuis et al. 2016).

Functional translational readthrough of peroxisomal dehydrogenases provides an intriguing example of how proteins achieve multiple localization by combination of stop codon recoding with a cryptic peroxisomal targeting signal.

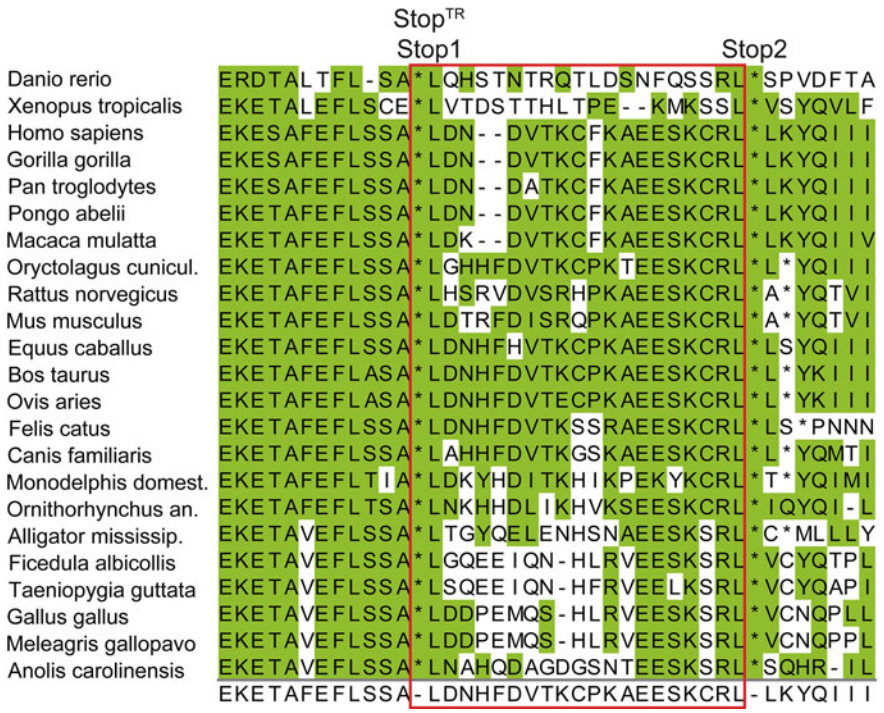

Fig. 4 The combination of a high-readthrough motif at the first stop codon and a downstream PTS1 in MDH1x is conserved in all vertebrates. In mammals, the consensus of the terminal tripeptide of the PTS1 is CRL, in non-mammalian vertebrates, the consensus is SRL. Figure modified from Hofhuis et al. (2016)

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Evolution of the Peroxisomal Proteome

Toni Gabaldón

Abstract Peroxisomes are single-membrane bound intracellular organelles that can be found in organisms across the tree of eukaryotes, and thus are likely to derive from an ancestral peroxisome in the last eukaryotic common ancestor (LECA). Yet, peroxisomes in different lineages can present a large diversity in terms of their metabolic capabilities, which reflects a highly variable proteomic content. Theories on the evolutionary origin of peroxisomes have shifted in the last decades from scenarios involving an endosymbiotic origin, similar to those of mitochondria and plastids, towards hypotheses purporting an endogenous origin from within the endomembrane system. The peroxisomal proteome is highly dynamic in evolutionary terms, and can evolve via differential loss and gain of proteins, as well as via relocalization of proteins from and to other sub-cellular compartments. Here, I review current knowledge and discussions on the diversity, origin, and evolution of the peroxisomal proteome.

Keywords Peroxisomes · Evolution · Peroxisomal proteome · Endomembrane system

Abbreviations

LECA	Last eukaryotic common ancestor
H_2O_2	Hydrogen peroxide
PEX	Peroxin
ER	Endoplasmic reticulum

T. Gabaldón (✉)
Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology (BIST), Dr. Aiguader, 88, 08003 Barcelona, Spain
e-mail: tgabaldon@crg.es

T. Gabaldón
Universitat Pompeu Fabra (UPF), 08003 Barcelona, Spain

T. Gabaldón
Institució Catalana de Recerca i Estudis Avançats (ICREA), Pg. Lluís Companys 23, 08010 Barcelona, Spain

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ERAD Endoplasmic reticulum associated decay
AGT Alanine:glyoxylate aminotransferase
PXA Peroxisomal ABC transporter
CTA Catalase
FOX Fatty acid oxidase
FAA Fatty acid CoA synthetase

1 Introduction

Peroxisomes were first described by Rhodin in 1954 as a particular type of cytoplasmic granules—which he named "microbodies"—in convoluted tubule cells from mouse kidney (Rhodin 1954). Similar structures, surrounded by a single membrane and showing a granular matrix, were described by electron microscopy by other researchers in several other mammalian tissues. However, a functional understanding of the possible roles of this newly discovered organelle was only gained after the Belgian Biochemist Christian de Duve and co-workers were able to isolate microbodies from rat liver and characterize them biochemically (De Duve and Baudhuin 1966; de Duve 1982; De Duve 1996). De Duve and his team noted the presence of several H_2O_2 producing oxidases (i.e. D-amino acid oxidase, uric acid oxidase), as well as large quantities of enzymes capable of reducing this H_2O_2 such as peroxidases and catalases (De Duve and Baudhuin 1966). Similar H_2O_2 producing oxidases were found in microbodies from other tissues and organisms, including the protist *Tetrahymena pyriformis*, which contained additional H_2O_2 producing oxidases acting on glycolate, L-hydroxy acids, and L-amino acids. Based on this clear preponderance of reactions involving hydrogen peroxide, de Duve proposed the name "peroxisome" for this organelle. Christian de Duve later obtained the Nobel Prize for his discovery of another subcellular organelle, the lysosome, but he should also be recognized by his instrumental contributions regarding the biochemical characterization of peroxisomes in diverse organisms as well as by his influential ideas on the evolution of these organelles, and of eukaryotes in general (de Duve 1982; Bowers 1998). In the more than sixty years since the discovery and initial characterization of the peroxisomes, we have exponentially increased our knowledge about this organelle in many ways. Although direct experimentation and comprehensive characterization is still limited to model organisms, the advent of high throughput technologies have allowed us to expand our knowledge on peroxisomes in diverse organisms from across the broad eukaryotic tree of life. Experimental advances in the separation of sub-cellular compartments coupled to proteomics analysis have enabled charting the protein content (i.e. the proteome) of peroxisomes in different organisms and tissues (Taylor et al. 2003; Andersen and Mann 2006). Furthermore, the increasing amount of fully sequenced genomes from the main clades of the eukaryotic domain coupled

to bioinformatics analyses has paved the way not only for predicting the proteomic content of peroxisomes from organisms not amenable to experimentation, but also to trace the evolution of the organelle (Gabaldón et al. 2006; Schlüter et al. 2007; Gabaldón 2010). Here, I will briefly survey the current status of our knowledge on the diversity of peroxisomes across the eukaryotic tree of life and discuss past and current ideas on the origin and evolution of these organelles.

2 Functional Diversity of Extant Peroxisomes

Peroxisomes generally contain enzymes involved in lipid metabolism and detoxification of reactive oxygen species. However, this is an overly simplistic view of the true diversity of peroxisomal metabolic functions. The list of taxon-specific functions of peroxisomes is constantly expanding, with functions ranging from the synthesis of specific secondary metabolites, to the involvement of peroxisomes in developmental processes. Furthermore, within a single species, peroxisomes from different tissues can show clearly distinct features and display radically different proteomic content. In some cases, this has led to the initial description of peroxisomes from certain species or tissues as different organelles. Such is the case of the peroxisomes in germinating plant seedlings, which were initially described as "glyoxysomes" for their characteristic presence of enzymes of the glyoxylate cycle (Breidenbach et al. 1968), or of peroxisomes in trypanosomatids, which were named "glycosomes" for the presence of glycolytic enzymes (Opperdoes and Borst 1977). Later, it became clear that glyoxysomes and glycosomes were just particular types of peroxisomes, although their specific names are still often used (Gabaldón et al. 2016). Peroxisomes were isolated from a growing number of species as catalase-containing, cytoplasmic particles surrounded from a single membrane which equilibrated in gradient centrifugations at densities different from mitochondria and plastids. Besides the presence of catalase, the list of peroxisome-harbored enzymes quickly expanded. Enzymes of the fatty-acid β-oxidation system were found associated with peroxisomes in several plant, fungal, and mammalian systems (Cooper and Beevers 1969; Lazarow and De Duve 1976). In contrast to the β-oxidation system that was known for mitochondria, where acyl-CoA dehydrogenase feeds electrons into the oxidative phosphorylation chain, the peroxisomal acyl-CoA oxydase transferred the electrons to molecular oxygen, thereby generating H_2O_2. Some organisms can have both mitochondrial and peroxisomal systems of fatty-acid β-oxidation, whereas others may exclusively show one of them. Various enzymes catalyzing oxidative reactions on different substrates can often be found in peroxisomes of certain organisms such as malate synthetase and glycolate oxidase in the budding yeast *Saccharomyces cerevisiae* (Szabo and Avers 1969). Transaminases and enzymes involved in photorespiration can be found in peroxisomes from plant leaves (Tolbert et al. 1968). Finally, most peroxisomes harbor enzymes involved in ether-lipid metabolism, the oxidative branch of the pentose-phosphate pathway, purine catabolism, or alcohol oxidation, among many

other oxidative processes (Smith and Aitchison 2013). Fungal organisms show a remarkable diversity in their peroxisomal enzymatic content. In addition to the common processes in H_2O_2 metabolism and fatty-acid oxidation, fungal peroxisomes also harbour enzymes for the synthesis of biotin and many products of the secondary metabolism, including antibiotics such as penicilin and toxins (van der Klei and Veenhuis 2013). Peroxisomes in microbial eukaryotic lineages show the broadest metabolic diversity (Gabaldón 2010; Gabaldón et al. 2016). Some pathways have a unique peroxisomal localization in some unicellular eukaryotes. This is the case of the presence of enzymes of the glycolytic and gluconeogenic pathways in the glycosomes of Kinetoplastea and Diplonemida (Gualdrón-López et al. 2012).

Besides their roles in the central or secondary metabolism of the cell, peroxisomes of different organisms have also been recruited to a variety of cellular signaling and developmental processes in various organisms. For instance, studies on the filamentous ascomycete *Podospora anserina* have established a role of peroxisomes in sexual development in this species (Terlecky et al. 2009). Similarly glycosomes of trypanosomatid parasites have been shown to regulate differentiation through the life-cycle (Szöör et al. 2014). However, perhaps one of the most extreme examples of specialization of peroxisomes is the so-called Woronin bodies present in filamentous ascomycetes (Jedd and Chua 2000). These, highly specialized peroxisomes are metabolically inactive, but have a structural role by blocking the septal pores, thereby preventing cytoplasmic bleeding in damaged hyphae.

3 Diverse but All the Same

Despite the above described versatility in metabolic and physiological roles and the extreme variability of their enzymatic content, all members of the organelle family of peroxisomes—peroxisomes, glyoxysomes, Woronin bodies, glycosomes—share several important features. First of all, all types of peroxisomes share the same route of biosynthesis, mediated by a protein machinery that is largely shared across diverse species (Pieuchot and Jedd 2012). Peroxisomes do not contain DNA, and all the proteins that function in the peroxisome are encoded in the nuclear chromosomes, synthesized on cytosolic ribosomes and directed, post-translationally to the organelles. This intracellular routing of peroxisomal proteins is achieved by the use of the same type of sequence-encoded signals (Pieuchot and Jedd 2012; Smith and Aitchison 2013). Although the proteins harboring peroxisomal targeting signals may be different in diverse organisms, the signals themselves are of the same kind, and are recognized by homologous sets of organelle biogenesis proteins, called peroxins (or PEX proteins) that mediate the import of matrix proteins and the insertion of membrane proteins. In addition, the transport of soluble metabolites through the peroxisomal membrane is mediated in a similar manner in widely different organisms: larger solutes such as acyl-CoAs and coenzymes are translocated by homologous ABC transporters and members of the mitochondrial carrier family, respectively, whereas smaller metabolites are transported through pores

created by proteins which, in most cases, remain to be identified (Antonenkov and Hiltunen 2012). Peroxisomes from diverse organisms also proliferate in the same manner by growth and division of pre-existing peroxisomes. However, this process is not entirely independent from the endoplasmic reticulum (ER) as some of the peroxisomal membrane proteins are first inserted into special domains of the ER, followed by transfer of budded vesicles containing lipids and these proteins to the growing peroxisomes. Finally, despite the above mentioned metabolic diversity of peroxisomes, there are several central features that are ubiquitously present in peroxisomes from diverse lineages. The common denominator of all extant peroxisomes could be described, in simple words, as a membrane-bound compartment, with a peroxin-based protein import machinery, which harbors one or several pathways that produce reactive oxygen species and at least one type of a detoxifying enzyme, generally catalase. However, this "universality", as other "universal rules" in biology, is not without anecdotal exceptions, some of which have been described in the above section.

4 Evolutionary Origin of Peroxisomes

Soon after their discovery, it became clear that peroxisomes were present across the diversity of eukaryotic lineages and thus should represent one ancestral feature in the evolution of eukaryotes. Peroxisomes, as mitochondria, the nucleus, the endomembrane system, and many other eukaryotic features have been inferred to be already present in the last common ancestor of all extant eukaryotes (LECA) (Gabaldón 2010). And, as with all these features, there has been controversy about how they have first originated, and in which order (Gabaldón 2014b). Christian de Duve was one of the most active thinkers about the evolution of peroxisomes, and of eukaryotes in general, and the one who conceived the first reasoned hypothesis on how peroxisomes may have come about. In his proposed scenario peroxisomes would have been originated through engulfment of a prokaryote into the eukaryotic cytosol at the time in which the level of oxygen on earth was raising (de Duve 1982). First eukaryotes were thought to have been anaerobic, and highly sensitive to the highly reactive oxygen. Incorporating an endosymbiont with the ability to detoxify highly reactive oxygen species would have allowed eukaryotes to survive the so-called "oxygen holocaust", as the raise of atmospheric oxygen levels at the beginning of the proterozoic period is sometimes referred to (Holland et al. 2006). Christian de Duve based his scenario on the fact that peroxisomes shared many features with mitochondria and plastids, including their ability to multiply by growth and division, and their ability to import proteins post-translationally. This resemblance, coupled to the rise of the serial endosymbiosis theory, which proposed that many eukaryotic features were the result of endosymbiotic events with prokaryotes, made de Duve's hypothesis an attractive one, and was quickly adopted by the scientific community. The fact that peroxisomes, contrary to plastids and mitochondria, do not harbor bacterial-like DNA, was interpreted as the result of a

more ancestral origin of this organelle as compared to mitochondria. The finding that some peroxisomal enzymes seemed to have a prokaryotic ancestry reinforced this idea.

The view of an endosymbiotic origin of peroxisomes was the predominant one in the decades that followed peroxisome discovery. It was not until the beginning of this century that the picture started to tremble, as evidence refuting an endosymbiotic origin started to accumulate. In 2006, two independent phylogenetic studies (Gabaldón et al. 2006; Schlüter et al. 2006) showed compelling evidence that core proteins involved in the biogenesis of peroxisomes had an evolutionary origin, through duplication and divergence, from the Endoplasmic Reticulum Associated Decay (ERAD) pathway. This protein complex sits in the ER membrane, and is responsible for transporting misfolded proteins outside the ER for their degradation in the cytoplasm. This process involves tagging mis-folded proteins with ubiquitin. In the peroxisome, homologous proteins are used to shuttle the PEX5 protein in and out the peroxisomal membrane, a process involving ubiquitination of PEX5 and transport of peroxisomal proteins (Erdmann and Schliebs 2005). These results were fitting previous experimental observations showing that *Saccharomyces cerevsiae* PEX5 mutants, which lack peroxisomes, could form peroxisomes de novo upon re-introduction of the wild type gene (Tabak et al. 2003). Remarkably, these newly formed peroxisomes started their life in the ER, where some peroxins were first targeted, to then bud off as peroxisomes.

Furthermore, one of the above mentioned phylogenetic studies (Gabaldón et al. 2006) reconstructed the so called ancestral peroxisomal proteome by combining phylogenetic analyses, and proteomic inventories of peroxisomes that were available by then: rat, human, the budding yeast *Saccharomyces cerevisiae*, and the plant *Arabidopsis thaliana*. The resulting reconstructed proto-peroxisomal proteome, supposedly present in the common ancestor of all these organisms, harbored a minimal set of 12 proteins (Fig. 1). This set included six peroxins (PEX1, PEX2, PEX4, PEX5, PEX10, PEX14 in their yeast nomenclature); two fatty acid transporters (PXA1, PXA2), catalase (CTA1), and three enzymes involved in fatty acid metabolism (FOX1, FOX2, FAA2). Remarkably, most of the proteins in the ancestral peroxisomal proteome had no prokaryotic ancestry, suggesting many of the prokaryotic derived pathways had been subsequently incorporated in the peroxisome. However, the origin of one of them (FOX2) could be traced back to an alpha-proteobacterial ancestor, suggesting an origin from the mitochondrion. Altogether, these results were supporting an alternative scenario for the origin of the peroxisomes, that of an endogenous origin from the endomembrane system. The emerging picture was that, similar to lysosomes or the Golgi apparatus, peroxisomes originated from within the endomembrane system and were evolutionary selected to deal with a diverse set of pathways, particularly those involving the production of reactive oxygen species. The idea of an oxygen-detoxifying endosymbiont was abandoned, although not entirely (De Duve 2007). However, many new questions emerged, particularly with regard to the initial selective pressure that drove the origin of peroxisomes from the endomembranous system.

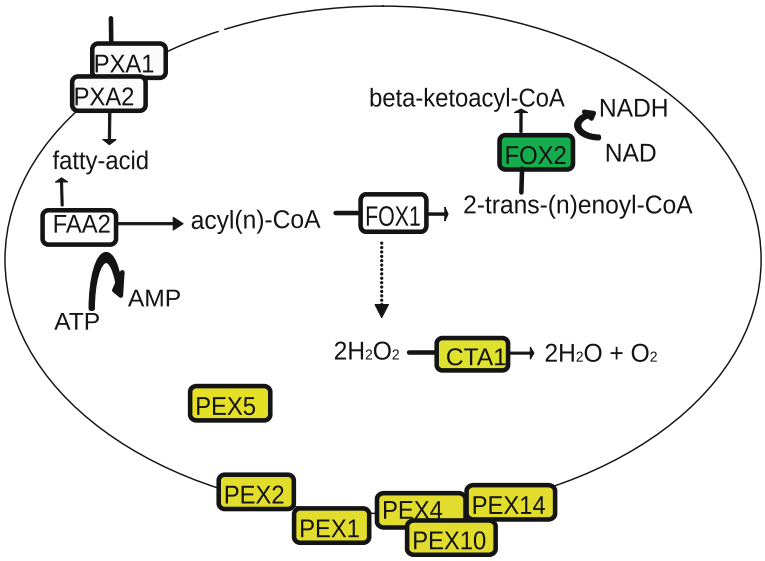

Fig. 1 Minimal ancestral proto-peroxisomal proteome as reconstructed in (Gabaldón et al. 2006). Rectangles correspond to proteins inferred to be present in the peroxisome in the last eukaryotic common ancestor. The yeast gene nomenclature is used. Colors indicate inferred origin (Yellow: eukaryotic origin; Green: alpha-proteobacterial origin; White: non-alphaproteobacterial origin)

5 Current Hypotheses on the Origin of the Peroxisome

In 2011, Speijer (2011), proposed that the driving force for the origin of peroxisomes was the selective advantage provided by localizing the beta-oxidation of very long fatty acids outside the mitochondrion. The oxidation of long chain fatty acids produces a relatively higher amount of $FADH_2$ per NADH molecule (this ratio tends to 0.5 in long fatty acids) as compared to the normal breakdown of glucose or lactate ($FADH_2$/NADH ratio of 0.2). Speijer argued that the consequent excess of $FADH_2$ produced during fatty acid oxidation would lead to unbalanced rations of ubiquinone and its reduced form, ubiquinol, which in turn will interfere with the normal progression of the mitochondrial respiratory chain, eventually leading to the reverse functioning of the electron transport chain. This reverse functioning leads to the production of highly reactive oxygen species and oxidative damage. Avoiding this damage would have supposed a selective advantage for driving the re-localization of specific beta oxidation enzymes to an alternative compartment. However, as it was originally proposed, this model lacked a possible explanation of how peroxisomes originated from the ER (Gabaldón 2014b; Gabaldón et al. 2016). Recently, some ideas into how these could have been progressed have been provided (Speijer 2017). In my view, however, a possible selective advantage of relocating fatty acid oxidation out of the mitochondrion does not necessarily imply that peroxisomes originated to allocate these enzymes. It remains possible that

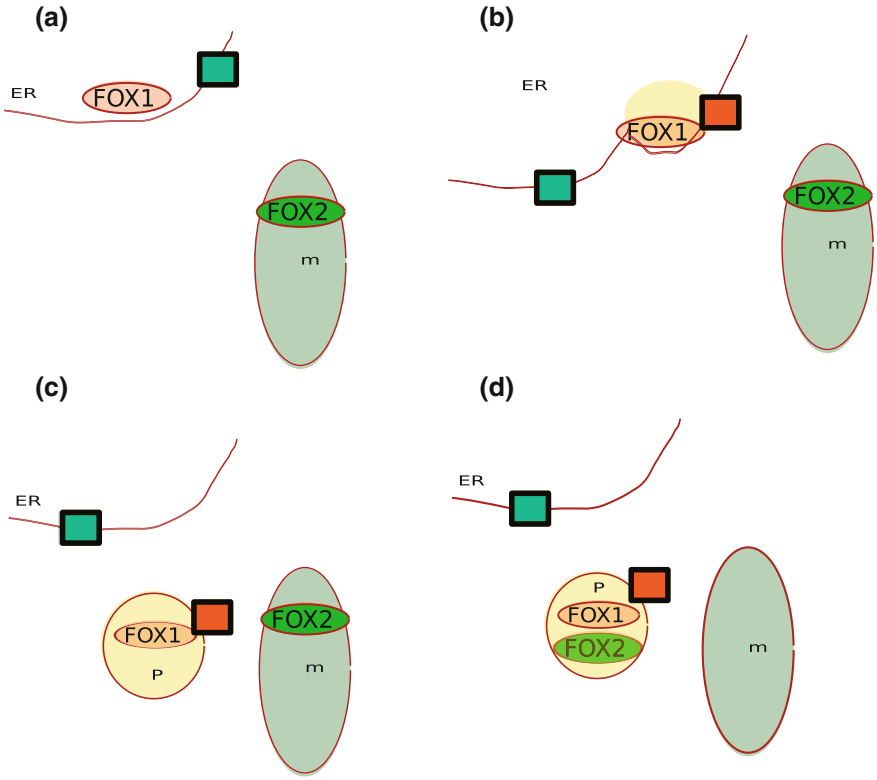

Fig. 2 Hypothetical stepwise scenario of the evolution of peroxisomes prior to the last eukaryotic common ancestor. **a** FOX1 sits at the Endoplasmic reticulum (ER), playing a role in fatty acid metabolism, the ERAD system (green square) works by pulling out misfolded proteins and targeting them for degradation in the cytoplasm. **b** FOX1 is targeted to specialized ER subcompartments (yellow) thanks to the specialization of an ERAD-like system into a transport system (pink square) via duplication and neofunctionalization from the ERAD system (green square). **c** These subcompartments gradually get more isolated form the ER until they get totally detached;, the ERAD-like transport system enables the import of any protein that acquires a targeting signal. **d** Fatty acid oxidation enzymes from the mitochondrion (m), such as FOX2, are among the first enzymes to be retargeted to the peroxisomes (P). Note that, although a mitochondrion is depicted since step A, the model is agnostic with regards to its presence before step D

peroxisomes were already there to accommodate that pathway, as they have accommodated many other pathways during evolution (Fig. 2).

In 2014, I proposed a metabolic scenario, which could explain how peroxisomes originated from the ER (Gabaldón 2014a). For this I tried to fit some key pieces of evidence. These could be summarized as (i) peroxisomes likely originate evolutionarily from the endoplasmic reticulum, co-opting the ERAD-system and (ii) the proto-peroxisomal proteome has mainly a eukaryotic origin, with notable exceptions (iii) a hydrogen peroxide producing enzyme involved in fatty acid metabolism

is among the ancestral peroxisomal proteins, and (iv) many steps of fatty acid metabolism take place in the ER. To join these dots, I put forward a metabolic scenario in which the selective force that allowed the evolution of peroxisomes was the progressive isolation from the rest of the ER processes, of H_2O_2 producing enzymes involved in fatty acid metabolism. Processes such as protein folding and modification which take place in the ER lumen and that are very sensitive to oxidative stress would be negatively affected by the presence of metabolic reactions producing reactive oxygen species, such as H_2O_2. Thus, the compartmentalization of such toxic reactions would be advantageous for the cell. An obvious candidate protein is FOX1, which is involved in fatty acid metabolism, predicted to have been present in the ancestral proto-peroxisome, and produces H_2O_2. This compartmentalization could have been progressive, starting first with targeting of the relevant enzymes to particular ER domains that eventually could be completely detached, once a system to directly import enzymes into the peroxisomal matrix had been developed. The development of this system could similarly have started by co-opting specialized ERAD-related complexes for targeting the relevant enzymes into the right ER domain without transiting through the ER. The origin of such transport system would have been enabled by duplication and divergence of the ERAD system. Contrary to Speijer's model, which implies that mitochondria predate peroxisomes, this scenario is agnostic regarding the relative order of appearance of peroxiomes and mitochondria, as both possibilities are compatible with an origin of peroxisomes from the ER and a subsequent retargeting of additional enzymes from the mitochondrion and elsewhere. Finally, this model is also compatible with the idea that the re-localization of fatty acid enzymes from the mitochondrion to another compartment was advantageous, which would provide an explanation for the driving force of this subcellular retargeting. In fact the retargeting of every new enzyme or pathway to the peroxisome have likely had some selective advantage at the time it occurred. However, a retargeting scenario is facilitated by the existence of the peroxisomal compartment with an already developed protein import machinery. Linking the retargeting of mitochondrial enzymes to the origin of the peroxisome itself poses the additional difficulty of explaining how the two events happened concomitantly, and why the new organelle had to be developed from within the ER. Importantly, the H_2O_2 producing ancestral peroxisomal enzyme, FOX1, has no alpha-proteobacterial origin and thus is not necessary to assume a mitochondrial provenance for this enzyme. This distinct origin also suggests that FOX1 had potentially a function out of beta-oxidation until the advent of the mitochondrial proteins. What seems also clear is that the origin of peroxisomes and the retargeting of mitochondrial beta oxidation was already in place in LECA, before the radiation of current eukaryotic groups.

6 Shaping the Peroxisomal Proteome Through Subcellular Retargeting

If peroxisomes originate from a common ancestral proto-peroxisome, how can they be so diverse in the different eukaryotic lineages? In his initial evolutionary scenario involving an endosymbiotic event, de Duve favored differential loss from a metabolic rich ancestor to explain the observed diversity (de Duve 1982). However, as the list of enzymes that can have a peroxisomal location expanded thanks to the exploration of diverse lineages, it became clear that acquisition of new pathways, in addition to loss of ancestral ones, must have been a relevant evolutionary process shaping the peroxisomal proteome. Subcellular re-targeting, as a route for expanding the repertoire of peroxisomal pathways was supported with several examples in the first computational reconstruction of the ancestral proto-peroxisome, and more recent intermediates. This re-targeting of proteins sometimes can occur through the acquisition of a peroxisomal targeting signal in one of the paralogs after duplication (Gabaldón et al. 2006). Remarkably, the mitochondrion seems to be a preferential source for proteins that are evolutionarily re-targeted to the peroxisome, with nearly two thirds of peroxisomal proteins having mitochondrial homologs or being dually targeted to this organelle (Gabaldón and Pittis 2015). This strong evolutionary connection reflects the many metabolic connections that exist between the metabolism of these two organelles. Of note, this evolutionary flux of proteins from the mitochondrion has been ongoing throughout the evolution of eukaryotes. This targeting likely started before the radiation of current eukaryotic groups, with at least one fatty acid enzymes of alpha-proteobacterial origin being present in the last common ancestor of all peroxisomes (Gabaldón et al. 2006). And has continued over time with examples of evolutionary recent re-targeting events such as alanine:glyoxylate aminotransferase (AGT) in the order carnivora (mammals) and the *S. cerevisiae* peroxisomal citrate synthase 2 (Cit2p) (Gabaldón et al. 2006; Gabaldón and Pittis 2015). Although this clear evolutionary connection between the mitochondrion and the peroxisome may seem to support an origin of the peroxisome from the mitochondrion, this is not necessarily the case. Firstly, a strong retargeting with the endomembrane system (48%) and the nucleus (58%) are also apparent from this analysis (Gabaldón and Pittis 2015), which underscores the capacity of the peroxisome to host pathways from many other different compartments. Secondly, retargeting events have occurred throughout the eukaryotic tree of life, with the fraction of proteins with alpha-proteobacterial origin actually increasing over time (Gabaldón et al. 2006). It is, nonetheless, clear that metabolic and evolutionary relationships between the peroxisome and the mitochondrion are certainly ancient, and were already present in LECA.

7 Secondary Loss of Peroxisomes

Peroxisomal pathways can also be lost from peroxisomes. This may result from the entire loss of function from the organism or the relocation to other subcellular compartments. For instance, trypanosomatids, such as *Trypanosoma* spp. and *Leishmania* spp., do not have catalase due to loss after the Kinetoplastea split into the Trypanosomatida and Bodonina (Gabaldón et al. 2016). In some parasitic protists peroxisomes seem to have been lost completely. The loss of peroxisomes is often associated with reduced metabolic capabilities related to adaptations to a parasitic life-style, frequently in organisms that lack typical mitochondria and display mitosomes or hydrogenosomes instead. This is the case for microsporidians, *Entamoeba* sp, *Trichomonas vaginalis*, *Giardia* spp, and some ciliates (Gabaldón 2010). Based on the absence of peroxisomal biogenesis (PEX) genes from their genomes, apicomplexan parasites were described as the first group of eukaryotes harboring typical mitochondria but lacking peroxisomes (Schlüter et al. 2006). However recent surveys indicated the presence of some hallmark peroxisomal proteins in *Toxoplasma gondii and Neospora caninum* (Gabaldón et al. 2016). These results have been recently confirmed and expanded, clarifying the evolutionary scenario of peroxisome loss in some alveolates (Ludewig-Klingner et al. 2018). Taken together the multiple independent losses of peroxisomes in eukaryotes seem to coincide with parallel extreme overall reduction of lipid metabolic pathways in these organisms.

8 Concluding Remarks

Peroxisomes are ancient eukaryotic organelles showing an extremely high metabolic and functional diversity across the eukaryotic tree of life. The peroxisomal proteome has been shaped by processes of gene gain, gene transfer and gene loss, as well as by subcellular retargeting of proteins that were initially in other subcellular compartments. In general, peroxisomes share a core set of proteins involved in their biogenesis and in the transport of proteins and solutes across its membranes. Moreover, they generally harbor enzymes catalyzing oxidative reactions, often involving the production of highly reactive oxygen species, and proteins involved in the detoxification thereof. In extreme cases, peroxisomes have been entirely lost or have been adapted to perform structural roles or functions unrelated with oxidative metabolism. Similarly, in multicellular organisms, peroxisomes can be involved in developmental or signaling processes. Further physiological diversity is likely hidden within the vast amount of microbial eukaryotic lineages that remain poorly unexplored. Peroxisomes originated before the diversification of extant eukaryotic lineages. Although the idea that peroxisomes had an endosymbiotic origin, just like mitochondria and plastids, gained widespread attention in the past, the established consensus is now that they likely had an endogenous origin from within the endomembranous system. The exact metabolic scenario of how this happened, however, remains controversial.

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Peroxisome Protein Prediction in *Drosophila melanogaster*

Matthew Anderson-Baron and Andrew J. Simmonds

Abstract As a laboratory animal, *Drosophila melanogaster* has made extensive contributions to understanding many areas of fundamental biology as well as being an effective model for human disease. Until recently, there was relatively little known about fly peroxisomes. There were early studies that examined the role of peroxisome enzymes during development of organs like the eye. However, with the advent of a well-annotated, sequenced genome, several groups have collectively determined, first by sequence homology and increasingly by functional studies, *Drosophila* Peroxins and related peroxisome proteins. Notably, it was shown that *Drosophila* peroxisome biogenesis is mediated via a well-conserved PTS1 import system. Although the fly genome encodes a Pex7 homologue, a canonical PTS2 import system does not seem to be conserved in *Drosophila*. Given the homology between *Drosophila* and *Saccharomyces cerevisiae* or *Homo sapiens* peroxisome biogenesis and function, *Drosophila* has emerged as an effective multicellular system to model human Peroxisome Biogenesis Disorders. Finally, *Drosophila* peroxisome research has recently come into its own, facilitating new discoveries into the role of peroxisomes within specific tissues, such as testes or immune cells.

Keywords *Drosophila melanogaster* · Peroxisome · Peroxins · Model system Homology prediction

Abbreviations

GAL4	Yeast galactose-responsive transcription factor
UAS	Yeast upstream activating sequence
dsRNA	Double-stranded ribonucleic acid
RNAi	RNA interference
XDH	Xanthine dehydrogenase (*CG7642*)
DAO	D-amino acid Oxidase (*CG12338*)

M. Anderson-Baron · A. J. Simmonds (✉)
Department of Cell Biology, Faculty of Medicine and Dentistry,
University of Alberta, 5-14 Medical Sciences, Edmonton, AB T6G 2H7, Canada
e-mail: andrew.simmonds@ualberta.ca

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SPCA	Secretory pathway Ca^{2+}-ATPase
SPoCk	Secretory pathway calcium ATPase (*CG32451*)
Pex	Peroxin
SOD1	Superoxide dismutase 1 (*CG11793*)
PTS	Peroxisome targeting sequence
FAR1/2	Fatty acyl-CoA reductase (*CG5065*)
ABCD1	ATP-binding cassette, subfamily D, member 1 (*CG2316*)
AGPS	Alkylglycerone phosphate synthase (*CG10253*)
PMP34	Peroxisomal membrane protein 34 (*CG32250*)
HACL1	2-hydroxyacyl-CoA lyase 1 (*CG11208*)
IDE	Insulin degrading metalloproteinase (*CG5517*)
PMVK	Phosphomevalonate kinase (*CG10268*)
MUL1	Mitochondrial E3 ubiquitin protein ligase 1 (*CG1134*)
ACAA1	B-ketoacyl-CoA thiolase (*CG9149*)
CCS	Copper chaperone for superoxide dismutase (*CG17753*)
GOT1	Glutamate oxaloacetate transaminase 1 (*CG8430*)
Acsl	Acyl-CoA synthetase long-chain (*CG8732*)
Rtnl1	Reticulon-like 1 (*CG33113*)
PBD	Peroxisome biogenesis disorders
VLCFA	Very long chain fatty acid
DNM1L	Dynamin-1-like protein
Drp1	Dynamin related protein 1 (*CG3210*)
AMP	Antimicrobial peptide
IMD	Immune deficiency
DPiM	*Drosophila* protein interaction map
S2	Schneider 2 cells
MSEA	Metabolite set enrichment analysis

1 Introduction: *Drosophila* Has Been Used in the Laboratory for over 100 Years

Drosophila melanogaster, the common fruit fly, was one of the first laboratory animals used for genetic research. References to *Drosophila* as an experimental organism first appeared in the mid-18th century. The first laboratory *Drosophila* strains were isolated by Thomas Hunt Morgan at Columbia University in the early 1900's (Morgan 1910). Flies have been used since to uncover fundamental biological processes and as a model for human disease. Some of the attributes that make *Drosophila* a valuable multicellular experimental system include: a short lifespan; amenability to forward genetic studies; a large number of progeny; a sequenced and annotated genome; and they are easy and inexpensive to maintain (Wangler et al. 2015; Kaufman 2017; Bilder and Irvine 2017).

Fertilized female *Drosophila* deposit eggs externally which hatch into wandering larvae after 24 h. When raised at 25 °C, the larval stage proceeds through three instars, separated by molts, over four days. During the larval stage, the precursors of the adult tissues are present as small sacs of cells known as imaginal discs. During the subsequent five-day pupal stage, most larval tissues are degraded and the adult forms from the imaginal discs. Adults are sexually mature within 24 h (Demerec 1994).

Fly researchers have developed many unique genetic tools, such as 'balancers', multiple inverted chromosomes that prevent homologous crossing over during meiosis. Some constitutively express green flourescent protein (GFP) at high levels fostering simple maintenance of deleterious mutant alleles and ready identification of homozygous animals at any stage (Casso et al. 1999). Another tool used often in fly studies is selective induction of chromosome recombination in somatic cells producing clones of homozygous mutant cells in a heterozygous animal (Xu and Rubin 1993). Transgenic tools such as the GAL4-UAS system enabled precise tissue-specific analyses in the context of whole animals (Rodriguez et al. 2012; Brand and Perrimon 1993). The GAL4/UAS system has become particularly useful in UAS-mediated RNAi amplicon expression of a large collection of transgenes targeting almost any *Drosophila* gene of interest (Rodriguez et al. 2012; Dietzl et al. 2007).

Despite the fact that *Drosophila* have been a laboratory workhorse, there has been little research of their peroxisome biology until recently. However, in the past decade, several studies have shown that the key processes of peroxisome biogenesis and function are conserved in *Drosophila* and that flies are an excellent platform to discover new roles for peroxisomes. *Drosophila* researchers have access to an extensive collection of cultured cells from multiple stage and tissue origins (Schneider 1972). The most commonly used are Schneider 2 (S2) cells, derived from dissociated *Drosophila* embryos (Deml and Wagner 1998; Schneider 1972). S2 cells are highly amenable to genetic manipulation by dsRNA treatment, which makes them effective for unbiased identification of factors involved in processes including peroxisome biogenesis, movement, fission and function (Wynant et al. 2014). When added to cultures of S2 cells, dsRNA amplicons of almost any size (generally 200–500 base pairs), are taken up and processed by the endogenous RNAi enzymes to induce robust gene silencing (Kao and Megraw 2004).

2 Early Studies of *Drosophila* Peroxisomal Proteins

2.1 Analysis of Potential Peroxisome Enzymes in Drosophila Organs

The first studies related to *Drosophila* peroxisome protein activity were published in the 1980s. These focused on two enzymes: xanthine dehydrogenase (XDH) (*CG7642*) and D-amino acid oxidase (DAO) (*CG12338*) and their role in

specific tissues (Beard and Holtzman 1987; St Jules et al. 1989, 1991, 1990; Reaume et al. 1991). The *rosy* gene encodes *Drosophila* XDH. The name *rosy* stems from the observation that mutation resulted in a brownish-red eye color phenotype (Reaume et al. 1991). Beard and Holtzmann analyzed the $rosy^{506}$ null mutation (Beard and Holtzman 1987). They found that XDH co-fractionated with other predicted peroxisome enzymes and while peroxisomes were observed in the gut of homozygous $rosy^{506}$ flies, they lacked xanthine oxidase activity (Beard and Holtzman 1987). Reaume et al. analyzed the tissue-specific expression of *rosy* by *in situ* hybridization and found high levels of expression in the gut and the malpighian tubules (Reaume et al. 1991), though be the insect analog of the mammalian kidney (Gautam et al. 2017). The highest levels of XDH activity were detected in the apical region of the eye and the protein was detected at the interface between the retina and the lamina using a *rosy* specific antibody (Reaume et al. 1991). Similar assays of *Drosophila* DAO activity identified membrane bound structures in the fat body and gut epithelium of both larvae and adult *Drosophila* (St Jules et al. 1989; St Jules et al. 1991). These structures were round, sometimes dumbbell-shaped, and 0.5–1.0 μm in diameter, which was considered consistent with peroxisome morphology (St Jules et al. 1989; St Jules et al. 1991). St Jules et al. used enzymatic assays to identify populations of reactive DAO bodies which were considered presumptive peroxisomes in the fat body and a sparse population in cells making up the eye (St Jules et al. 1990).

2.2 *Release of the Drosophila Genome Sequence and Peroxisome Protein Predictions*

The first version of an assembled and annotated sequence of the *Drosophila* euchromatic genome was released in the early 2000s, and this facilitated the first computational prediction of *Drosophila* peroxisome protein homologues (Adams et al. 2000). Note that *Drosophila* genes are uniquely identified by a CG (Celara Genomics) identification number which is retained until the gene is named based on its function or mutant phenotype (Adams et al. 2000). Oba et al. (2004) characterized the predicted product of the *CG6178* gene and found similarities to luciferase (Oba et al. 2004), a known peroxisome targeted enzyme in fireflies (Gould et al. 1987). CG6178 was found to possess long-chain fatty acyl-CoA synthesis activity, consistent with the canonical functions of a peroxisomal enzyme (Oba et al. 2004). In addition, the predicted CG6178 protein had a serine-lysine-leucine (SKL) C-terminal motif (Oba et al. 2004), the canonical peroxisomal targeting sequence (PTS1) found in *Saccharomyces cerevisiae* and *Homo sapiens* (Gould et al. 1989).

As multiple *Drosophilid* genome sequences became available, a comparative evolutionary analysis of the predicted subcellular localization of predicted proteins identified 544 unique proteins that could potentially localize to peroxisomes (Hazkani-Covo et al. 2004). Comparison of *Drosophila*, *S. cerevisiae* and

H. sapiens identified the homologue of Ca^{2+}/Mg^{2+} ATPase (SPCA), named Secretory Pathway Calcium ATPase (*SPoCK*) (*CG32451*) in *Drosophila* (Southall et al. 2006). Notably, *SPoCK* encodes three protein isoforms, only one of which localized to peroxisomes (Southall et al. 2006). Peroxisome localization of the C-isoform of SPoCK was confirmed by colocalization with a Catalase antibody in S2 cell peroxisomal membrane preparations (Southall et al. 2006). Similar to other previously characterized *Drosophila* peroxisome enzymes, the C-isoform of SPoCK was found to be highly expressed in malpighian tubules and given the function of SPoCK, it was hypothesized that peroxisomes may have an important role in calcium signalling in malpighian tubules (Southall et al. 2006).

3 Systematic Analysis of the *Drosophila* Peroxisome Proteome

Beginning in 2011, several groups took advantage of the improved quality and annotation associated with each successive versions of the *Drosophila* genome (Hoskins et al. 2015) to perform systematic validation and analysis of the predicted *Drosophila* peroxisome proteome (Faust et al. 2012; Baron et al. 2016; Mast et al. 2011). Figure 2 summarizes the predicted roles of the currently known *Drosophila* Pex protein homologues during peroxisome biogenesis and fission (Table 1).

Table 1 Names by Faust et al. and Baron et al. for peroxisome proteins and the corresponding names listed in Flybase

Drosophila peroxisome proteins		
Faust et al. Baron et al.	Flybase	Name
NUDT19	CG10194	
AGPS	ADPS	Alkyldihydroxyacetonephosphate synthase
PMVK	CG10268	
HMGCR	Hmgcr	HMG Coenzyme A reductase
HMGCL	CG10399	
CRAT	CRAT	Carnitine *O*-Acetyl-Transferase
DHSR4/PECR	CG10672	
PMP22	CG11077	
NUDT7	CG11095	
HACL1	CG11208	
MUL1	Mul1	Mitochondrial E3 ubiquitin protein ligase 1
TMEM135	CG11737	
SOD1	Sod1	Superoxide dismutase 1

(continued)

Table 1 (continued)

Drosophila peroxisome proteins		
Faust et al. Baron et al.	Flybase	Name
PEX6	Pex6	Peroxin 6
DDO	CG12236	
DAO	CG12338	
MP17	CG12355	
CROT	CG12428	
ACSF2	CG12512	
PMP70	Pmp70	Peroxisomal membrane protein 70 kDa
PEX11C	CG13827	
PEC1	CG13890	
PHYH	CG14688	
PEX5	Pex5	Peroxin 5
PRDX1	Jafrac1	Thioredoxin peroxidase 1
LKAP	CG17018	
FIS1	Fis1	Fission, mitochondrial 1
SCPx	CG17597	
CCS	Ccs	Copper chaperone for superoxide dismutase
HAO1/2	CG18003	
ACSF3	CG18155	
EPHX2	CG1882	
ABCD1/2	CG2316	
DRS7B	CG31548	
PMP34	Pmp34	Peroxisomal membrane protein 34
ATP2CD1	SPoCk	Secretory pathway calcium atpase
MVK	CG33671	
DBP	Mfe2	peroxisomal multifunctional enzyme type 2
TYSND1*	CG3589	
PEX12	Pex12	Peroxin 12
DECR2	CG3699	
AGXT	Spat	Serine pyruvate aminotransferase
SERHL	kraken	kraken
PEX16	Pex16	Peroxin 16
ACSL1	CG3961	
ACSL5/6	CG3961	
PEX14	Pex14	Peroxin 14
LBP	Mtpα	Mitochondrial trifunctional protein α subunit
SLC27A2	CG44252	
SLC27A4	Fatp	Fatty acid (long chain) transport protein
GNPAT	Dhap-at	Dihydroxyacetone phosphate acyltransferase
PEX13	Pex13	Peroxin 13

(continued)

Table 1 (continued)

Drosophila peroxisome proteins		
Faust et al. Baron et al.	Flybase	Name
ACAD11	CG4860	
ACOX1	CG5009	
FAR1/2	CG5065	
PEX19	Pex19	Peroxin 19
MDH1	Mdh1	Malate dehydrogenase 1
IDE	Ide	Insulin degrading metalloproteinase
HSDL2	CG5590	
IDI1/2	CG5919	
SLC22A5	Orct	Organic cation transporter
PEX7	Pex7	Peroxin 7
NOS2	Nos	Nitric oxide synthase
PEX1	Pex1	Peroxin 1
PEX3	Pex3	Peroxin 3
CAT	Cat	Catalase
PEX2	Pex2	Peroxin 2
URO	Uro	Urate oxidase
IDH1	Idh	Isocitrate dehydrogenase
PRDX5	Prx5	Peroxiredoxin 5
XDH	ry	Rosy
PEX10	Pex10	Peroxin 10
PAOX	CG8032	
PEX11A/B	Pex11	Peroxin 11
GOT1	Got1	Glutamateoxaloacetatetransaminase1
ACSL3	Acsl	Acyl-CoAsynthetase long-chain
LONP	Lon	Lon protease
SOD2	Sod2	Superoxidedismutase2 (Mn)
ACAA1	CG9149	
AMACR	CG9319	
ACOX3	CG9527	
ECH1	CG9577	

3.1 Identification of Drosophila Peroxisome Biogenesis Protein Homologs

Peroxisome studies in *Drosophila* were aided by development of a S2 cell line that stably express GFP fused to the canonical PTS1 signal at its C-terminus (GFP-SKL), serving as a peroxisome marker compatible with live cell imaging (Fig. 1) (Kural et al. 2005). Mast et al. performed double-stranded RNA (dsRNA) knock of the expression of each of the *Drosophila* genes homologous to

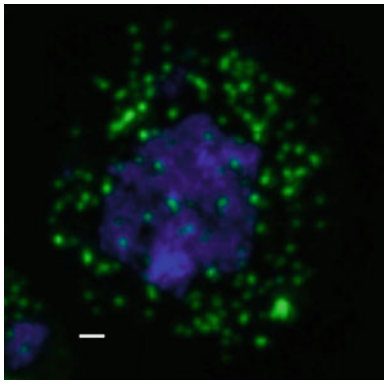
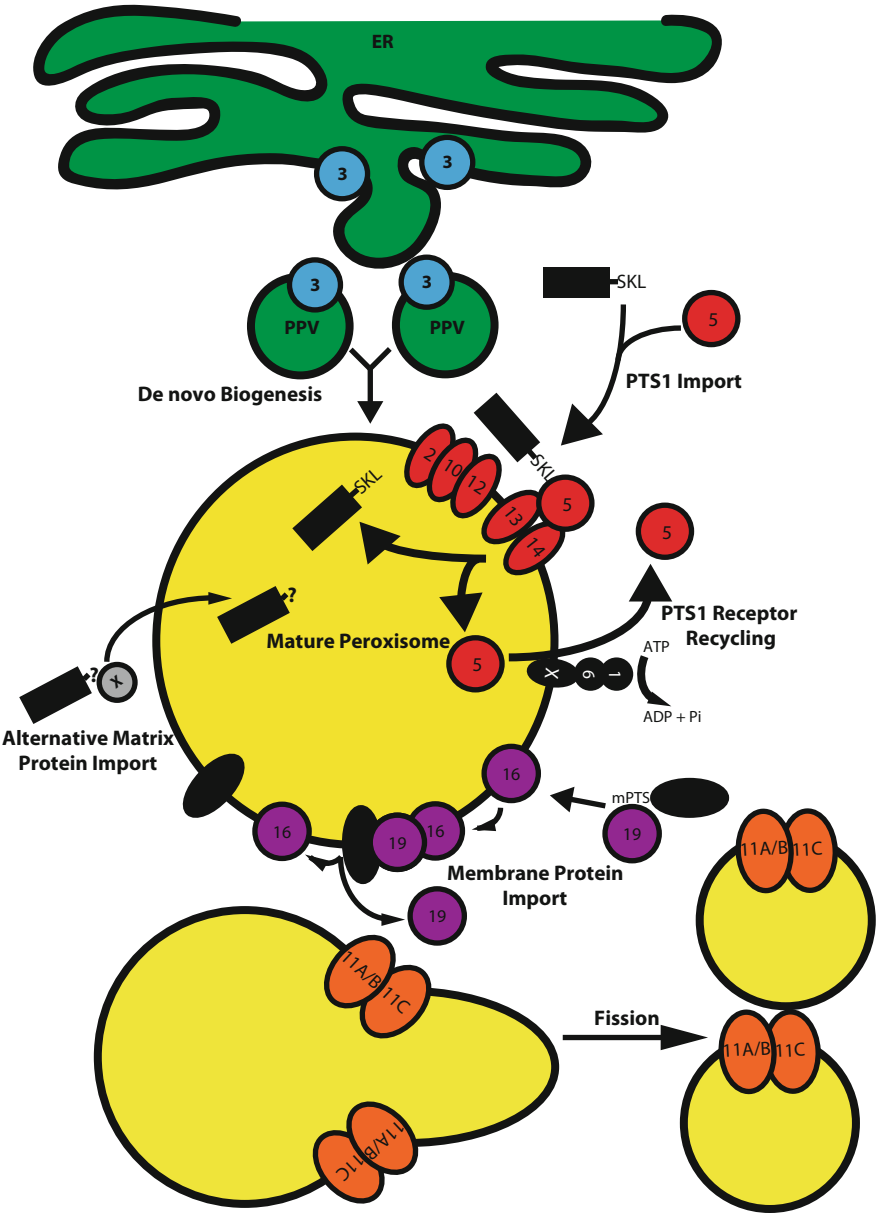


Fig. 1 Schneider 2 (S2) cells stably expressing GFP-PTS1. Shown is an individual *Drosophila* S2 cells that stably expresses GFP-PTS1, which targets the GFP molecule to the matrix of the peroxisome. This marks the organelle, shown in green. DAPI marks the nucleus, shown in blue. Scale bar represents 1 μm

S. cerevisiae and *H. sapiens* Pex genes (Mast et al. 2011). Knockdown of *Pex1*, *Pex5*, *Pex13*, and *Pex16* abolished the punctate GFP-positive signal indicating that proteins encoded by these genes were involved in PTS1-mediated import (Mast et al. 2011). Similarly, dsRNA knockdown of *Pex2*, *Pex3*, *Pex6*, *Pex12*, and *Pex14* induced a cytosolic GFP signal with a few peroxisomes remaining, also confirming that these genes likely encoded Pex proteins (Mast et al. 2011). Lastly, knockdown of *Pex11* and *Pex19* resulted in fewer and larger GFP-positive peroxisomes, whereas knockdown of *Pex20* and *Pex23* resulted in smaller and more numerous peroxisomes again, in keeping with the function of these Pex genes in *S. cerevisiae* and *H. sapiens* (Mast et al. 2011) (Fig. 2).

Faust et al. made the initial encompassing prediction of the *Drosophila* peroxisome proteome (Faust et al. 2012). Potential *Drosophila* peroxisome proteins predicted by sequence similarity to known vertebrate homologs (Faust et al. 2012). Known peroxisome proteins from *H. sapiens* were used to BLAST the *Drosophila* proteome and PTS1 locating software was used to identify any other predicted fly proteins that possessed the canonical PTS1 or some variation. This analysis identified 82 potential peroxisomal proteins, including the *Pex* gene homologs identified Mast et al. (Faust et al. 2012; Mast et al. 2011). They also noted the *Drosophila* genome encoded three isoforms of *Pex11* and two splice isoforms of *Pex5* were

Fig. 2 Peroxisome biogenesis in *Drosophila.*De novo biogenesis occurs at the endoplasmic reticulum (ER) whereby pre-peroxisomal vesicles (PPV) bud off via Pex3. The PPVs then fuse to form a mature peroxisome, shown in yellow. The PTS1 import pathway, shown in red, occurs via the soluble cargo receptor, Pex5, which binds to the PTS1 signal of cargo (SKL). Pex5 and its cargo dock at the peroxisomal membrane via Pex13 and Pex14, which also forms a pore through which Pex5 and its cargo pass through. Pex5 dissociates from its cargo inside the matrix and Pex5 is recycled back out into the cytosol via Pex1 and Pex6. In addition, an alternative to the PTS1 import pathway exists in Drosophila, however, it is unclear by what process this occurs. Membrane protein import occurs when Pex19 recognizes mPTS containing cargo and docks via Pex16 at the membrane. Once the protein is inserted into the membrane, Pex19 is recycled back out into the cytosol. Peroxisome fission occurs via Pex11A/B and Pex11C whereby the membrane is elongated and undergoes scission to form 2 daughter organelles ▶


ER
3
3
3
3
PPV
PPV
SKL
5
PTS1 Import
De novo Biogenesis
SKL
SKL
2
10
12
13
14
5
5
?
Mature Peroxisome
5
PTS1 Receptor
Recycling
ATP
ADP + Pi
X
6
1
?
X
Alternative Matrix
Protein Import
16
mPTS
16
19
16
19
19
Membrane Protein
Import
11A/B
11C
11A/B
11C
Fission
11A/B
11C
11A/B
11C

noted, which may correspond to the long and short isoforms found in mammalian systems although this has not yet been confirmed experimentally (Faust et al. 2012).

3.2 *Functional Characterization of Predicted Drosophila Pex Proteins*

Fakieh et al. characterized intracellular sorting of Pex3 in *Drosophila* S2R+ cells (Fakieh et al. 2013), an adhesive variant of S2 (Yanagawa et al. 1998). When yeast ScPex3 was expressed in S2R+ cells, it colocalized with the peroxisome marker mRFP-PTS1, indicating a conservation of Pex3 transport in *Drosophila* (Fakieh et al. 2013). Localization of ScPex3 to the peroxisome required either RSR or RHRGK motifs at the N-terminus of the protein as the double mutant appeared to be trapped in the endoplasmic reticulum (ER) (Fakieh et al. 2013). In addition, the N-terminus of the *Drosophila* Pex3 was used to replace the N-terminus of the yeast ScPex3 and the resulting chimera was found to properly localize to peroxisomes in wild-type yeast cells, confirming that the sorting of Pex3 from the ER to the peroxisome is evolutionarily conserved (Fakieh et al. 2013).

Baron et al. and Faust et al. both performed functional examination of the trafficking of each predicted peroxisomal protein relative to a peroxisomal marker (Baron et al. 2016; Faust et al. 2012). The majority of the *Drosophila* Pex homologues were found to strongly overlap with GFP-SKL when tagged with either RFP or FLAG in S2 cells. FLAG-Pex5 and FLAG-Pex7 showed a significant cytosolic signal and formed punctate structures that overlapped with GFP-SKL (Baron et al. 2016). This was considered consistent with their known functions as cytosolic cargo receptors for the PTS1 and PTS2 import pathways, respectively (Baron et al. 2016; Gould et al. 1988; Faber et al. 1995) in *S. cerevisiae* and *H. sapiens*. Immunofluorescent (IF) detection of FLAG-Pex19 produced a signal that overlapped weakly with GFP-SKL. Instead, FLAG-Pex19 had a prominent cytosolic signal, which was also considered consistent with its conserved function as the receptor for membrane proteins targeted to the peroxisome (Baron et al. 2016; Fujiki et al. 2006). Baron et al. extended the previous analysis of Pex3 sorting showing that IF detection of FLAG-Pex3 produced a signal the smallest degree of overlap with GFP-SKL peroxisome reporter compared to the other Pex homologues (Baron et al. 2016). Tagged Pex3 formed a reticular-like pattern, which suggests that when over-expressed, it becomes trapped inside the ER (Baron et al. 2016). This would be consistent with a known function in the de novo peroxisome biogenesis pathway (Mayerhofer 2016).

Detection of tagged putative peroxisomal Acyl-CoA oxidase, encoded by *CG17544*, showed that it strongly colocalized with peroxisomes (Faust et al. 2012). The *CG4389* gene, encoding Enoyl CoA hydratase (LBP) has several predicted splice isoforms. Some LBP isoforms localized to peroxisomes while others localized to other subcellular structures (Faust et al. 2012). Only the B-isoform appeared to strongly overlap with peroxisomes (Faust et al. 2012). In addition, superoxide

dismutase 1 (SOD1), encoded by *CG11793*, expresses—AKV at its C-terminus, a variation of PTS1, which localizes to both peroxisomes and the cytosol (Faust et al. 2012). When the AKV was mutated to AKL, SOD1 localization shifted to completely peroxisomal (Faust et al. 2012). When the last 3 residues were deleted (ΔAKV), the fusion protein was completely cytosolic (Faust et al. 2012). This suggests that in *Drosophila*, the non-canonical AKV signal interacts weakly with the Pex5 soluble cargo receptor for PTS1 mediated import.

Baron et al. also systematically examined the functional conservation of the PTS1, PTS2, and membrane import pathways by dsRNAs knockdown of *Pex5*, *Pex7*, and *Pex19*, and looking at the transport of homologs of several known peroxisome matrix proteins rather than a fluorescent protein reporter (Baron et al. 2016). Knockdown of *Pex5*, but not *Pex7*, disrupted the transport of RFP-D-Bifunctional Protein (DBP) to peroxisomes, which possesses a canonical PTS1 at its C-terminus (Baron et al. 2016). Conversely, knockdown of neither *Pex5* nor *Pex7* disrupted transport of a Fatty acyl-CoA reductase 1/2 (FAR1/2), (encoded by *CG5065*) fusion protein to the peroxisome (Baron et al. 2016). FAR1/2 does not possess a canonical PTS1 at its C-terminus, which suggests *Drosophila* employs an alternative to the PTS1 pathway for these proteins (Baron et al. 2016). Lastly, knockdown of *Pex19* resulted in the mis-localization of the ATP-binding cassette, subfamily D, member 1 (ABCD1) (*CG2316*), a peroxisomal membrane protein, to the plasma membrane (Baron et al. 2016). Overall, this suggests that the PTS1 and membrane protein import pathways are conserved in *Drosophila* and they are dependent on Pex5 and Pex19, respectively. In addition, it appears an alternative to the PTS1 protein import pathway is active in *Drosophila* cells, but that this pathway is not dependent on *Pex7*.

3.3 *Drosophila Does not Seem to Employ PTS2 Mediated Protein Trafficking*

Drosophila do not seem to use a canonical PTS2 import pathway for peroxisome matrix protein targeting. A homologue for Pex7, the soluble cargo receptor for PTS2 containing cargo, was identified in the predictions made by Mast et al. (2011), Faust et al. (2012) and Baron et al. (2016). Mutations in human *PEX7* result in AGPS mislocalization and manifests as rhizomelic chondrodysplasia punctata (Aubourg and Wanders 2013; Fujiki 2016). Alkylglycerone phosphate synthase (AGPS) catalyzes the exchange of fatty acids for long-chain fatty alcohols in the peroxisome (Malheiro et al. 2015). Human AGPS is directed to peroxisomes by the N-terminal PTS2 pathway. *CG10253* encodes the predicted homologous *Drosophila* enzyme, also named AGPS but this protein has a canonical PTS1 sequence at its C-terminus (Faust et al. 2012). C-terminal fusions of mCherry to AGPS were constructed to expose a potential PTS2 and mask the PTS1 sequence. When expressed in *Drosophila* S2 cells, no peroxisomal trafficking was observed (Faust et al. 2012). However, when mCherry was fused to the N-terminus to expose the predicted PTS1, it strongly overlapped with

PMP34, showing that *Drosophila* AGPS utilizes the PTS1 import pathway (Faust et al. 2012). the N-terminus (residues 1–72) of human AGPS (hAGPS) fused to mCherry localizes to peroxisomes in COS-7 cells [mammalian fibroblast-like cells derived from monkey kidney tissue (Jensen et al. 1964)] but not in S2 cells (Faust et al. 2012). Faust et al. concluded that, although the *Drosophila* genome encodes a Pex7 homologue, fly peroxisomes rely solely on the PTS1 pathway for the import of cargo into the matrix.

Despite the apparent lack of PTS2-mediated protein import in flies, there seems to be some role for Pex7 during peroxisome biogenesis. *Pex7* dsRNA knockdown does have mild effects on peroxisome size and number and other peroxisome related activities (Baron et al. 2016; Di Cara et al. 2017). Some clues about the role of Pex7 have emerged from large-scale screening efforts supported by the *Drosophila* community that systematically examine the role of each gene in the genome. For example, the MODEncode consortium has performed basic tissue-expression characterization of most *Drosophila* genes (Roy et al. 2010). The expression of *Pex7* was noteworthy in that it was highly expressed in CNS derived cell lineages (Roy et al. 2010). Similar support for a role for *Pex7* in *Drosophila* comes from unbiased forward-genetic screens. Whole-genome RNAi screens in S2 cells, looking for factors affecting phagocytosis (Stroschein-Stevenson et al. 2006) or screening *Drosophila* mutants that enhance phenotypes associated with fly homologs of genes that are linked to human intellectual disability (Inlow and Restifo 2004) both identified *Pex7*.

3.4 *Conservation of Drosophila Peroxisome Membrane Protein (PMP) Trafficking*

While there has been considerable effort spent on the predicted *Drosophila* Pex homologs, less is known about the other peroxisome membrane proteins. Faust et al. generated a S2 line expressing a PMP34-cerulean fusion protein (Faust et al. 2012). PMP34 formed donut-shaped structures that coalesce around the periphery of SKL positive puncta in S2 cells (Faust et al. 2012). ECFP-tagged Peroxisome Membrane Protein 70 (PMP70) has also been shown to mark the presumptive peroxisome membrane (Nakayama et al. 2011). Currently, these are the only validated non-Pex proteins that are targeted to peroxisome membranes in *Drosophila* cells.

3.5 *Drosophila Peroxisome Matrix Proteins*

Of the remaining 68 predicted peroxisome genes functionally validated by Baron et al., the localization patterns of the encoded proteins were grouped into three categories based on the degree of colocalization with a GFP-SKL peroxisome

marker (Baron et al. 2016). Of the 34 proteins analyzed that strongly colocalized (>60%) with GFP-SKL, the majority possessed a canonical PTS1 or some variation of it (Baron et al. 2016). This supported the conclusion that *Drosophila* has a robust PTS1 mediated peroxisome-targeting system. The few peroxisome localized proteins that do not possess a PTS1, such as solute carrier family 22 member 5 (SLC22A5), seem to represent membrane proteins that utilize the membrane protein import pathway to target the peroxisome (Baron et al. 2016). Twenty-six of the proteins analyzed showed little to no overlap with GFP-SKL (<30%) (Baron et al. 2016). Of those 26, two possess a canonical PTS1 signal at their C-terminus: 2-hydroxyacyl-CoA lyase 1 (HACL1) (*CG11208*) and insulin degrading enzyme (IDE) (*CG5517*) (Baron et al. 2016). It is possible that both proteins are post-translationally modified and their C-terminus is cleaved, removing a peroxisome-targeting signal. Others have also reported that IDE is localized to the cytosol and at the plasma membrane where it functions as a metalloprotease (Galagovsky et al. 2014). The remaining eight proteins showed a moderate degree of colocalization (30–60%) with GFP-SKL (Baron et al. 2016). One such protein, LBP, showed a high degree of colocalization with GFP-SKL (59.6%) and formed punctate structures that were independent of the peroxisome marker (Baron et al. 2016). These structures could represent other organelles, such as mitochondria or lipid droplets, although this remains to be tested. Baron et al. also revisited the XDH enzyme initially studied in the 1980s. They found that XDH formed punctate structures that overlapped with GFP-SKL but also had a significant cytosolic signal (Baron et al. 2016). It is possible that this third class of proteins characterized have multiple organellar targeting signals, with PTS1 being one of them.

4 The Effects of Overexpressing *Drosophila* Peroxisome Proteins

Baron et al. also measured the effect of overexpression in S2 cells of each of the 82 predicted *Drosophila* peroxisomal proteins on peroxisome volume or number. Overexpression of phosphomevalonate kinase (PMVK) (*CG10268*) and Mitochondrial E3 ubiquitin protein ligase 1 (MUL1) (*CG1134*) fusion proteins had the most significant effect on peroxisome volume, with an increase of roughly 0.173 μm^3 under normal conditions, to volumes exceeding 400 μm^3 (Baron et al. 2016). Both of these proteins showed a moderate degree of colocalization with GFP-SKL (30–60%) (Baron et al. 2016). It is possible that the overexpression of these proteins directly influences peroxisome volume because they partially localize to the peroxisome. However, it is equally as possible that these effects are indirect through their non-peroxisomal localization. A small number of fusion proteins caused a decrease in peroxisome volume when overexpressed, such as Pex11A/B, which would be consistent with its proposed function as a regulator of peroxisome fission in *S. cerevisiae* and *H. sapiens* (Baron et al. 2016). One unique observation to S2 cells was that overexpression of Mpv like protein-17 (Mp17), results in an

increased number of peroxisomes per cell (Baron et al. 2016). Mp17 is proposed to regulate mitochondrial fission, so its effects on the peroxisome population may be indirect (Baron et al. 2016). Overexpression of several other predicted *Drosophila* peroxisome proteins including: Superoxide dismutase 1 (SOD1), β-ketoacyl-CoA thiolase (ACAA1) (*CG9149*), and MUL1 also resulted in a significant decrease in the number of peroxisomes per cell (Baron et al. 2016). It is unclear how overexpression of these proteins would cause a decrease in peroxisome number. However, it is possible that the overexpression of these proteins interferes with peroxisome biogenesis and/or fission (Baron et al. 2016).

5 Screening for Peroxisome-Associated Proteins in *Drosophila*

5.1 Large Scale Screens for Novel Protein Interactions

The availability of a sequenced genome incorporating validated transcript sequencing for *Drosophila* has fostered several large-scale mass-spectrometry protein-interaction studies. This includes the *Drosophila* Protein interaction Map (DPiM) project, based on mass-spectrometry of proteins co-affinity purified with 3488 different bait proteins expressed in S2R+ cells. DPiM interactions were scored statistically to define a high-quality interaction map (Guruharsha et al. 2011). DPiM identified known peroxisome-assocaited interactions like Pex5 and Pex14 (Guruharsha et al. 2011). This study also showed high-confidence interactions between Pex5/Pex14 and Ribosomal protein S15Ab, β-Tubulin at 85D and Histone H3.3B (Guruharsha et al. 2011). The significance of these interactions is not currently known. In addition, extensive interaction networks for glutamate oxaloacetate transaminase (GOT1) (*CG8430*) were identified by DPiM, identifying its interaction with SOD1, copper chaperone for superoxide dismutase (CCS) (*CG17753*), as well as ribosomal proteins such as Rps21 (Guruharsha et al. 2011). In a separate study, O'Sullivan et al. reported an interaction between Acyl-CoA synthetase long-chain (Acsl), a peroxisomal enzyme, and Reticulon-like 1 (Rtnl1) (*CG33113*), a structural protein found in the ER and other organelles such as the lipid droplets (O'Sullivan et al. 2012). This suggests that *Drosophila* peroxisome proteins interact with proteins normally found in other organelles like the ER or lipid droplets.

5.2 Proteins Regulating Peroxisome Dynamics in Drosophila S2 Cells

The availability of S2 cells stably expressing the GFP-SKL peroxisome reporter has facilitated several live cells studies of peroxisome dynamics (Kural et al. 2005;

Kim et al. 2007; Kulic et al. 2008). The first systemic RNAi screens in S2 cells expressing GFP-SKL found that peroxisomes require Kinesin and cytoplasmic Dynein for movement, as RNAi knockdown of genes encoding either protein abolishes the bidirectional movement of peroxisomes along microtubules (Kim et al. 2007). The overexpression of dominant-negative inhibitors to Dynein-dependent processes were found to significantly inhibit peroxisome movement (Kim et al. 2007). Furthermore, it was found that Kinesin and Dynein are not antagonistic in peroxisome movement, but rather, oligomers of each protein cooperatively coordinate peroxisome movement along microtubules in vivo (Kural et al. 2005). Kulic et al. monitored peroxisome movement in GFP-SKL S2 cells and found that populations moved in unison over long time frames and showed correlations with microtubule tip positions (Kulic et al. 2008). Two different peroxisome populations were observed: (i) relatively stationary peroxisomes that move <100 nm over a 5 s window whose trajectories do not follow microtubule lengths; and ii) rapidly moving peroxisomes whose trajectories were parallel to microtubule processes (Kulic et al. 2008). In addition, motor-dependent longitudinal microtubule oscillations affect peroxisome movement throughout the cell (Kulic et al. 2008). Thus, it was concluded that peroxisome mobility is a result of both movement along microtubule tracks in addition to movement of the microtubule tracks themselves (Kulic et al. 2008).

6 Developing *Drosophila* as a Model for Human Peroxisome Biogenesis Disorders

With a conserved cohort of Pex proteins and confirmation that most other aspects of peroxisome biogenesis are conserved, *Drosophila* has been successfully developed as a small animal model for Peroxisome Biogenesis Disorders (PBDs), linking defects in peroxisome function to effects on specific organ systems.

6.1 Phenotypes Associated with Defective Peroxisome Biogenesis in Drosophila

Chen et al. reported that peroxisomes are important for spermatocyte development in the testes of *Drosophila* (Chen et al. 2010). Homozygous mutant *Pex2* males showed a growth defect and were sterile (Chen et al. 2010). During normal spermiogenesis, immature spermatids move away from the testis wall and differentiate into mature spermatocytes (Lin et al. 2000). The immature spermatids of the *Pex2* mutants were indistinguishable from the wild-type, however, very few mature spermatoctyes were detected (Chen et al. 2010). The same phenotype was observed in *Pex10* and *Pex12* mutants (Chen et al. 2010), which is consistent with the fact the

Pex2, Pex10, and Pex12 function in a complex during peroxisome biogenesis (Prestele et al. 2010; Platta et al. 2009). Spermatocytes in the mutants were found to be defective during differentiation with failed cytokinesis during meiotic division. The mutant testis were found to express low levels of nuclear *cyclin A*, which suggests that the spermatocytes do not reach the G2/M transition during meiosis (Chen et al. 2010). In addition, low levels of *don juan*, a spermatocyte arrest gene (Santel et al. 1997), were detected (Chen et al. 2010). This suggests that peroxisome deficiencies lead to cell cycle arrest in spermatocytes of *Drosophila.* It is possible that a disruption to the energy metabolism due to peroxisome deficiency within the spermatocytes leads to cell cycle arrest. Membrane protein import appears normal in the *Pex* mutants, however, matrix protein import is dysfunctional as GFP-SKL does not form punctate structures (Chen et al. 2010). Consistent with what is observed in PBD patients, *Pex10* and *Pex12* mutant *Drosophila* had elevated levels of very long chain fatty acids (VLCFAs), which were exacerbated when raised on a high-fat diet and become even more elevated with age (Chen et al. 2010).

Nakayama et al. reported that some pathophysiological effects associated with Zellweger Syndrome, the most common form of PBD (Fujiki 2016), were mirrored in *Drosophila* with homozygous loss of function *Pex3* or *Pex16* mutations (Nakayama et al. 2011). *Pex3* mutants were homozygous larval lethal, but *Pex16* mutants survived to adulthood (Nakayama et al. 2011). In order to analyze the peroxisome population in fly tissues, EGFP-SKL and ECFP-Peroxisome Membrane Protein 70 (PMP70) were overexpressed to mark the peroxisome matrix and membrane, respectively (Nakayama et al. 2011). Both signals were absent in the *Pex3* mutant (Nakayama et al. 2011). Both signals were present in the malpighian tubules of the *Pex16* homozygous mutant; however, the peroxisome number appeared greatly reduced (Nakayama et al. 2011). *Pex16* mutants recapitulated many symptoms of Zellweger syndrome: growth defects (despite normal feeding rates), reduced lifespans, elevated VLCFA, and locomotor defects (Nakayama et al. 2011; Fujiki 2016). In addition, they also displayed the *rosy* eye color phenotype (Nakayama et al. 2011). Structural defects were also observed in the dendritic trees of the optic lobe (Nakayama et al. 2011). These defects began at the pupal stage, did not worsen after 10 days, and could be rescued by the overexpression of Pex16 in the fat body or specific neurons (Nakayama et al. 2011). In addition, similar to the findings of Chen et al., *Pex16* mutants exhibited arrested spermatocyte development and the absence of mature sperm resulting in male sterility (Nakayama et al. 2011).

Also Mast et al. analysed the developmental phenotypes associated with a homozygous *Pex1* mutant and found considerable similarities to symptoms of Zellweger patients with a corresponding null *PEX1* mutation (Mast et al. 2011). Mutant *Pex1* larvae were significantly smaller than their wild-type counterparts and did not survive past second instar (Mast et al. 2011). They also displayed locomotor defects, but showed no defects in their musculature when analyzed by IF using muscle-specific antibodies (Mast et al. 2011). When the nervous system was analyzed using neuron-specific antibodies, significant abnormalities were observed in the number of cells and organization of both the peripheral nervous system and the central nervous system, which is likely responsible for the locomotor defects

observed (Mast et al. 2011). In addition, the structure of the malpighian tubules appeared abnormal in the *Pex1* mutants (Mast et al. 2011).

Wangler et al. generated *Pex2* and *Pex16* mutant *Drosophila* via P-element insertion and found both exhibited similar phenotypes to previously described *Pex* mutants (Wangler et al. 2017). Homozygous *Pex2* and *Pex16* mutants had elevated VLCFA, cells with cytoplasmic GFP-SKL in multiple tissues, shortened lifespans, and locomotor defects (Wangler et al. 2017). The authors performed a Metabolite Set Enrichment Analysis (MSEA) (analogous to Gene Set Enrichment Analysis) in which sets of metabolites are explored for enriched alterations (Xia and Wishart 2010, 2011). MSEA revealed an increase in phospholipid precursors, and a corresponding decrease in phospholipid degradation products (Wangler et al. 2017). This suggested a defect in synthesis and a reduction in the breakdown of membrane lipids. MSEA also consistently showed altered carbohydrate metabolism, specifically in the pentose phosphate pathway, starch and sucrose metabolism, and glycolysis (Wangler et al. 2017). When the mutant flies were raised on a low-sugar diet, their lifespan was significantly decreased (Wangler et al. 2017). While wild-type flies showed decreased activity when fed low-sugar food, *Pex2* and *Pex16* mutants showed increase activity (Wangler et al. 2017). This was proposed to be an elevated foraging response as result of decreased carbohydrate levels. There was also significant co-regulation of regulating peroxisome genes and genes involved in carbohydrate metabolism in *Drosophila* (Wangler et al. 2017). Overall, this suggests that peroxisome activity is intimately linked to carbohydrate metabolism in both *Mus musculus* (Peeters et al. 2011) and *Drosophila.*

Bulow et al. recently reported *Pex19* mutants could be linked to lipotoxicity and mitochondrial damage in *Drosophila* (Bulow et al. 2018). An analysis of the larval CNS of *Pex19* null mutants revealed neurodegeneration and upon examination of the fat profile revealed elevated lipolysis and lipotoxicity (Bulow et al. 2018). VLCFA levels were increased, whereas medium and long chain fatty acids were decreased (Bulow et al. 2018). The increase in lipolysis was attributed to an upregulation of *lipase3* and *hepatocyte nuclear factor 4* (*Hnf4*) (Bulow et al. 2018). In addition, it was found that *Pex19* mutants showed increased beta-oxidation, elevated ROS levels, and exhibited defects in mitochondrial morphology (Bulow et al. 2018).

6.2 *Analysis of the Drosophila Whole-Animal Response to Pex1 Deficiency*

Mast et al. (2011) was the first to use flies to examine the underlying organismal response to peroxisome deficiency. A comparative genome-wide transcriptome analysis between wild-type and homozygous *Pex1* mutant larvae revealed 551 genes that were three-fold differentially expressed in the *Pex1* mutants (Mast et al. 2011). Gene ontology analysis revealed an overrepresentation of genes involved in

neural development (Mast et al. 2011), consistent with the observed defects of both the PNS and the CNS. In addition, there were defects associated with other pathways, such as the antibacterial response; purine base metabolic processes; VLCFA metabolism, developmental hormone response; eye pigmentation (Mast et al. 2011). Association with purines were first found in the pea leaf, where it was discovered that peroxisomes could catabolize xanthine to uric acid and allantoin (Corpas et al. 1997). In addition, some steroid hormones, such as β-endorphin and β-lipotropin, are localized to peroxisomes in certain human tissues (Weinhofer et al. 2013), which suggests that peroxisomes are involved in hormone metabolism. One other response was genes involved in regulation of alternative RNA splicing, suggesting that this may play a role in peroxisome regulation (Mast et al. 2011). This demonstrates the efficacy of the fly model in probing systemic genetic responses to peroxisome deficiencies.

6.3 *Modelling Single Peroxisome Enzyme Deficiencies Using Drosophila*

Changes in peroxisome phenotype have been observed in *Drosophila* mutants of non-peroxisomal genes, shedding new light on the physiological connections between peroxisomes and other organelles. Chao et al. analyzed the effect of perturbing *Dynamin 1-like* (*Drp1*) (*CG3210*), the homologue of human Dynamin-1-like protein (DNM1L), in wandering third instar larvae (Chao et al. 2016). DNM1L is a mitochondrial GTPase protein involved in mediating mitochondrial fission (Chang and Blackstone 2007). Mutations in DNM1L cause infantile encephalopathy, which is typically characterized as a mitochondrial disease (Uziel et al. 2011). Although overexpression of *Drp1* had no effect, 2 different point xmutations in *Drp1* had a significant effect on the peroxisome population in L3 larvae (Chao et al. 2016). Point mutations in similar regions in DNM1L, A395D and G350R, have been previously characterized as disease causing in humans (Waterham et al. 2007; Chao et al. 2016). Both mutations caused an increase in peroxisome size and altered cellular distribution (Chao et al. 2016). The increase in peroxisome size showed a concomitant decrease in peroxisome number per cell, which suggests mutant Drp1 causes a defect in peroxisome fission (Chao et al. 2016).

Long-chain acyl-CoA synthetase 4 (ACSL4) is an enzyme involved in lipid metabolism that has 2 variants: a short-form that is ubiquitously expressed and a long-form that is expressed primarily in the brain (Mercade et al. 2005). Mutations in human ACSL4 are associated with non-syndromic intellectual disability in humans (Longo et al. 2003). In Huang et al. (2016) analyzed the effects of a mutation of the single *Drosophila* gene encoding *Acyl-CoA synthetase long-chain* (*Acsl*). They found that Acsl localizes to the ER, mitochondria, and peroxisomes in neuronal cells, but localizes primarily to peroxisomes in non-neuronal cells of the

Drosophila brain (Huang et al. 2016). In addition, the brain lobes of *Acsl* mutants were significantly smaller than wild-type brain lobes, which could be rescued by the overexpression of human ACSL4 (Huang et al. 2016). Homozygous *Acsl* mutants showed disrupted neural synaptic activity, possibly due to a change in the lipid composition of the brains, which suggests that *Acsl* plays a role in regulating these activities, consistent with peroxisome function (Huang et al. 2016).

7 Identification of Novel Peroxisome Functions Using *Drosophila*

A recent publication by Di Cara et al. revealed novel peroxisome functions by demonstrating that they are involved in the immune response in *Drosophila* (Di Cara et al. 2017). RNAi mediated targeting of *Pex5* or *Pex7* in S2 cells induced reduced phagocytosis of bacteria (Di Cara et al. 2017). Note that S2 cells are thought to be of a hemocyte lineage, the primary macrophage lineage in flies (Schneider 1972). Peroxisome deficient cells do not exhibit the membrane protrusions found in wild-type cells when bacteria are being engulfed, which suggests defects in the cytoskeleton (Di Cara et al. 2017). These defects extended to cell motility, as *Pex5* and *Pex7* knockdown cells were unable to repopulate an area in liquid culture as well as their wild-type counterparts (Di Cara et al. 2017). *Pex5* and *Pex7* knockdown cells also show lysosomal defects, as observed lysosomes were larger and fewer, with a mislocalization of Lamp1 (Di Cara et al. 2017), a late endosomal or lysosome marker (Shapiro et al. 2007; Rohrer et al. 1996). In wild-type cells, it was observed that peroxisomes coalesce around the site of phagocytosis when bacteria are being engulfed, which suggests that they play a role in this process (Di Cara et al. 2017). It has been shown that reactive oxygen species are involved in signaling for the immune response to bacterial infection (Underhill and Ozinsky 2002; Pinegin et al. 2017). Pioneering studies in plant cells found the presence of SOD in peroxisomes and they were discovered as sites of superoxide radical production (del Río et al. 2006; del Río 2011; del Río and López-Huertas 2016). Wild-type cells showed a 10-fold increase in hydrogen peroxide levels in response to bacterial infection, whereas the hydrogen peroxide response was significantly attenuated in *Pex5* and *Pex7* knockdown cells (2.5 fold) (Di Cara et al. 2017). In addition, steady-state hydrogen peroxide levels in uninfected *Pex5* and *Pex7* knockdown cells were significantly higher than wild-type cells, which suggests a dysregulation of hydrogen peroxide metabolism (Di Cara et al. 2017). Overexpression of Catalase, partially rescued the phagocytic defect in *Pex7* knockdown cells, but not *Pex5* knockdown cells (Di Cara et al. 2017). In adult flies, knockdown of both *Pex5* and *Pex7* resulted in a lower survival rate in response to infection of both gram-negative and gram-positive bacteria (Di Cara et al. 2017). Anti-microbial peptides (AMP) are produced and secreted when the IMD pathway is activated (Myllymaki et al. 2014). AMP production was not observed in either *Pex5* or *Pex7* knockdown cells (Di Cara et al. 2017). Together, this identified a new

role for peroxisomes in immune cells, activating the immune response to bacterial infection (Di Cara et al. 2017).

8 Summary

With the development of what is now a comprehensive 'toolbox' of reagents to study peroxisome formation and function, *Drosophila* will undoubtedly continue to contribute to the field of peroxisome biology. The open questions surrounding the role of Pex7 in terms of peroxisome biogenesis or functions need to be addressed. Additionally, confirmation of the largely conserved nature of the *Drosophila* peroxisome proteome will allow flies to be used as they are consistently used best, for forward/reverse screening. The ability to easily perform screens in S2 cells as well as in individual fly organs, will undoubtedly uncover additional players in *Drosophila* peroxisome regulation or function, that are conserved in other metazoans.

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Part III
Analysis of Peroxisome Proteome Interaction Networks

Using Pull Down Strategies to Analyze the Interactome of Peroxisomal Membrane Proteins in Human Cells

Jessica Klümper, Silke Oeljeklaus, Bettina Warscheid, Ralf Erdmann and Wolfgang Schliebs

Abstract Different pull-down strategies were successfully applied to gain novel insight into the interactome of human membrane-associated proteins. Here, we compare the outcome, efficiency and potential of pull-down strategies applied to human peroxisomal membrane proteins. Stable membrane-bound protein complexes can be affinity-purified from genetically engineered human cells or subfractions thereof after detergent solubilization, followed by size exclusion chromatography and analysis by mass spectrometry (MS). As exemplified for Protein A-tagged human PEX14, one of the central constituents of the peroxisomal matrix protein import machinery, MS analyses of the affinity-purified complexes revealed an unexpected association of PEX14 with other protein assemblies like the microtubular network or the insertion apparatus for peroxisomal membrane proteins comprising PEX3, PEX16 and PEX19. The latter association was recently supported by using a different pull-down strategy following in vivo proximity labeling with biotin, named BioID, which enabled the identification of various membrane proteins in close proximity of PEX16 in living cells.

Keywords Peroxisomal membrane proteins · BioID · AP-MS
PEX14 · PEX16

Abbreviations

AP	Affinity purification
BioID	Biotin identification
DDM	N-dodecyl-β-D-maltoside
ER	Endoplasmic reticulum

J. Klümper · R. Erdmann · W. Schliebs (✉)
Institute of Biochemistry and Pathobiochemistry, Ruhr-University Bochum, 44780 Bochum, Germany
e-mail: wolfgang.schliebs@rub.de

S. Oeljeklaus · B. Warscheid
Faculty of Biology and BIOSS Centre for Biological Signalling Studies, Institute for Biology II, Biochemistry and Functional Proteomics, University of Freiburg, 79104 Freiburg, Germany

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IP Immunoprecipitation
LC Liquid chromatography
MS Mass spectrometry
PEX Peroxin
PMP Peroxisomal membrane protein
PPI Protein-protein interaction

1 Membrane Protein Complexes in Human Peroxisomes

The knowledge about the protein composition of the human peroxisomal membrane expanded over the last few years. Up to now, 31 integral peroxisomal membrane proteins have been identified with a remarkable diversity of functions, including membrane and matrix protein import into peroxisomes, metabolite transport, peroxisome maintenance, proliferation, and inheritance, innate immunity, signal transduction, and the formation of contact zones of peroxisomes with other organelles (Fig. 1).

1.1 Biogenesis I—de Novo Formation, Proliferation and Division

De novo formation of peroxisomes can exclusively originate from the ER (Kim et al. 2006), or, as recently suggested, by fusion of mitochondrial derived vesicles and ER-derived pre-peroxisomes (Sugiura et al. 2017). Alternatively, peroxisomes proliferate via growth and division (Motley and Hettema 2007; Delille et al. 2010). The initial steps of peroxisome biosynthesis involve PEX3 (Soukupova et al. 1999), PEX16 (Honsho et al. 1998) and PEX19 (Matsuzono et al. 1999b) representing together the peroxisomal membrane protein (PMP) import machinery. All three peroxins display at least a dual localization at the ER and the peroxisomal membrane (Dimitrov et al. 2013). Absence or dysfunction of one of these proteins leads to a complete loss of peroxisomal membrane remnants ('ghosts') (Shimozawa et al. 2000; Honsho et al. 1998; Matsuzono et al. 1999a).

Division of peroxisomes is at least a three-step process, consisting of membrane elongation, membrane constriction and final fission of the peroxisome (Delille et al. 2010). Members of the PEX11 family (PEX11α (PMP28)/PEX11β/PEX11γ) (Abe and Fujiki 1998; Tanaka et al. 2003) function as membrane elongation factors and ectopic expression leads to formation of juxtaposed elongated peroxisomes (Koch et al. 2010). Conversely, loss of murine PEX11β causes a reduced number of peroxisomes (Li and Gould 2003).

Upstream of the elongation process, mitochondria and peroxisomes share the same fission machinery. The mitochondrial fission factor [MFF (Gandre-Babbe and

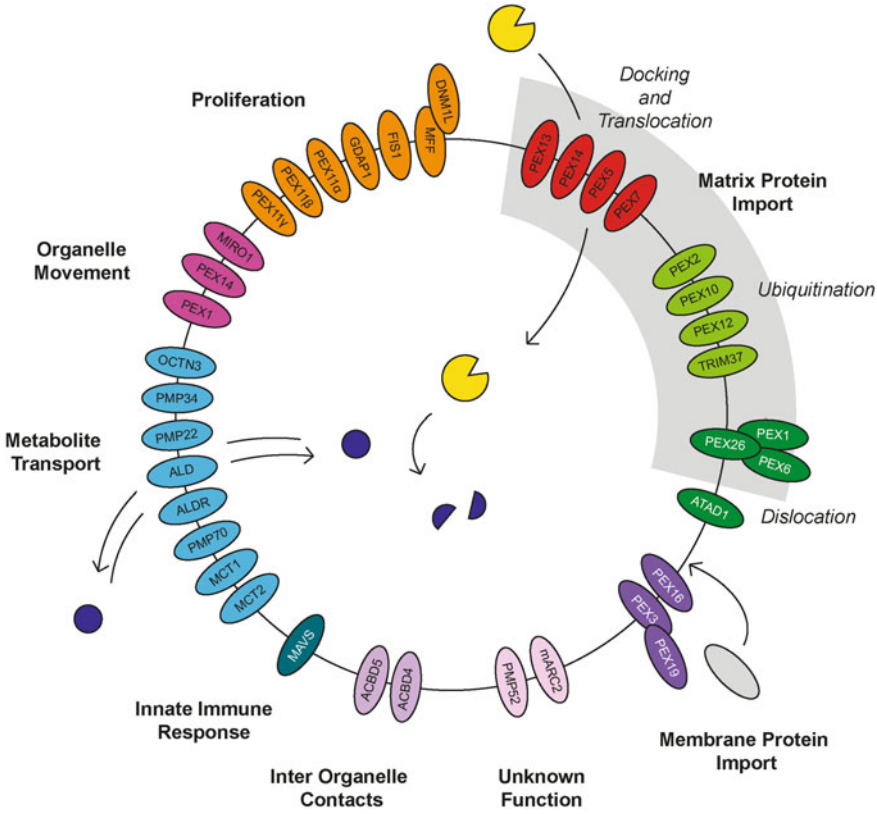

Fig. 1 Functional organization of peroxisomal membrane proteins. The peroxisomal membrane harbours at least 31 integral membrane proteins, which can be arranged in eight functional groups visualized by color. The membrane protein import machinery consists of PEX16, PEX3 and PEX19. Matrix protein import is divided in three steps: translocation and docking (PEX14, PEX13, PEX5, PEX7), ubiquitination (PEX2, PEX10, PEX12, TRIM37) and dislocation (PEX26, PEX1, PEX6). The dislocase ATAD1 does not seem to be related to matrix protein import but quality control of mistargeted tail-anchored membrane proteins. Additional import and export of metabolites and cofactors is crucial for peroxisomal functionality. For this purpose, several transporters namely OCTN3, PMP34, PXMP2, ALD (ABCD1), ALDR (ABCD2), PMP70 (ABCD3), MCT1 and MCT2 have been identified. Mature peroxisomes can divide which requires the proliferation machinery consisting of PEX11α, PEX11β, PEX11γ, GDAP1, FIS1, MFF and DNM1L. Additional proteins enable peroxisomes to maintain inter-organelle contacts (ABCD4, ABCD5, PEX11), function in innate immune response (MAVS) and move along the tubulin network (PEX14, PEX1, MIRO1). It has been shown that PMP52 and mARC2 localize to peroxisomes, but their function still has to be studied. Abbreviations: ATP-binding cassette sub-family D member (ABCD), acyl-CoA binding domain protein (ACBD), Adrenoleukodystrophy protein (ALD), Adrenoleukodystrophy-related protein (ALDR), ATPase family AAA (ATPase associated with various cellular activities) domain-containing protein 1 (ATAD1), dynamin-1-like protein (DNM1L), Ganglioside-induced differentiation-associated protein 1 (GDAP1), mitochondrial amidoxime reducing component 2 (mARC2), monocarboxylate transporters (MCT), mitochondrial fission 1 protein (FIS1), mitochondrial Rho GTPase 1 (MIRO1), mitochondrial antiviral-signaling protein (MAVS), mitochondrial fission factor (MFF), organic cation/carnitine transporter 3 (OCTN3), peroxin (PEX), peroxisomal membrane protein (PMP), Tripartite motif-containing protein 37 (TRIM37)

van der Bliek 2008)] recruits the dynamin-1-like protein [DNM1L (Koch et al. 2003)] to the peroxisomal membrane. Other proteins involved in peroxisomal fission are the mitochondrial fission 1 protein [FIS1 (Koch et al. 2005)] and ganglioside-induced differentiation associated protein 1 [GDAP1 (Huber et al. 2013)]. However, the mechanism and chronology of the fission initiation process is poorly understood. Membrane constriction and fission is then triggered by self-oligomerization of DNM1L into spirals that resemble collar structures encircling the peroxisome (Shin et al. 1999). DNM1L catalyzes membrane fission and vesicle release in a GTP hydrolysis-dependent manner, relying on the GTPase-activating function of PEX11 (Williams et al. 2015).

1.2 Biogenesis II—Membrane and Matrix Protein Import

The insertion of the majority of PMPs into peroxisomal membranes depends on the cytosolic receptor protein PEX19 (class I PMPs), which interacts at the peroxisomal membrane with its docking factor PEX3 (Fang et al. 2004). Alternatively, PMPs can be imported in a PEX19-independent pathway via the ER (class II PMPs). As shown for PEX3 and PMP34, this pathway requires direct interaction with ER-associated PEX16 (Aranovich et al. 2014), which is inserted co-translationally into the ER membrane (Kim et al. 2006).

The core of the peroxisomal matrix protein import machinery consists of the docking complex PEX13/PEX14 and a set of cytosolic receptors (PEX5, PEX7), which recognize proteins destined for the peroxisomal lumen by specific targeting sequences. Both receptors are cycling between the peroxisomal membrane and the cytosol. In mammalian cells, an isoform of PEX5 targets cargo-loaded PEX7 to the membrane (Braverman et al. 1998; Otera et al. 2000). PEX5 associates with the peroxisomal membrane during the import cycle (Gouveia et al. 2003), either becoming an integral constituent of a translocation pore, as shown for yeast (Meinecke et al. 2010), or by transient interaction with a large cavity-forming membrane complex of unknown identity (Dias et al. 2017).

Prior to export into the cytosol, the cycling receptor PEX5 becomes ubiquitinated. Therefore a set of peroxins with ubiquitin-protein isopeptide ligase activity, the so-called RING-Finger complex [PEX2, PEX10 and PEX12 (Biermanns et al. 2003; Okumoto et al. 1998; Okumoto and Fujiki 1997)] functions together with the cytosolic ubiquitin-conjugating enzymes E2D1/2/3 [UBCH5a/b/c (Grou et al. 2008)]. Recently, an additional E3 ligase called TRIM37, required for stabilization of PEX5, was discovered (Wang et al. 2017). Receptor dislocation into the cytosol is facilitated by the AAA^+ ATPases PEX1 and PEX6, anchored to the peroxisomal membrane via PEX26 (Matsumoto et al. 2003). USP9X has been identified as a cytosolic enzyme with PEX5 de-ubiquitinating activity (Grou et al. 2012). However, it is not clear whether de-ubiquitination takes place at the membrane or at a later step of the receptor cycle.

1.3 PMPs with Other Functions

1.3.1 Metabolite Transport

Since peroxisomes are involved in a plethora of metabolic functions, metabolite transporters are needed to regulate exchange of metabolites, cofactors and inorganic ions. Molecules smaller than 300 Da, like the known metabolites glyoxalate, pyruvate and 2-ketoglutarate, can diffuse freely across the peroxisomal membrane (Rokka et al. 2009), probably via the PXMP2 channel [SLC25A17 (Wylin et al. 1998)]. Bulky cofactors such as ATP, CoA, FAD and NAD^+, which are synthesized in the cytosol and exceed the size limit of the PXMP2 channel, have been shown to be transported into the peroxisomal lumen via PMP34 (Agrimi et al. 2012; Visser et al. 2002). The monocarboxylate transporters MCT1 (SLC16A1) and MCT2 (SLC16A7) were predicted to function as lactate/pyruvate shuttles (Valença et al. 2015). A human orthologue of the mouse peroxisome-located carnitine transporter OCTN3 [SLC22A21 (Lamhonwah et al. 2003)] has been identified.

The transport of fatty acids and bile acid precursors is performed via members of the ATP-binding cassette subclass D (ABCD) transporters. The peroxisomal membrane is known to harbor three of these transporters: ABCD1 [ALDP (Mosser et al. 1993)], ABCD2 [ALDRP (Lombard-Platet et al. 1996)] and ABCD3 [PMP70 (Kamijo et al. 1990)]. The localization of ABCD4 (PMP69) is still controversially discussed. It has been previously annotated as peroxisomal but actually seems to be localized to the ER (Kashiwayama et al. 2009).

1.3.2 Inter-Organelle Contact Sites

In recent years, there has been an increasing interest in inter-organelle membrane contact sites. However, most of the work has been done in yeast and the knowledge about inter-organelle contacts, especially peroxisomal ones, in mammals is still in its infancy. For a detailed review, see (Islinger et al. 2015). The peroxisome/ER and the peroxisome/mitochondria connections seem to be mediated via protein-protein interactions (PPIs), whereas peroxisome/lysosome contacts are mediated by phospholipids in the peroxisomal membrane, which are bound by lysosomal synaptotagmin (Chu et al. 2015). Recently, the two tail-anchored acyl-CoA-binding domain-containing proteins ACBD4 and ACBD5 have been reported to function as peroxisome/ER tethers. ACBD4/5 interact with the ER-localized VAPA and VAPB (Costello et al. 2017a, b).

Peroxisomes and mitochondria share not only their fission machinery (FIS1, DNM1L, GDAP1, MFF) and metabolic pathways like β-oxidation but also some other membrane proteins, like ATAD1 and MAVS (see below). A close physical connection of peroxisomes and mitochondria has so far only been revealed in yeast. There, Pex11p could be identified to act as a tether and interact with Mdm34p,

a member of the ERMES complex (Mattiazzi Ušaj et al. 2015). Whether PEX11 fulfills such a function also in human cells has to be investigated.

Peroxisomes also contact the cytoskeleton, which contributes to their homogeneous distribution throughout the cytoplasm and is important for their inheritance. In human cells, peroxisomal motility comes in two flavors: vibrational movement and long range directional movement. Interestingly, the vibrational mode is increased by silencing of the ER tethering factor ACBD5 (Costello et al. 2017a). Peroxisomal long range movements occur along the microtubular network (Rapp et al. 1996). An involvement in organelle motility has been attributed to PEX14, PEX1 and the mitochondrial Rho GTPase 1 (MIRO1). Accordingly, PEX14 binds directly to β-tubulin (Bharti et al. 2011), PEX1 is indirectly attached to microtubules by interacting with KifC3, an adaptor protein belonging to the family of kinesin binding proteins (Dietrich et al. 2013). Only recently a function for peroxisomal MIRO1 as an adaptor for microtubule-based peroxisomal motility was proposed (Castro Inês et al. 2018; Okumoto et al. 2018).

1.3.3 Novel Functions of PMPs

Based on the observation that viral proteins can associate with peroxisomes, a role of the organelle in innate immune response has been anticipated. In support of this notion, it has been shown that MAVS, a RIG-I-like receptor adaptor protein, is localized at peroxisomes and promotes the rapid antiviral response pathway (Dixit et al. 2010).

A peroxisome-associated membrane protein with unknown function is a molybdenum-containing enzyme called mitochondrial amidoxime reducing component (mARC) that comes in two isoforms, mARC1 and mARC2. mARC2 (synonymous MOSC2) has been shown to be dually localized at mitochondria (Wahl et al. 2010) and peroxisomes (Wiese et al. 2007). In the latter study, also PMP52 and the ATPase family AAA^+ domain-containing protein 1 (ATAD1) were identified as novel peroxisomal membrane proteins. Whereas the AAA^+ ATPase has been characterized as a dislocase for mistargeted tail-anchored proteins (Wohlever et al. 2017), the function of PMP52 is still unknown.

2 Proteomic Strategies Applied to the Characterization of PMPs from Human Cells

PMPs are located at the interface between peroxisomes and the surrounding cellular milieu. Consequently, they play crucial roles in mediating peroxisomal protein import/export, the exchange of metabolites as well as interactions and communication with other cellular compartments. To fully understand a PMP's function, knowledge about its protein-protein interactions (PPIs), i.e. the "interactome" of the

PMP, the molecular organization of PMP (sub)complexes, the dynamics of their composition as well as condition- or disease-dependent changes is of great importance. Such knowledge will ultimately improve our understanding of molecular mechanisms underlying peroxisome-related processes on the organellar as well as the cellular level.

For the analysis of PMPs from human cells, three main strategies using pull-down techniques have been employed to date: (i) co-immunoprecipitation (Co-IP) combined with Western blot analysis, (ii) affinity purification of an epitope-tagged protein of interest ("bait") combined with high-resolution MS (AP-MS), and (iii) proximity-dependent biotin identification (BioID). For future studies, a combination of protein pull-down techniques with cross-linking MS may present a promising strategy to enable the identification of transient and weak interactions as well as interaction partners of low stoichiometry. In addition, this approach allows for identifying intermolecular contact sites in native PMP complexes. Furthermore, as an alternative to BioID, proximity labeling using a modified ascorbate peroxidase, referred to as APEX (Rhee et al. 2013) or APEX2 (Lam et al. 2014), can be used to explore the microenvironment of a PMP of interest.

The traditional biochemical method for the identification of PPIs, Co-IP, relies on the use of antibodies specifically recognizing the protein of interest and Western blot analysis to detect co-precipitated proteins. This method has successfully been employed to many peroxins such as PEX26 (Tamura et al. 2014) (see also the Chapter by Fujiki and colleagues in this volume). Co-IP enables to target the native, unmodified protein expressed at endogenous levels (as opposed to epitope tag-based pull-down strategies). Thus, proteins and their interaction partners are purified from in vivo conditions. For low abundant PMPs, however, this may result in low yield. A critical step for Co-IPs of membrane proteins is the solubilization of the target protein and associated interaction partners. The choice of detergent has a crucial influence on the integrity of the interactome that is pulled down. Even mild detergents such as digitonin may interfere with weak PPIs and, thus, impair the identification of transient interaction partners. Furthermore, the success of the Co-IP method strictly depends on the availability of antibodies specifically binding to the protein of interest without exhibiting cross-reactivity to other proteins. In particular, for integral PMPs, the generation of specific antibodies can be very difficult. From the conceptual point of view, the identification of PPIs by Co-IP and Western blotting is generally a hypothesis-driven approach and requires assumptions about putative interaction partners as well as a selection of suitable antibodies to detect these proteins.

AP-MS is recognized as a most powerful strategy to identify PPIs (Gingras et al. 2007; Oeljeklaus et al. 2009; Meyer and Selbach 2015). The use of MS for the identification of proteins associated with the bait represents an unbiased approach allowing for unanticipated discoveries. AP-MS-based studies of PMPs have greatly contributed to the characterization of distinct PMPs and to an improved understanding of peroxisome-associated processes in yeast [e.g., (Agne et al. 2003;

Grunau et al. 2009; Oeljeklaus et al. 2012; Chan et al. 2016; Wróblewska et al. 2017; David et al. 2013)]. An alternative strategy for the study of PPIs is proximity-dependent biotin identification (BioID). This method relies on the expression of the protein of interest fused to a biotin ligase and biotinylation of proteins in close proximity to the fusion protein (Roux et al. 2012). AP-MS and BioID have successfully been used to decipher the interactomes of PEX14 (Bharti et al. 2011) and PEX16 (Hua et al. 2017), respectively, in human cells.

2.1 MS-Based Methods for the Characterization of Human PMP Complexes

2.1.1 Affinity Purification Combined with High-Resolution Mass Spectrometry (AP-MS)

A generic AP-MS workflow for PMPs comprises the affinity purification of an epitope-tagged version of the PMP of interest and associated proteins from detergent-treated crude membrane fractions, proteolytic digestion of purified proteins, liquid chromatography (LC)-MS analysis of the resulting peptide mixture, and computational data analysis to identify proteins co-purified with the bait. For higher sensitivity, subfractionation on protein or peptide level may be included. As control, a "mock" purification using cells expressing the native, non-tagged version of the PMP is performed and analyzed in parallel. Since protein complexes cannot be purified to homogeneity and due to the high sensitivity of modern MS instruments that enable the detection of minute amounts of protein, the discrimination between specific interaction partners and co-purified background proteins can be a challenge. This task is facilitated by incorporating quantitative MS techniques into the workflow, which include label-free as well as metabolic or chemical stable isotope labeling approaches (Bantscheff et al. 2012) and allow to determine differences in the abundance of individual proteins between the purified PMP complex and control sample with high reliability and accuracy (Oeljeklaus et al. 2012). True interaction partners are specifically enriched with the bait, resulting in complex-to-control abundance ratios significantly higher than 1. Non-specifically co-purified background proteins, which are equally present in the purified complex and the control sample, exhibit abundance ratios of approximately 1.

The mere identification of a protein by AP-MS as part of a protein complex does not provide information about the nature of the interaction, which can be stable or transient and dynamic. This knowledge, however, may be essential to fully understand the protein's function within the complex. Warscheid and co-workers performed dual-track quantitative AP-MS studies using stable isotope labeling by amino acids in cell culture [SILAC (Ong et al. 2002)] that allowed the discrimination between stable core components and transient interaction partners of Pex14p and Pex30p in yeast (Oeljeklaus et al. 2012; David et al. 2013), a strategy that is

also applicable to study PMP interactomes in human cells. Cells expressing epitope-tagged Pex14p or Pex30p were grown in the presence of unlabeled "light" arginine and lysine, while control cells expressing the untagged, native version of the proteins were metabolically labeled by growth in medium containing the stable isotope-coded "heavy" counterparts. Protein affinity purification was either performed prior to mixing ("AP-PM") or after mixing of differentially labeled samples ("AP-AM"). Following the AP-PM track, all purification steps were carried out separately for the differentially labeled cell populations before the eluates were combined for joint LC-MS analysis, and interaction partners were identified based on their light-to-heavy protein abundance ratios. In contrast, in AP-AM experiments, equal amounts of light and heavy labeled cells were mixed directly after harvesting and subsequent purification and analysis steps were performed together for differentially labeled protein complexes. This workflow results in the exchange of labeled and unlabeled interaction partners that only transiently interact with the protein complex during the process of the affinity purification. As a consequence, transient interaction partners may exhibit complex-to-control abundance ratios of approximately 1 and, thus, are misclassified as co-purified contaminants. To exemplify, the cytosolic receptor Pex5p that cycles between cytosol and peroxisomal membrane where it associates with Pex14p was identified as specific Pex14p interaction partner in AP-PM experiments but classified as "contaminant" in AP-AM experiments (Oeljeklaus et al. 2012). Thus, the integration of interaction data obtained by AP-AM and AP-PM ultimately enables to discriminate between stable core components of a protein complex and transient or dynamic interaction partners.

2.1.2 Proximity-Dependent Biotin Identification (BioID)

The proximity labeling strategy BioID requires the expression of the PMP of interest fused to a mutant form of the biotin ligase BirA from *Escherichia coli* [BirA* (Roux et al. 2012)]. In the presence of biotin, BirA* promotes the biotinylation of accessible lysine side chains in proteins that are located in close proximity of the PMP in vivo [labeling radius of approx. 10 nm (Kim et al. 2014)]. These include direct interaction partners as well as proteins present in the nano-environment of the PMP but not directly interacting with it. Biotinylated proteins can be affinity-purified from cell lysates using streptavidin and subsequently analyzed by LC-MS. Inclusion of appropriate control experiments (e.g., cells expressing PMP-BirA* not treated with biotin, cells not expressing the BirA* fusion protein treated with biotin) and quantitative MS facilitates the discrimination between "true" PMP-proximal proteins and nonspecifically co-purified background proteins as described above for the identification of true interaction partners by AP-MS. Commonly co-purified contaminants in BioID experiments are carboxylases that contain biotin as cofactor and are biotinylated by endogenous

protein-biotin ligases. An improved BioID method using a smaller promiscuous biotin ligase, BioID2 (27 vs. 35 kDa of BirA*), with enhanced labeling efficiency has recently been reported (Kim et al. 2016).

The strength of the BioID method lies in the in vivo-labeling of proteins present in the nano-environment of the target protein. As a consequence, the detection of interaction partners and other proximal proteins does not depend on maintaining the integrity of protein complexes or entire interactomes during the purification, which makes this approach amenable for stringent lysis and purification procedures. This is particularly advantageous for the study of membrane proteins that need to be extracted from the membrane for a comprehensive analysis. Furthermore, BioID enables to capture transient or week interactions, which are often lost during conventional affinity purification processes, and it provides information about the spatial environment of the target protein, which is also lost when proteins are affinity-purified from crude membrane fractions but may be a crucial aspect to reveal a protein's biological function.

2.2 Critical Aspects to Consider for Experimental Design

The introduction of a stably expressed tagged protein of interest is the first step to enable large scale affinity purification of membrane complexes out of mammalian cells. Today several techniques exist to generate stably expressing cell lines. A common method is to integrate fusion genes coding for tagged bait proteins into the human genome. The Flp-In™ System (Invitrogen) is an efficient tool to create isogenic cell lines by making use of Flp recombinase-mediated DNA recombination at the Flp Recombination Target (FRT) site. The correct integration of the target gene can be verified by a set of growth tests using hygromycine selection, zeocine sensitivity, lack of β-galactosidase activity and protein expression. For both pull-down approaches used so far for human PMPs, Protein A-tag affinity purification and BioID, the Flp-In™ methodology has been used to isolate cell lines expressing the bait proteins PEX14 and PEX16. In contrast to Co-IP, the additional expression of a marker gene, which is required to monitor the genomic integration, and the control of the gene of interest by a foreign promotor can cause artefacts such as mislocalization and non-physiological posttranslational modifications and, thus, must be controlled carefully. In the case of tagged PEX14, patient cell lines defective in the respective genes could be used to verify functional complementation and peroxisomal localization of the bait proteins.

Another critical parameter for affinity purification of membrane complexes concerns the solubilization procedure, in particular the choice of detergent has a significant impact on the amount, composition and integrity of isolated protein complexes. Frequently, non-ionic and zwitter-ionic detergents are chosen to solubilize membrane proteins with retention of function (Seddon et al. 2004). For the isolation of Protein A-tagged peroxisomal membrane complexes from yeast, the nonionic detergents DDM, CYMAL-4, Triton X-100, or digitonin were compared

(Agne et al. 2003). Only Pex14p extraction by 1% digitonin preserved association with all known binding partners at the peroxisomal membrane. Reguenga et al. solubilized human peroxisomal membranes using either 1% digitonin or a mixture of non-ionic NP40 and anionic sodium deoxycholate and determined the molecular size of PMP assemblies by Blue Native gel electrophoresis (Reguenga et al. 2001). Using the mild detergent digitonin, the core constituents of the matrix protein import machinery PEX5, PEX14, PEX10 and PEX12 retain as subunits of a membrane complex larger than 750 kDa. Using the harsh detergent mixture, the same peroxins were detected at a molecular size of about 250 kDa, suggesting that subunits of the heteromeric complex were lost during the preparation. Similar sizes for PEX5 containing membrane complexes were obtained in other studies using 1% Triton X-100 (Itoh and Fujiki 2006). Thus, 1% digitonin is recommended for affinity purification aimed to characterize the size and function of native complexes. For BioID approaches, the detergent can be chosen based on solubilization efficacy of PMPs.

In addition, one should be aware that AP-MS and BioID coupled to MS are complementary strategies to characterize a protein's interactome and subcellular environment (Lambert et al. 2015; Hesketh et al. 2017). While AP-MS in clever combination with metabolic labeling has the potential to discriminate between core components and transient interaction partners of a protein assembly, weakly interacting or low abundance proteins may be lost during the purification process and elude detection by MS. In addition, AP-MS is prone to artifacts originating from abundant cellular proteins or from pooling proteins from different subcellular localizations during cell lysis resulting in artificial interactions. This kind of erroneous data is prevented in BioID experiments since labeling of protein interaction partners and proteins of the nano-environment with biotin occurs prior to cell lysis in living cells. Results of BioID studies, however, do not allow the discrimination whether proteins are part of a distinct interactome or just located in close proximity. Thus, the integration of data obtained in AP-MS and BioID experiments enables a most comprehensive characterization of proteins. It should further be noted that neither AP-MS nor BioID provides information about direct or indirect PPIs. Further studies are required to ascertain direct interactions between individual proteins (e.g. by yeast two-hybrid/split-ubiquitin assay, in vitro binding studies, fluorescence-based microscopy techniques).

3 Towards a PMP Interactome

3.1 The PEX14 Complex Analysis Revisited

One of the best studied interactomes at the peroxisome membrane refers to Protein A-tagged PEX14, which could be isolated together with associated binding partners from yeast and man (Bharti et al. 2011; Oeljeklaus et al. 2012).

In the proteomic approach by Bharti et al., a C-terminally Protein A-tagged version of PEX14 was integrated into the genome of Flp-In^{TM}-293 cells (Invitrogen) and affinity-purified from 1% digitonin-solubilized membranes. In total, more than 200 associated proteins were identified by MS. Among these were 22 proteins with annotated peroxisomal localization, including 14 annotated membrane-associated proteins (Fig. 1). All PEX14-associated peroxins are involved in protein import into peroxisomes: PEX5, PEX13, and the RING-finger peroxins PEX2, PEX10, PEX12 and the AAA^{+}-peroxin PEX1 are required for matrix protein import, while PEX19, PEX3 and PEX16 accomplish membrane protein targeting. Noteworthy, other highly abundant membrane-bound peroxins like the proliferation factor PEX11ß were not detected, thereby demonstrating the specificity of the pull-down approach.

Several of the identified peroxins are supposed to interact directly with PEX14, in particular the soluble receptor for matrix enzymes, PEX5, and the PMP receptor PEX19 (Will et al. 1999; Schliebs et al. 1999; Neufeld et al. 2009; Fransen et al. 2002). Physical interaction of mammalian PEX13 and PEX14 had been reported (Fransen et al. 1998; Will et al. 1999; Itoh and Fujiki 2006). However, other studies, i.e. two hybrid analyses, failed to detect complex formation between human PEX14 and PEX13 (Fransen et al. 2002; Will et al. 1999).

Surprisingly, ß-tubulin could be identified as an additional interacting partner of the conserved N-terminal domain of PEX14 by Bharti et al. (2011). Independent binding assays with recombinant PEX14 variants and purified tubulin supported a direct protein-protein interaction between the N-terminal domain of PEX14 and microtubular filaments (Bharti et al. 2011; Theiss et al. 2012).

The fact that human PEX5, PEX19 and ß-tubulin bind to the same domain of PEX14, and they even compete with each other for in vitro interaction (Bharti et al. 2011), suggests that distinct PEX14 subcomplexes may exist at the peroxisomal membrane. Accordingly, Bharti et al. showed by size exclusion chromatography that PEX14-complexes cover a broad range of molecular masses ranging from the size exclusion limit (about 2 MDa) to 150 kDa (Bharti et al. 2011). Only minor amounts of PEX14 were detected at lower molecular weight regions, suggesting that PEX14 does not primarily functions as a monomeric protein. Within the high-molecular weight range, the peaks of tubulin, PEX5, and PEX19 were clearly separated, suggesting that the peroxisomal membrane harbors at least three heteromeric PEX14 subcomplexes with different composition and function. The complex containing PEX5 and PEX14 displays a molecular mass between 800 and 1000 kDa. The size of this complex correlates with a previous finding by Reguenga et al. using Blue Native gel electrophoresis from digitonin-solubilized rat liver peroxisomes (Reguenga et al. 2001). Here, it was demonstrated that PEX5, PEX14, PEX10 and PEX12 are subunits of a membrane complex with a size larger than 750 kDa. Bharti et al. identified another PEX14 assembly with a molecular mass between 600 and 800 kDa, which co-fractionated with PEX19 (Bharti et al. 2011). It is still an open question whether the PEX14/PEX19 complex represents a PMP in transit or if PEX14 even fulfills another not yet identified function in conjunction with PEX19, PEX3 and PEX16 (see Sect. 3.3). The largest PEX14-containing

subcomplex with a size above 1 MDa contains tubulin and, most likely, other microtubule-associated proteins. The existence of large oligomeric PEX14 assemblies in peroxisomes of mammalian cells has previously also been shown by Itoh and Fujiki (Itoh and Fujiki 2006) (see also the Chapter by Fujiki and colleagues in this book/volume). By subjecting Triton X-100-solubilized peroxisomes to glycerol gradient centrifugation, they could detect the major fraction of PEX14 with a size above 1 MDa, clearly separated from PEX5 at 200–300 kDa and PEX19 below 66 kDa.

To analyze PEX14-associated proteins in conjunction with the microtubular network in greater detail, PEX14-Protein A affinity purification was carried out in the presence of the inhibitor nocodazole, which decreases stability of microtubules in vivo and in vitro (Bharti et al. 2011; Vasquez et al. 1997). This treatment drastically reduced the amount of tubulin and microtubule-associated proteins (Bharti et al. 2011). In addition, numerous organellar (mitochondria, ER) proteins, which were found in solubilisates of untreated cells, were not detected in the complexes from the nocodazole-treated cells, suggesting that many unspecific proteins identified in the PEX14 affinity eluate represent vesicular and organellar cargos of the microtubular network. The complete list of PEX14-associated proteins co-purified using nocodazole-treated cells is shown in Table 1. PEX5 and PEX19, the known binding partners of PEX14, were detected in the complex. However, other peroxins, especially those which are supposed to bind indirectly to PEX14, were not detected, suggesting some unknown effects of the inhibitor on PEX14 assemblies.

Most of the PEX14-interacting proteins with highest abundance are cytosolic proteins belonging to the super families of chaperones and other folding assists, especially members of the HSP70 and HSP90 class. Indeed, a role for HSP70 in PEX5 targeting to the peroxisomal membrane had been suggested previously (Harano et al. 2001). Even earlier, Walton et al. showed that microinjection of anti HSP70 antibodies into human fibroblasts impaired peroxisomal protein import (Walton et al. 1994). In this study, HSP70 molecules were localized to the outer surface of peroxisomal membranes. Another cytosolic binding partner of interest is the de-ubiquitinating hydrolase USP9X which is by far the most active cytosolic de-ubiquitinase acting on an artificial Ub-PEX5 fusion construct (Grou et al. 2012). This enzyme, which was not denoted by Bharti et al. (2011), was identified from eluate fractions of all four affinity purifications with or without nocodazole. This suggests that the process of de-ubiquitanation takes also place at the peroxisomal membrane, maybe at a state of the receptor cycle where PEX5 is still attached with PEX14.

3.2 PEX16 Interactome Defined by BioID

The group by Peter Kim used the advanced pull-down technology BioID (Roux et al. 2012) to identify proteins which are in close vicinity or potentially interact with PEX16 (Hua et al. 2017). In brief, PEX16 was fused to the prokaryotic biotin

Table 1 PEX14 interaction partners identified by AP-MS following inhibition of the PEX14-tubulin interaction using nocodazole

Gene symbol	UniProt ID	Protein	M_W (kDa)	Sequ. cov. (%)	Mascot score	Enr. factor	SC PEX14
PEX14	O75381	Peroxisomal membrane protein PEX14 (isoform 2)	36.7	50.6	1644.4	33.3	200
HSPA1A; HSPA1B	P0DMV8; P0DMV9	Heat shock 70 kDa protein 1A; Heat shock 70 kDa protein 1B	70.1	41.5	2225.9	3.4	103
HSPA8	P11142	Heat shock cognate 71 kDa protein (isoform 1)	70.9	36.8	2462.1	6.8	81
HSP90AA1	P07900	Heat shock 90 kDa protein 1, alpha (isoform 2)	98.2	26.2	1567.0	2.8	73
PEX5	P50542	Peroxisomal targeting signal 1 receptor (isoform 3)	69.8	31.4	1576,2	13.2	66
HSP90AB1	P08238	Heat shock protein HSP 90-beta	83.3	24.2	1156.3	2.6	58
CAD	P27708	Putative uncharacterized protein CAD	243.0	12.2	883.4	spec.	43
HSPA2	P54652	Heat shock-related 70 kDa protein 2	70.0	20.5	703.3	4.4	40
n/a	B4DJ30	cDNA FLJ61290, highly similar to Neutral alpha-glucosidase AB	112.9	18.2	855.8	32.0	32
RUVBL1	Q9Y265	RuvB-like 1	50.2	25.4	817.1	spec.	29
HSPA5	P11021	HSPA5 protein	72.3	24.1	786.8	4.5	27
DAW1	Q8N136	Dynein assembly factor with WDR repeat domains 1	45.8	9.9	85.3	2.2	24
DYNC1H1	Q14204	Cytoplasmic dynein 1 heavy chain 1	532.4	3.7	832.0	21.0	21
PRKCSH	P14314	Glucosidase 2 subunit beta	59.4	19.6	670.2	spec.	21
CENPJ	Q9HC77	Centromere protein J	153.0	0.9	587.4	2.1	21
RUVBL2	Q9Y230	RuvB-like 2	51.1	28.7	401.7	spec.	17
CCT2	P78371	T-complex protein 1 subunit beta	57.5	22.6	320.5	14.0	14
FASN	P49327	Fatty acid synthase	273.4	5.7	361.0	6.5	13
PEX19	P40855	Peroxisomal biogenesis factor 19	32.8	25.1	250.8	4.0	12
n/a	B7Z809	cDNA FLJ56016, highly similar to C-1-tetrahydrofolate synthase	110.5	11	231.1	5.0	10
TTC28	Q96AY4	Tetratricopeptide repeat protein 28	270.9	3.4	217.3	spec.	8

(continued)

Table 1 (continued)

Gene symbol	UniProt ID	Protein	M_W (kDa)	Sequ. cov. (%)	Mascot score	Enr. factor	SC PEX14
GNPAT	O15228	Dihydroxyacetone phosphate acyltransferase	77.2	8.2	200.5	spec.	8
PHGDH	O43175	D-3-Phosphoglycerate dehydrogenase	56.7	10.8	196.0	spec.	8
RPS27A; UBC; UBB	P62979 P0CG48 P0CG47	Ubiquitin-40S ribosomal protein S27a; Polyubiquitin-C; Polyubiquitin-B	9.1 (Ubiquitin)	35.4	143.8	2,7	8
PFKP	Q01813	ATP-dependent 6-phosphofructokinase, platelet type	85.6	8.5	247.2	spec.	7
PGK1	P00558	Phosphoglycerate kinase 1	44.6	12.5	181.3	3.0	6
RPL12	P30050	60S ribosomal protein L12 (isoform 1)	17.8	23.6	127.6	3.0	6
RPS13	P62277	40S ribosomal protein S13	17.2	33.8	253.9	5.0	5
ERP44	Q9BS26	Endoplasmic reticulum resident protein 44	47.0	8.6	172.0	spec.	5
GCN1	Q92616	eIF-2-alpha kinase activator GCN1	292.8	1.8	169.9	spec.	5
PCBP1	Q15365	Poly(rC)-binding protein 1	37.5	7.3	153.9	2.5	5
PHB2	Q99623	Prohibitin-2	33.3	12.7	64.4	4.0	4
RPL5	P46777	60S ribosomal protein L5	34.4	11.4	1671.9	spec.	4
LYZ	P61626	Lysozyme C	16.5	14.2	1145.4	spec.	4
PKM2	P14618	Pyruvate kinase	57.9	9.8	53.7	4.0	4
BAG6	P46379	Large proline-rich protein BAG6	119.4	6.2	615.8	spec.	4
RPS18	P62269	40S ribosomal protein S18	17.8	19.1	632.7	3.0	3
RCN2	Q14257	Reticulocalbin-2	36.9	7.3	275.4	spec.	3
UBL4A	P11441	Ubiquitin-like protein 4A	17.8	16.6	119.2	spec.	3
VPS52	Q8N1B4	Vacuolar protein sorting-associated protein 52 homolog	82.2	4.0	232.5	spec.	3
USP9X	Q93008	ubiquitin specific protease 9, X-linked (isoform 2	290.5	0.5	197.3	spec.	3
RPL11	P62913	60S ribosomal protein L11 [Ribosomal protein L11]	20.3	8.6	159.4	spec.	3
UACA	Q9BZF9	Uveal autoantigen with coiled-coil domains and ankyrin repeats	162.5	1.1	74.7	spec.	3

Abbreviations: ID Identification; *Mw* Molecular weight; *Sequ. Cov.* Sequence coverage; *Enr. factor* Enrichment factor; *SC* Spectra counts; *spec.* NOT identified in the Control pull-down

protein ligase BirA*. The fusion protein was stably expressed in Flp-In™ T-REx™ cells, which were grown in biotin-supplemented medium. Membranes were collected by sedimentation and solubilized with 1% Triton X-100. Subsequently, all biotinylated proteins were affinity-purified using streptavidin Sepharose beads and identified by MS. As demonstrated by Kim and co-workers, BioID allows the detection of PPIs between different membranes. In this case, they identified ER-located VAPA and peroxisomal ACBD5 as main constituents of the ER-peroxisome contact zone.

The validity of this method to detect proximal or interacting proteins was demonstrated by the identification of other known peroxisomal membrane proteins, like the half-transporter PMP70 and the putative channel PMP52, signal-transducing MAVS, the proliferation factors MFF, DNML1 and PEX11ß. The best hits, as based on the number of spectral counts (i.e. the number of MS/MS fragmentation spectra assigned to a given protein, which is used as a measure for protein abundance in a sample) derived from MS analysis were known peroxisomal binding partners of PEX16, in particular PEX19 and PEX3. Surprisingly, PEX14, PEX5 and PEX13 were identified as closely attached proteins with similar numbers of spectral counts as shown for PEX19 and PEX3. This is in agreement with the results obtained by PEX14-Protein A affinity purification, suggesting that a close association between the import machineries for matrix proteins and membrane proteins exists (Fig. 2). A direct interaction between PEX19 and PEX14 has been shown previously (Neufeld et al. 2009; Fransen et al. 2004; Sacksteder et al. 2000). Noteworthy, the PEX14 binding site of PEX19 is not overlapping with the PEX3- and the general PMP binding domain (Fransen et al. 2005). For this reason, it has been speculated that the PEX14 association with PEX16 and PEX3 is bridged by PEX19 in transit (Bharti et al. 2011). However, the failure to detect other highly abundant PMPs, like PEX11, that binds with higher affinity than PEX16 to the same binding region of PEX19 (Schueller et al. 2010), argues against this hypothesis. Although the functional meaning for the association of PEX16/PEX19/PEX3 complex with PEX14/PEX5 at the peroxisomal membrane is not clear, it should be noted that a specific role for PEX19 in assembly of the PTS receptor complex has been suggested previously (Fransen et al. 2004).

The BioID approach also proved that the receptor dislocation complex consisting of the AAA^+ peroxins PEX1 and PEX6, and their membrane anchor PEX26 are in close proximity to the membrane and matrix protein import machineries. Assuming that the number of spectral counts correlates with true distances, quantification could provide some insight into the structural organization of subcomplexes. Using PEX16 as bait (Hua et al. 2017), PEX1 was detected with the highest number of peptide counts. PEX6 was also detected, but with lower yield, while PEX26, the membrane anchor of AAA^+-peroxins, could not be identified at all. The dominant abundance of PEX1 correlates with findings of the PEX14 complex isolation and might reflect a direct binding of ubiquitinated PEX5 with PEX1. Indeed, a physical interaction between a homo-oligomer of PEX1 and PEX5 was reported (Tamura et al. 2014). Based on structural similarities, Shiozawa et al. reports on a putative ubiquitin-binding domain at the N-terminal region of PEX1

(Shiozawa et al. 2004). However, non-detection of PEX26 in the pull-down approaches is difficult to reconcile with another finding by Tamura et al. (2014). In their study, PEX26 directly binds PEX14 (CHO and HEK cells, 1% Triton-X) and this interaction is modulated by the AAA^+ peroxins and also by PEX5. Therefore, it seems possible that the PEX26/PEX14 interaction reflects an initial interaction, which is lost at later steady-states of protein import.

3.3 Comparing the Peroxin Network of Yeast and Man

The interactome of Protein A-tagged Pex14p has been studied in the yeast *Saccharomyces cerevisiae* by affinity purification of digitonin-solubilized membrane complexes. SILAC combined with high-resolution MS led to the identification of 9 core components and 12 additional transient binding partners (Oeljeklaus et al. 2012). The results obtained resemble those of the human PEX14 affinity approach in the way that PEX14 and PEX13 together with the RING-finger peroxins are forming a core complex of the importomer. In addition, Pex5p, which is forming a proteinaceous pore together with Pex14p (Meinecke et al. 2010), could be identified in the yeast Pex14p-interactome, albeit as a transient binding partner.

The most striking differences when comparing yeast and human PEX14 interactome analyses concern the lack of distinct peroxins. While a PEX16 homolog has not yet been identified in bakers' yeast, homologs of Pex8p and Pex17p which are both constituents of the core complex in yeast do not seem to exist in mammalian cells. While the role of Pex17p is not clearly defined, Pex8p has been shown to connect the receptor docking complex to the RING-finger complex (Agne et al. 2003) in yeast cells. It is tempting to speculate that analogous proteins, maybe human peroxins themselves, perform this function in human cells.

Another remarkable difference concerns the association of AAA^+ peroxins with the translocation apparatus. In yeast cells, the AAA^+-complex anchoring Pex15p was detected as transient binding partner of Pex14p, whereas both AAA proteins, Pex1p and Pex6p, were not stably associated with the protein import machinery. In contrast, in human cells an association between the AAA^+ peroxin PEX1 and PEX5 was reported. As discussed above, this species-dependent differences could reflect different binding affinities and dynamics of association of the receptor dislocation machinery.

One remarkable feature of the human peroxin interactome is the association of the PEX5/PEX14 complex with the PEX3/PEX16/PEX19 network, which is required for membrane biogenesis (Fig. 2). In contrast, neither Pex19p nor its associated binding partner Pex3p are components of the yeast Pex14p-complex, not even as transient interactors. As discussed above, in human cells PEX19 is a suitable candidate to bridge these two assemblies. Interestingly, the high-affinity PEX14-binding motif of human PEX19, which competes with the PEX14 binding

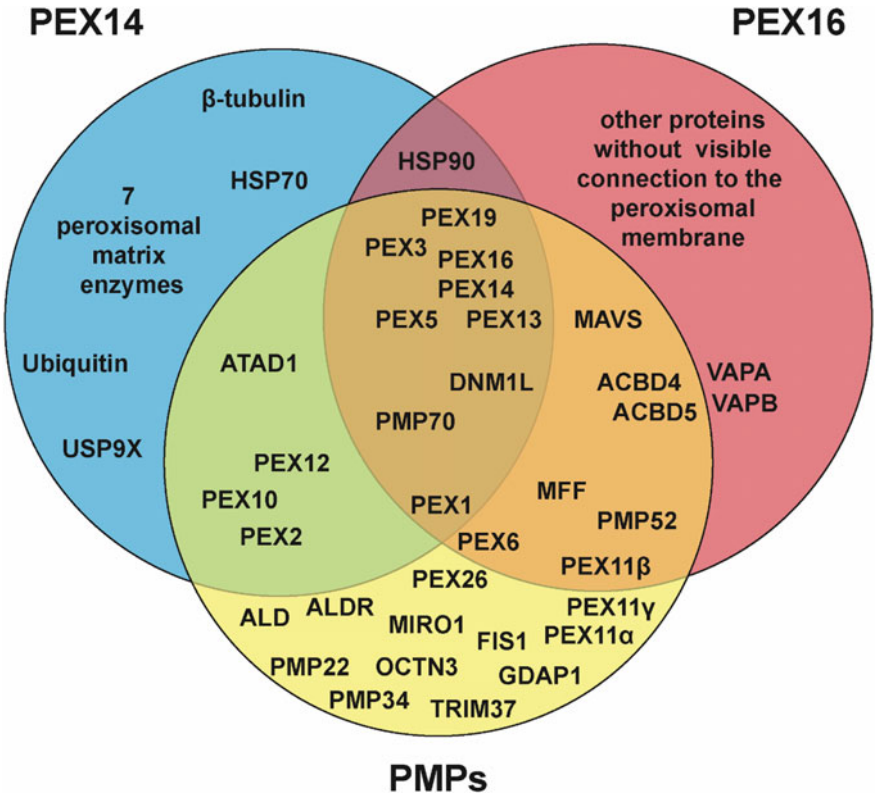

Fig. 2 Overlap of proteins copurifying with PEX14-Protein A, BirA*-PEX16 and the peroxisomal membrane proteome. Constituents of PEX14 and PEX16 complexes were compared to the human peroxisomal membrane proteome and displayed as Venn diagram. Bharti et al. used isolated membranes for affinity purification of digitonin-solubilized PEX14- Protein A complexes and mass spectrometry to identify PEX14 interaction partners (Bharti et al. 2011). A different approach called BioID was used by Hua et al. to target transient and stable interaction partners of PEX16 as well as proximal proteins (Hua et al. 2017). Abbreviations (only those not defined in Fig. 1): heat shock protein (HSP), x-linked ubiquitin specific protease 9 (USP9X), vesicle-associated membrane protein-associated protein (VAP)

sites of PEX5, is not conserved in yeast (Neufeld et al. 2009). Although highly speculative, the close association of complexes facilitating matrix protein import and insertion of membrane proteins might be correlated with an increased efficacy in biogenesis of peroxisomes.

4 Outlook

Studies of the peroxisomal interactome using pull-down approaches is a new field in molecular and cell biology of human cells. The fusion of bait proteins with either pull-down tags (Protein A) or catalytic domains allowing in vivo labeling of proximal proteins (BioID) depends on genomic engineering of human cells and methods which were not well established in the past. Novel technologies like the Flp-In™ technique allowed stable expression of bait proteins in cultured cells. However, several limitations of this method are due to the expression of additional non-tagged copies of the bait proteins under control of their own promotors which could compete for interactions. These problems can be avoided in future research using the CRISPR/Cas9 technique to either knock-out endogenous copies of the gene of interest or, even better, to manipulate target genes at authentic alleles. Use of CRISPR/Cas9 technology will also allow to distinguish between indirect and physical interaction of two proteins. To this end, a potentially bridging protein could be deleted, resulting in a different set of identified proteins. If this can be carried out in a systematic way, organization of protein-protein networks could be analyzed. Multiple PPIs are strongly regulated by post-translational modifications like phosphorylation, ubiquitylation or proteolytic processing, Thereby, the growing number of available specific compounds, like kinase or proteasome inhibitors, will help to analyze the peroxisomal interactome in even higher spatio-temporal resolution.

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Identification of Peroxisomal Protein Complexes with PTS Receptors, Pex5 and Pex7, in Mammalian Cells

Kanji Okumoto, Non Miyata and Yukio Fujiki

Abstract Pex5 and Pex7 are cytosolic receptors for peroxisome targeting signal type-1 (PTS1) and type-2 (PTS2), respectively, and play a pivotal role in import of peroxisomal matrix proteins. Recent advance in mass spectrometry analysis has facilitated comprehensive analysis of protein-protein interaction network by a combination with immunoprecipitation or biochemical purification. In this chapter, we introduce several findings obtained by these methods applied to mammalian cells. Exploring Pex5-binding partners in mammalian cells revealed core components comprising the import machinery complex of matrix proteins and a number of PTS1-type cargo proteins. Biochemical purification of the Pex5-export stimulating factor from rat liver cytosol fraction identified Awp1, providing further insight into molecular mechanisms of the export step of mono-ubiquitinated Pex5. Identification of DDB1 (damage-specific DNA-binding protein 1), a component of CRL4 (Cullin4A-RING ubiquitin ligase) E3 complex, as a Pex7-interacting protein revealed that quality control of Pex7 by CRL4A is important for PTS2 protein import by preventing the accumulation of dysfunctional Pex7. Furthermore, analysis of binding partners of an intraperoxisomal processing enzyme, trypsin-domain containing 1 (Tysnd1), showed a protein network regulating peroxisomal fatty acid β-oxidation.

K. Okumoto · N. Miyata
Department of Biology, Faculty of Sciences, Kyushu University, Nishi-ku, Fukuoka 819-0395, Japan

K. Okumoto
Graduate School of Systems Life Sciences, Kyushu University, Nishi-ku, Fukuoka 819-0395, Japan

Y. Fujiki (✉)
Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan
e-mail: yfujiki@kyudai.jp

Present Address
N. Miyata
Department of Chemistry, Faculty of Sciences, Kyushu University, Nishi-ku, Fukuoka 819-0395, Japan

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1 Introduction

Peroxisome is an essential intracellular organelle functioning in many important metabolic pathways. In mammals, peroxisomal β-oxidation is required for chain-shortening of very long chain fatty acids that cannot be dealt with mitochondrial β-oxidation. Mammalian peroxisomes also play an essential role in synthesis of plasmalogens, docosahexaenoic acids, and bile acids as well as in degradation of D-amino acids and polyamine (Wanders 2014). Physiological consequence of peroxisomes are highlighted by fatal human severe, genetic disorders named peroxisome biogenesis disorders (PBDs), represented by Zellweger spectrum disorders (Fujiki et al. 2014; Waterham et al. 2016). So far, about 40 *PEX* genes including over 30 in several yeast species, 16 in a plant *Arabidopsis thaliana*, and 14 in mammals are identified. Their gene products, named peroxins, are essential for peroxisome biogenesis including membrane assembly, matrix protein import, division, and inheritance (Liu et al. 2012; Hasan et al. 2013; Fujiki et al. 2014; Hayashi and Nishimura 2006; Cross et al. 2016; Reumann and Bartel 2016) Isolation of the causal genes of human PBDs contributes to better understanding of the molecular mechanisms underlying pathogenesis of PBD (Fujiki et al. 2014; Waterham et al. 2016).

Peroxisomal functions rely on the strictly and spatiotemporally regulated compartmentalization of the enzymes catalyzing respective reactions. Both peroxisomal membrane proteins (PMPs) and matrix proteins are synthesized on cytoplasmic free ribosomes and posttranslationally translocated into peroxisomes. Two types of topogenic peroxisome-targeting signals (PTSs) has been identified in peroxisomal matrix proteins; PTS1 is a C-terminal tripeptide of the sequence SKL and its derivatives (Gould et al. 1989; Miura et al. 1992) found in the majority of matrix proteins and PTS2 is an N-terminal cleavable nonapeptide presequence (Osumi et al. 1991; Swinkels et al. 1991).

Pex5 and Pex7 function as the specific cytosolic receptors for PTS1 and PTS2, respectively. After recognizing newly synthesized PTS1 proteins in the cytosol, Pex5 targets the PTS1 proteins to peroxisomal membrane by docking onto a PMP, Pex14. Translocation of PTS1 proteins across peroxisomal membrane is mediated by coordinated action of Pex5 with "import machinery" in the peroxisomal membrane, including a docking complex comprising Pex14 and Pex13, and a translocation complex of RING peroxins Pex2, Pex10, and Pex12. Pex5 in the import machinery complex is then mono-ubiquitinated at a conserved cysteine residue in the N-terminal region in a manner dependent on RING peroxin complex and is exported to the cytosol by AAA peroxins Pex1 and Pex6 and their recruiter Pex26. Therefore, Pex5 functions as a shuttling receptor between the cytosol and peroxisomes [reviewed in (Liu et al. 2012; Hasan et al. 2013; Fujiki et al. 2014)].

In mammals, two isoforms of Pex5, Pex5S and Pex5L with a 37-aa insertion form homo- and hetero-oligomers, mainly a tetramer (Braverman et al. 1998; Otera et al. 1998). Although either Pex5S or Pex5L is sufficient for PTS1 protein import, Pex5L, not Pex5S, specifically binds Pex7 via the insertion and its proximal region of Pex5L (Otera et al. 2000; Matsumura et al. 2000), thus translocates the PTS2-Pex7 complex into peroxisomes (Otera et al. 2002; Mukai and Fujiki 2006). Despite some variations, the mechanisms underlying the peroxisomal import of both matrix proteins and PMPs are basically the same between species including yeast, plants, and mammals (Liu et al. 2012; Hasan et al. 2013; Fujiki et al. 2014; Hayashi and Nishimura 2006; Cross et al. 2016; Reumann and Bartel 2016).

Since identification of a series of *PEX* genes, recent advances in our understanding of peroxisome biogenesis has been largely taken by characterization of individual peroxins. Furthermore, powerful and efficient methods are now available for analysing a wide range network of protein-protein interactions. In this chapter, we introduce several peroxisomal protein complexes involved in peroxisomal matrix protein import in mammalian cells.

2 Approaches to Identifying Peroxisomal Protein Import Complexes in Mammalian Cells

As compared to studies using a yeast system that has great advantage of genetic approach, investigation with use of mammalian cells needs other strategies in search for a protein-protein interaction network. Recent improvement in mass spectrometry techniques enables us to identify a larger number of proteins from a subtle amount of samples. Isolating protein of interest by immunoprecipitation with higher specificity becomes a useful and efficient method for comprehensive analysis of protein-protein interaction (Fig. 1). We also applied a combination of biochemical fractionation and purification to identify any factors that accelerate the export of Pex5 from peroxisomes to the cytosol. In all cases, higher specificity and/or purity and with significant difference from the control is critical to demonstrate efficient and convincing results.

3 Identification of Peroxisomal Protein Complexes by Immunoprecipitation

3.1 Complexes with PTS1 Receptor Pex5

To gain mechanistic insight into peroxisomal matrix protein import, a HEK293 cell line stably expressing FLAG-tagged Pex5L (FL-Pex5L) was established and subjected to immunoprecipitation with anti-FLAG IgG-agarose (Okumoto et al. 2011a)

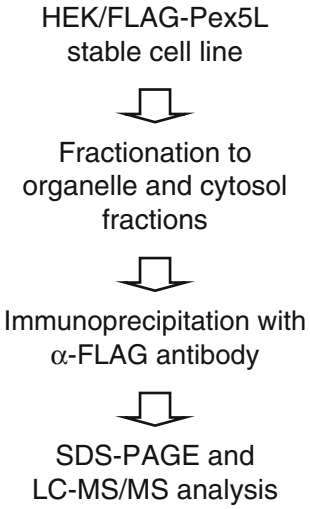

Fig. 1 Outline of identification of Pex5L-binding proteins. HEK293 cell line stably expressing FLAG-Pex5L (HEK/FLAG-Pex5L) was fractionated by centrifugation to organelle and cytosol fractions. FLAG-Pex5L was immunoprecipitated from respective fractions with anti-FLAG antibody. Immunoprecipitates of FLAG-Pex5L were separated by SDS-PAGE and all of the discernible bands were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis (Okumoto et al. 2011a)

(Fig. 1). FL-Pex5L was immunoprecipitated from separate cytosol and organelle fractions, which mainly and partially contains FL-Pex5pL, respectively, as in the case of endogenous Pex5p in HEK293 cells. Peptides by trypsin digestion resulting from all of the co-immunoprecipitated discernible bands in respective fractions were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) measurement. Totally 19 PTS1-containing proteins were identified, including nine enzyme proteins involved in peroxisomal fatty acid β-oxidation such as acyl-CoA oxidase 1 (AOx) and peroxisomal D- and L-bifunctional proteins (DBP and LBP, respectively) (Okumoto et al. 2011a) (Fig. 2). Two serine protease domain-containing proteins, trypsin domain containing 1 (Tysnd1) and peroxisomal Lon protease (PsLon) are included in Pex5-interacting proteins harboring PTS1 (Fig. 2). Furthermore, five membrane peroxins, including Pex14, Pex13, three RING peroxins Pex2, Pex10, and Pex12, and soluble PTS2 receptor Pex7, are also identified (Fig. 2). This is in good agreement with earlier reports that Pex5 interacts with the import complex during matrix protein import (Otera et al. 1998; Mukai and Fujiki 2006). Interestingly, Pex1, Pex6, and Pex26 are not detectable in the Pex5-interactors, suggesting that the ternary complex involving Pex5 export from the peroxisomal complex interacts with Pex5, but weakly and/or transiently.

Peroxisomal thiolase, a PTS2 protein, catalyzing the last step of peroxisomal fatty acid β-oxidation, is not recovered in the immunoprecipitate of Pex5, despite the fact that Pex5L interacts with PTS2 receptor Pex7. This implies that affinity of

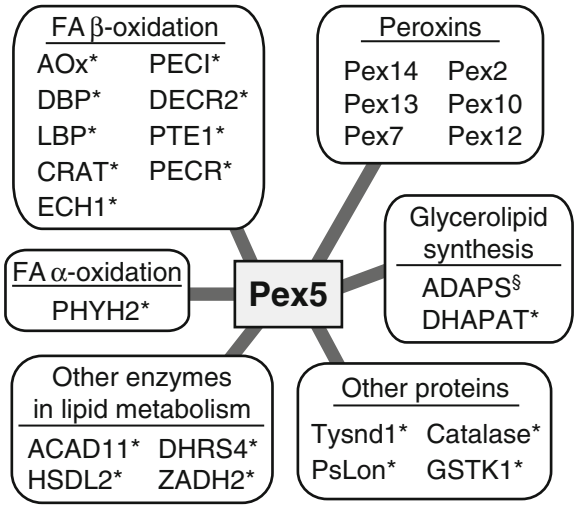

Fig. 2 Identified proteins bound to FLAG-Pex5L. Proteins interacting with Pex5L are searched as in Fig. 1 (Okumoto et al. 2011a). * and § show proteins harboring PTS1 and PTS2, respectively. Abbreviations: ACAD11, acyl-CoA dehydrogenase family, member 11; ADAPS; alkyldihydroxyacetonephospate synthase; AOx, acyl-CoA oxidase; CRAT, carnitine O-acetyltransferase; DBP, D-bifunctional protein; DECR2, 2,4-dienoyl CoA reductase 2; DHAPAT, dihydroxyacetonephosphate acyltransferase; DHRS4, dehydrogenase/reductase SDR (short-chain dehydrogenase/reductase) family member 4; ECH1, enoyl-CoA hydratase 1; FA, fatty acid; GSTK1, glutathione S-transferase kappa 1; HSDL2, hydroxysteroid dehydrogenase like 2; LBP, L-bifunctional protein; PECI, peroxisomal 3,2-trans-enoyl-CoA isomerase; PECR, peroxisomal trans 2-enoyl CoA reductase; PHYH2, phytanoyl-CoA hydroxylase 2; PsLon, peroxisomal Lon protease; PTE1, peroxisomal acyl-CoA thioester hydrolase 1; Tysnd1, trypsin-domain containing 1; ZADH2, zinc binding alcohol dehydrogenase domain containing 2

Pex7 to PTS2 proteins even in the presence of Pex5L is much lower than that of Pex5L to PTS1 proteins. Only alkyldihydroxyacetonephospate synthase (ADAPS), a PTS2-containing enzyme catalyzing the second step in plasmalogen synthesis (Honsho and Fujiki 2017), appears to be exceptionally identified as a Pex5-binding protein (Fig. 2), presumably due to co-purification with a PTS1 protein, the first step enzyme dihydroxyacetonephosphate acyltransferase (DHAPAT) that forms stable enzyme complex with ADAPS (Thai et al. 1997; Biermann et al. 1999).

The search for Pex5-interacting proteins clearly provides a list of the stable binding-partners of Pex5 in mammalian cells. This strategy would be also applicable to studies on the function of Pex5 and other peroxisomal proteins in more specialized or differentiated cells such as hepatocyte and neuronal cells.

3.2 Complexes with PTS2 Receptor Pex7

The PTS2 receptor Pex7 is a hydrophilic soluble protein that contains seven WD40 motif repeats (Braverman et al. 1997; Mukai et al. 2002). Molecular mechanisms of Pex7-Pex5L-mediated PTS2 protein import are elucidated (Braverman et al. 1997; Lazarow 2006; Mukai and Fujiki 2006; Kunze and Berger 2015). However, little is known about the mechanisms by which the quality and function of Pex7 are controlled. We for the first time found that Pex7 is rapidly degraded by an ubiquitin-proteasome system within several hours in mammalian cells, implicative of an ubiquitin ligase for Pex7 (Miyauchi-Nanri et al. 2014). To identify a potential ubiquitin ligase involved in the degradation of Pex7, stable cell line expressing FLAG-tagged Pex7 (FL-Pex7) in a *PEX7*-defective CHO mutant ZPG207 was established and subjected to immunoprecipitation with anti-FLAG antibody. Mass spectrometry analysis of the immunoprecipitates of FL-Pex7 identified DDB1 (damage-specific DNA-binding protein 1) as a potential Pex7-interacting protein, in addition to Pex5L and Pex14, both previously reported to be Pex7-associated proteins (Mukai and Fujiki 2006). Earlier studies showed that DDB1 serves as a substrate adaptor recruiting its substrates into the CRL4 (Cullin4A-RING ubiquitin ligase) E3 complex comprised of Cul4A, DDB1 and a RING finger protein Rbx1 and facilitating CRL4-mediated ubiquitination (Jiang et al. 2011; Nakagawa and Xiong 2011). Further biochemical analyzes confirmed that Pex7 interacts with CRL4 E3 complex via direct binding to DDB1. Knockdown of DDB1 and an inhibitor of Nedd8 modification that inactivates CRL4 increase the half-life of Pex7, showing that CRL4A is a major E3 ligase regulating Pex7 stability.

We also found that both DDB1 depletion and inactivation of CRL4A impair PTS2 protein import (Miyauchi-Nanri et al. 2014). Furthermore, dysfunctional Pex7-G212R, the orthologue of *PEX7* harboring a mutation frequently identified in patients with RCDP (rhizomelic chondrodysplasia punctata) (Braverman et al. 1997; Motley et al. 1997; Purdue et al. 1997), is more readily degraded by CRL4A complex. This result shows that dysfunctional Pex7 is removed by the CRL4A system to maintain normal PTS2 protein import. Therefore, CRL4A functions as an E3 ligase involved in the quality control of Pex7 to prevent the accumulation of dysfunctional Pex7 (Fig. 3). Our results define the mechanism underlying Pex7 homoeostasis and highlight its importance for regulating the PTS2 import.

3.3 Substrates of Intraperoxisomal Processing Enzyme, Tysnd1

Fatty acid β-oxidation is one of the essential functions of peroxisomes from yeast to humans (Fujiki et al. 2012; Wanders 2014). In mammals, peroxisomal β-oxidation is required for chain-shortening of very long fatty acids ($>C_{22}$) that cannot be catalyzed by β-oxidation system in mitochondria. Tysnd1 is a typical PTS1-type

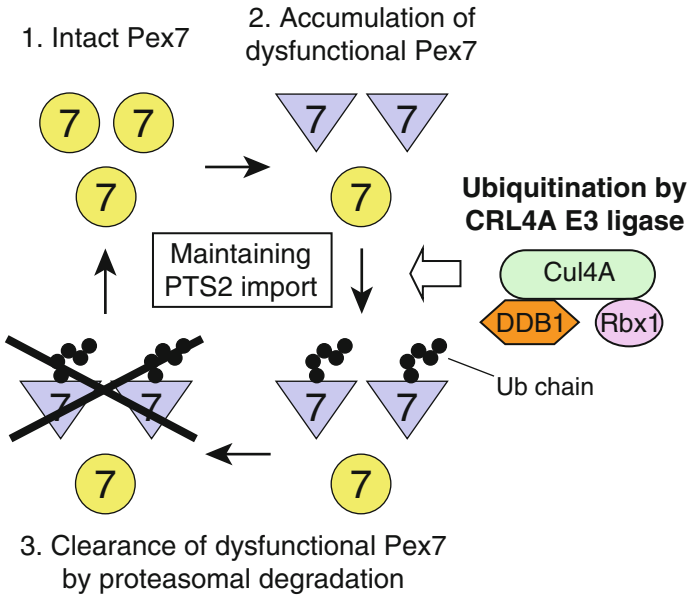

Fig. 3 A schematic model of the CRL4A-mediated quality control of Pex7 in maintaining PTS2 protein import. Pex7 intrinsically deteriorates with time, resulting in accumulation of dysfunctional Pex7 and impairment of PTS2 import. Dysfunctional Pex7 is rapidly ubiquitinated by the CRL4A E3 ligase complex and then degraded by the proteasomes, thereby leading to maintaining efficient PTS2 import by intact Pex7p. The number seven in circles and triangles indicates intact and dysfunctional Pex7, respectively. Ub, ubiquitin

peroxisomal matrix protein with a serine protease-like domain, which was originally identified by in silico search as one of the candidates of PTS1-containing peroxisomal proteins (Kurochkin et al. 2005). Tysnd1 is shown to be responsible for processing of several PTS1-containing proteins involved in the peroxisomal fatty acids β-oxidation pathway, including AOx, DBP, and sterol carrier protein x (SCPx) (Kurochkin et al. 2007; Okumoto et al. 2011a). Tysnd1 also cleaves off N-terminal PTS2 presequence from the precursor of thiolase (Kurochkin et al. 2007; Okumoto et al. 2011a).

To further investigate the substrates and the function of Tysnd1 in peroxisomes, FLAG-tagged Tysnd1 (FL-Tysnd1) is immunoprecipitated from cells, followed by mass spectrometry analysis (Okumoto et al. 2011a). To efficiently identify Tysnd1-interacting proteins, organelle fraction from HEK293 cells stably expressing FL-Tysnd1-SA, the protease-inactive mutant with a Ser^{481}-to-Ala substitution in the active site, is subjected to immunoprecipitation using anti-FLAG antibody. Several proteins are specifically identified in the co-immunoprecipitates with FL-Tysnd1-SA, including PTS1 proteins AOx and DBP that are substrates of Tysnd1 (Kurochkin et al. 2007; Okumoto et al. 2011a). Furthermore, nonspecific lipid transfer protein (ns-LTP, also called sterol carrier protein 2) and peroxisomal

trans-enoyl-CoA isomerase (peroxisomal Δ^3, Δ^2-enoyl-CoA isomerase, PECI) are also identified (Okumoto et al. 2011a). ns-LTP is a cleavable PTS2-containing protein (Wirtz 1997; Otera et al. 2001), thus presumably isolated as a substrate of Tysnd1. PECI is a PTS1-type peroxisomal enzyme that is essential for the peroxisomal β-oxidation of unsaturated fatty acids (Geisbrecht et al. 1999). No processing of PECI is observed (Geisbrecht et al. 1999), suggesting that PECI might associates with a putative protein complex consisting of peroxisomal β-oxidation enzymes as suggested (Wouters et al. 1998) for efficient catalytic reaction.

PsLon is also identified in the co-immunoprecipitates of FL-Tysnd1-SA (Okumoto et al. 2011a). Biochemical analyzes reveal that protease activity of Tysnd1 is inactivated by intermolecular one-cleavage self-conversion of the full-length form into its N- and C-terminal parts, which are then readily degraded by PsLon. Moreover, peroxisomal β-oxidation of very long fatty acids is significantly and partially decreased by knockdown of either Tysnd1 or PsLon (Okumoto et al. 2011a). Collectively, these findings demonstrate a molecular network in peroxisomal matrix, where Tysnd1 behaves as a key regulator of the peroxisomal β-oxidation pathway via proteolytic processing of β-oxidation enzymes.

4 Biochemical Purification of Awp1

To elucidate molecular mechanism underlying peroxisomal protein import, an in vitro system for shuttling reaction of the PTS1 receptor, Pex5, by mainly using post nuclear supernatant (PNS) fraction as a source of cytosol and peroxisomes has been used (Miyata and Fujiki 2005; Rodrigues et al. 2016). The assay system is robust and can reconstitute virtually entire steps of the shuttling of Pex5 between the cytosol and peroxisomes, including ATP-independent Pex5 import into peroxisomes, Cys-monoubiquitination of Pex5, and ATP- and Pex1/Pex6-dependent Pex5 export back to the cytosol. Miyata et al. found that heat-treated cytosol did not stimulate the export of Pex5 from peroxisomes, suggesting that a cytosolic factor(s) was involved in the Pex5 export (Miyata and Fujiki 2005).

By a combination of biochemical fractionation and purification of rat liver cytosol and in vitro Pex5 export assay, Awp1 is identified as a Pex5-export stimulating factor (Miyata et al. 2012). Purified recombinant Awp1 partially stimulates the Pex5 export in the absence of cytosol. Furthermore, antiserum raised against Awp1 strongly inhibits Pex5 export. These results confirm that Awp1 stimulates Pex5 export. Awp1 harbors two zinc-finger domains, an A20-type and an AN1 type. The A20-type zinc-finger domain is known to bind ubiquitin and play an important role in regulation of NF-κB (Chang et al. 2011; Enesa and Evans 2014). A20-type zinc-finger domain that also has an important role in Pex5 export, as assessed by a Cys to Ala mutation in the A20-type zinc-finger domain abolishes the in vitro Pex5 export-stimulating activity.

A highly conserved cysteine residue at N-terminus of Pex5 is mono-ubiquitinated by thioester bond (Okumoto et al. 2011b; Grou et al. 2012).

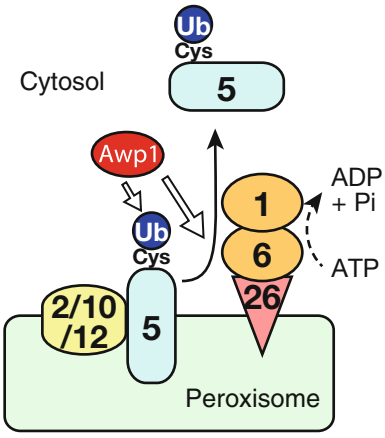

Fig. 4 A schematic view of the role of AWP1 in Pex5 export. At the terminal step of Pex5 shuttling for PTS1 protein import, Pex5 in peroxisomal membrane is mono-ubiquitinated at a conserved cysteine residue (Cys, Cys^{11} in mammals) by RING peroxins and exported to the cytosol in a manner dependent on ATP hydrolysis by Pex1–Pex6 AAA ATPases complex. AWP1 interacts with Pex6 and ubiquitinated Pex5 (open arrows), thereby stimulating export of ubiquitinated Pex5 in a concerted manner with the Pex1–Pex6 complex. The numbers designate individual peroxins. Ub, ubiquitin

Mono-ubiquitination of Pex5 is essential for the Pex5 export. Awp1 binds to Pex6 and mono-ubiquitinated Pex5. The Awp1 binding to Pex6 and mono-ubiquitinated Pex5 is also abolished by the Cys-to-Ala mutation in the A20-type zinc-finger domain. Thus, Awp1 more likely functions as a linking factor between Pex1/Pex6 complex and mono-ubiquitinated Pex5 to promote the Pex5 export (Fig. 4).

5 Concluding Remarks

Recent technical improvement of mass spectrometric analysis allows us to comprehensively investigate protein network in peroxisome biogenesis. Although studies described above were performed in normal condition, quantitative proteomic analyses such as SILAC (stable isotope labeling by amino acids in cell culture) method (Ong et al. 2002; Ong and Mann 2007) may also be useful to examine the response of peroxisomal protein network to the changes in intracellular conditions and extracellular environment. Furthermore, new procedures to identify protein-protein interaction such as BioID, a method for proximity-dependent labeling of proteins as in the identification of Pex16-binding proteins (Hua et al. 2017), could also facilitate understanding of protein network involved in peroxisome homeostasis.

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Unraveling of the Structure and Function of Peroxisomal Protein Import Machineries

Vishal C. Kalel and Ralf Erdmann

Abstract Peroxisomes are dynamic organelles of eukaryotic cells performing a wide range of functions including fatty acid oxidation, peroxide detoxification and ether-lipid synthesis in mammals. Peroxisomes lack their own DNA and therefore have to import proteins post-translationally. Peroxisomes can import folded, co-factor bound and even oligomeric proteins. The involvement of cycling receptors is a special feature of peroxisomal protein import. Complex machineries of peroxin (PEX) proteins mediate peroxisomal matrix and membrane protein import. Identification of PEX genes was dominated by forward genetic techniques in the early 90s. However, recent developments in proteomic techniques has revolutionized the detailed characterization of peroxisomal protein import. Here, we summarize the current knowledge on peroxisomal protein import with emphasis on the contribution of proteomic approaches to our understanding of the composition and function of the peroxisomal protein import machineries.

Keywords Peroxisome · Peroxin · Peroxisome targeting signal
Piggyback protein import · Cycling import receptors · Peroxisomal import pores

Abbreviations

AAA	ATPase Associated with diverse cellular Activities
DUBs	De-ubiquitinating enzymes
mPTS	membrane Peroxisome Targeting Signal
PBDs	Peroxisome Biogenesis Disorders
PEX	Peroxin

V. C. Kalel (✉) · R. Erdmann
Department of Systems Biochemistry, Faculty of Medicine,
Institute of Biochemistry and Pathobiochemistry,
Ruhr University Bochum, Universitätsstr. 150,
44801 Bochum, Germany
e-mail: vishal.kalel@rub.de

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PMPs Peroxisomal Membrane Proteins
PTS Peroxisome Targeting Signal
RING Really Interesting New Gene
SH3 Src Homology 3
TEV Tobacco Etch Virus protease
TPA TEV cleavage site—protein A
TPR Tetratrico-Peptide Repeat

1 Peroxisomal Protein Import

Peroxisomes are the ubiquitous organelles of eukaryotic cells, which compartmentalize diverse metabolic functions, including fatty acid oxidation and detoxification of reactive oxygen species. Peroxisomes are dynamic organelles, which can adapt their protein composition to the cellular needs. This feature of peroxisomes contributes to the capacity of cells to adapt to environmental changes. Peroxisome-like organelles are present in certain organisms and include Woronin bodies in filamentous fungi, glyoxysomes in plants and glycosomes in trypanosomatid parasites. Peroxisomes are single membrane bound organelles devoid of their own DNA, requiring that all peroxisomal proteins are encoded in the nucleus and that they have to be targeted to peroxisomes. Accordingly, peroxisomal matrix proteins are synthesized on free ribosomes in the cytosol and imported post-translationally. Hallmark features of peroxisomal protein import are the involvement of cycling receptors and the import of folded, co-factor bound even oligomeric proteins/complexes (Fig. 1).

Proteins involved in the biogenesis of peroxisomes are termed peroxins (PEX). In the early 90s, forward genetic screening of yeast mutants (Erdmann et al. 1989)

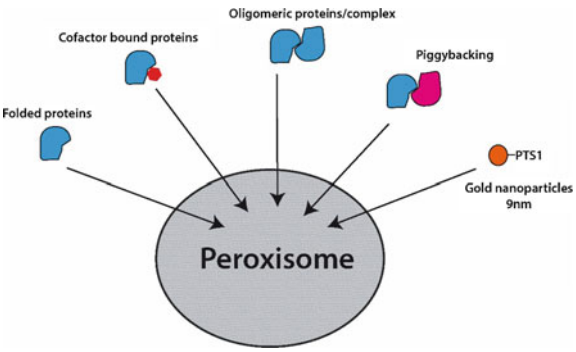

Fig. 1 Features of peroxisomal protein import.Peroxisomes can post-translationally import folded proteins, co-factor bound proteins, homo- or heteromeric protein complexes, even gold nanoparticles coated with peroxisome targeting signals. Some proteins lack a peroxisome targeting signal but can enter peroxisomes by piggybacking. These proteins hitchhike to peroxisomes by binding to PTS-containing proteins in the cytosol

or mutant mammalian cells (Tsukamoto et al. 1990) defective in peroxisome assembly was instrumental for the identification of majority of PEX genes. Later on, further PEX genes were originally identified by a complementary reverse genetic approach based on the isolation of peroxisomal membrane proteins and partial protein sequencing and the use of the sequence information for cloning of the corresponding genes by PCR using degenerated primers (Erdmann and Blobel 1995). This reverse genetic approach to study peroxisome biogenesis requires the isolation of purified peroxisomal membranes or PEX protein-complexes. The strategies for these are reviewed in this issue by Islinger et al. (Chap. 4), Klümper et al. (Chap. 11), and Okumoto et al. (Chap. 12). Genetic defects in PEX genes are responsible for several severe inborn disorders in humans, like the Zellweger syndrome. These patients usually die in early infancy. Glycosomes are peroxisome-like organelles of trypanosomatid parasites, which compartmentalize glycolysis inside the organelle. Glycosomes are essential for parasite survival and differences in peroxisome and glycosome biogenesis recently have been exploited as drug targets against trypanosomatid infections (Dawidowski et al. 2017).

So far, 36 peroxins have been identified in yeast, 14 in humans and 16 in plants. Of these, three peroxins are involved in membrane protein targeting, while majority play a role in targeting of peroxisomal matrix proteins (detailed in later sections) or other aspects of peroxisome maintenance (Table 1). Targeting of peroxisomal matrix proteins to the organelle depends on the presence of peroxisomal targeting signals (PTS), or piggybacking on proteins, which harbor PTS. Likewise, membrane proteins destined for peroxisomal membrane contain membrane peroxisome targeting signal (mPTS).

Peroxisomal protein targeting involves distinct steps; cargo recognition by the targeting receptor, docking of the cargo-receptor complex at the peroxisomal membrane, translocation of matrix proteins or insertion of membrane proteins and recycling of the receptor (Fig. 2). These steps involve the interaction of distinct sets of peroxins. Here, we review the current knowledge of peroxisomal protein import machineries, with emphasis on the proteomic approaches, which contributed to our understanding of their composition and function.

2 Peroxisomal Matrix Protein Import

2.1 Cargo Recognition

Proteins destined for import into peroxisomal matrix usually contain a type 1 (PTS1) or type 2 (PTS2) peroxisome targeting signal. The originally identified PTS1 is a conserved C-terminal tripeptide with the sequence SKL, first identified in Firefly luciferase (Gould et al. 1987). Sequence analysis of PTS1-containing proteins from different organisms and species led to a refined PTS1 consensus sequence [S/A/C][K/R/H][L/M] (Lametschwandtner et al. 1998). Additional

Table 1 Components of the peroxisomal protein import machinery

Function		Yeast	Human	Plant	Trypanosomatids
Peroxisomal matrix protein import					
Import receptors	PTS1 receptor	Pex5p Pex9p	PEX5S PXE5L	PEX5	PEX5
	PTS2 receptor	Pex7p	PEX7	PEX7	PEX7
	PTS2 co-receptor	Pex18p Pex20p Pex21p	PEX5L	PEX5	PEX5
Docking	Docking complex	Pex13p Pex14p Pex17p Pex33p	PEX13 PEX14	PEX13 PEX14	PEX13.1 PEX13.2 PEX14
	Importomer assembly	Pex8p Pex3p			
Receptor export	RING finger ligase complex (E3)	Pex2p Pex10p Pex12p	PEX2 PEX10 PEX12 TRIM37	PEX2 PEX10 PEX12 DSK2a/b	PEX2 PEX10 PEX12
	Ubiquitin conjugation (E2)	Pex4p Pex22p Ubc1/4/5	UbcH5a/b/c	PEX4 PEX22	PEX4 PEX22
	Deubiquitination	Ubp15p	USP9x		
	AAA dislocase	Pex1p Pex6p	PEX1 PEX6	PEX1 PEX6	PEX1 PEX6
	AAA dislocase anchor	Pex15p	PEX26	APEM9	
Peroxisomal membrane protein import					
Import receptor		Pex19p	PEX19	PEX19A/B	PEX19
Docking		Pex3p	PEX3	PEX3A/B	
Import complex		Pex36	PEX16	PEX16 (SSE1)	PEX16
Other functions					
Peroxisome proliferation		Pex11p, Pex25p, Pex27p	PEX11α, β, γ	PEX11a-e	PEX11 GIM5A GIM5B
Maintenance		Pex28p-32p, Pex34p, Pex35p			
Inheritance		Inp1p, Inp2p			

Peroxins (PEX) and accessory proteins involved in the peroxisomal matrix or membrane protein import and other functions such as proliferation and inheritance. The known peroxins from yeast, human, plant (*Arabidopsis*) and trypanosomatid parasites are grouped according to their molecular function

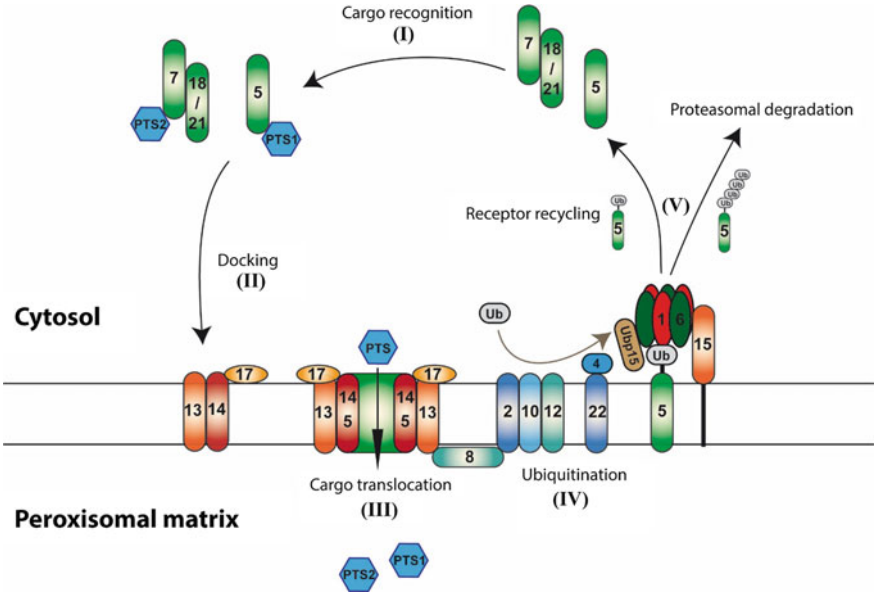

Fig. 2 Peroxisomal matrix protein import cycle of *S. cerevisiae*.Peroxisomal matrix proteins are synthesized in the cytosol on free ribosomes and imported post-translationally into peroxisomes. Peroxisomal protein import requires peroxin (PEX) proteins (individual PEX protein shown in bold numbers) and involves different steps (Roman numerals in brackets): (**I**) Cytosolic import receptors Pex5p and Pex7p along with co-receptor Pex18p/Pex21p recognize the cargo proteins via peroxisome targeting signals, PTS1 and PTS2 respectively. (**II**) The cargo-receptor complexes are transported to the peroxisomal membrane by binding to the docking complex comprised of Pex14p, Pex13p and Pex17p. In this scheme, emphasis is on the import of PTS1 proteins. (**III**) Association of Pex14p with Pex5p or Pex18p results in the formation of respective distinct transient PTS1 and PTS2 protein import pores. After the translocation of cargo proteins into the peroxisomal lumen, the import receptors are released from the peroxisomal membrane and recycled for the next round of protein import. (**IV**) Receptor recycling involves ubiquitination by the ubiquitin-conjugating (E2) enzyme Pex4p and its membrane anchor Pex22p, as well as the complex of RING (Really Interesting New Gene) finger domain containing E3 ubiquitin ligases Pex2p, Pex10p, Pex12p. Intra-peroxisomal Pex8p connects the docking complex to the RING-finger complex. (**V**) Mono-ubiquitination of Pex5p signals its ATP-dependent extraction from the membrane by the hetero-hexameric AAA-complex (Pex1p-Pex6p), which is anchored to the peroxisomal membrane by Pex15p. Poly-ubiquitination of Pex5p serves as a quality control mechanism that directs a supposedly non-functional receptor to proteasomal degradation

residues upstream of the PTS1 tripeptide are less conserved but turned out to be relevant for recognition of the PTS1 signal (Brocard and Hartig 2006; Hagen et al. 2015). Pex5p is the cytosolic receptor for PTS1-containing proteins and it recognizes the PTS1-signal via its carboxy-terminal tetratricopeptide repeat (TPR) domains (Terlecky et al. 1995).

The type 2 peroxisome targeting signal (PTS2) is a nonapeptide located near the N-terminal part of the proteins (Swinkels et al. 1991) with consensus sequence R[L/V/I/Q]xx[L/V/I/H][L/S/G/A]x[H/Q][L/A] (Petriv et al. 2004). Pex7p is the

cytosolic receptor for PTS2 containing proteins and the targeting signal is recognized by tryptophan-aspartate (WD-40) repeats of the receptor (Marzioch et al. 1994; Pan et al. 2013). Compared to PTS1, only few mammalian peroxisomal proteins harbor PTS2 and only two PTS2 proteins are known in yeast, thiolase (Fox3p) and glycerol phosphate dehydrogenase (Gpd1p) (Glover et al. 1994; Jung et al. 2010). However, one-third of peroxisomal proteins in plants contain a PTS2 signal (Reumann 2004).

Reumann et al. (2007) isolated highly purified peroxisomes from *Arabidopsis thaliana* leaves and performed proteomic analysis using gel-based and gel-independent approaches. The study identified 78 non-redundant proteins of which 42 novel proteins were previously not known to be associated with plant peroxisomes. Sequence analysis and fluorescence microscopy of full-length fusion proteins led to the identification of functional PTS1-related peptides in plants. The PTS1 signal typically harbors a positively charged residue in the middle of the PTS1-tripeptide (Lametschwandtner et al. 1998). Plant peroxisomes could import proteins terminating with SSL, SSI, and ASL residues, thus demonstrating that PTS1-signal in plants can be highly degenerated (Reumann et al. 2007). The PTS2 signal is normally localized close to the N-terminus of proteins (Swinkels et al. 1991). The proteomic study by Reumann et al. (2007) also identified an internal PTS2-like signal in transthyretin-like protein (TLP, At5g58220). TLP is a bifunctional protein and the PTS2-like signal is located at position 182-190 between the N-terminal decarboxylase and the C-terminal hydrolase domain. Deletion of the internal PTS2-like signal in TLP abolished its peroxisomal targeting, suggesting that an internal sequence can function as PTS2 signal (Reumann et al. 2007).

Some proteins that are imported into peroxisomes in a Pex5p-dependent manner lack a typical PTS1 signal. Of these, acyl-CoA oxidase (*S. cerevisiae*, *Y. lipolytica*) and alcohol oxidase (*H. polymorpha*) interact with the N-terminal domain of Pex5p (Schäfer et al. 2004; van der Klei and Veenhuis 2006), however, our knowledge on the molecular details of this cargo recognition is still scarce. Another mode of peroxisomal import of non-PTS proteins is the piggy-back transport. Here, proteins that lack a PTS hitch-hike to peroxisomes via binding to proteins that harbor PTS signals. This is possible as peroxisomes can import folded and oligomeric proteins, as pointed out earlier. In yeast, enoyl-CoA isomerase (Eci1p) lacking its own PTS1 signal is imported into peroxisomes by oligomerizing with its PTS1-containing homolog Dci1p (Yang et al. 2001). Mammalian Cu/Zn superoxide dismutase 1 (SOD1) lacks an endogenous PTS but is imported by piggy-backing on its natural PTS1-containing binding partner the copper chaperone of SOD1 (CCS) (Islinger et al. 2009). Also the PTS2-import machinery allows the piggy-back import of non-PTS containing proteins. Examples include hitch-hiking of PTS2-truncated thiolase by dimerizing with endogenous thiolase in *S. cerevisiae* (Glover et al. 1994) and artificial cargo proteins in plants (Flynn et al. 1998).

A recent proteomic study identified a natural piggy-back import via the PTS2-import machinery (Effelsberg et al. 2015). Pnc1p is a nicotinamidase, which localizes to peroxisomes in Pex7p-dependent manner although it lacks a typical PTS2 signal (Anderson et al. 2003). In response to osmotic stress, yeast PTS2

protein Gpd1p shows a tripartite localization in peroxisomes, cytosol and nucleus. Pnc1p also shows similar dynamic localization pattern (Jung et al. 2010). Effelsberg et al. (2015) isolated Gpd1p from the soluble fractions of high-salt grown yeast cells expressing Gpd1-TPA by affinity purification. The isolated Gpd1-complex contained an additional protein, which was identified as Pnc1p by mass-spectrometry. Size-exclusion chromatography of Gpd1p TEV-eluate revealed the presence of a heterodimeric complex of Gpd1p-Pnc1p. This study showed that the piggyback peroxisomal import of Pnc1p depends on the presence of PTS2 signal in Gpd1p (Effelsberg et al. 2015).

Pex5p recognizes the carboxy-terminal PTS1 tripeptide in cargo proteins and the amino acid residues upstream of PTS1 signal also influence the cargo recognition by Pex5p (Lametschwandtner et al. 1998; Brocard and Hartig 2006). To gain further structural insights into the binding interface of Pex5p and cargo protein Pcs60p, Hagen et al. (2015) performed in vitro photo-crosslinking studies of recombinant proteins and mass-spectrometric analysis of the cross-linked products. The study revealed that Pcs60p lacking the C-terminal tripeptide of the PTS1 is still cross-linked to Pex5p, indicating that also other regions of the cargo are in close proximity to the receptor. High-resolution mass spectrometry of the cross-linked products revealed that the amino acids adjacent to the C-terminal tripeptide contribute to the interaction and the study also identified the corresponding binding interface of Pex5p. Further biophysical and biochemical analysis revealed two modes of Pex5p-Pcs60p interaction. The high affinity binding occurs between the PTS1 tripeptide and Pex5p, while the amino acids upstream of the PTS1 provide a low affinity second binding site and interact with a distinct region of the receptor (Hagen et al. 2015).

2.1.1 Pre-import Complexes

Prior to peroxisomal targeting, the receptor cargo complex is assembled in the cytosol (pre-import complex). In contrast to the PTS1-receptor Pex5p, Pex7p requires additional cytosolic peroxins as co-receptors for PTS2-protein import. The co-receptor family includes Pex18p and Pex21p (*S. cerevisiae*) and Pex20p (other yeasts and filamentous fungi). In mammals and rice, alternative splicing leads to two isoforms of PEX5, the longer isoform PEX5L and the shorter PEX5S. *Arabidopsis* and Trypanosomatids encode a single PEX5 transcript, which resembles the mammalian long isoform. Mammalian PEX5L and plant as well as *T. brucei* PEX5 also act as co-receptor for PEX7. The common theme across Pex7p co-receptors is the presence of Pex7p binding domain (reviewed in Schliebs and Kunau 2006).

To investigate the early steps of the import of PTS2-proteins, Grunau et al. (2009) performed isolation of the pre-import complexes and the mass-spectrometric analysis of their composition. Authors isolated cytosolic complexes of Protein-A tagged co-receptors Pex18p and Pex21p, as well as the PTS2-cargo protein Fox3p. Mass spectrometric analysis identified Fox3p as the only abundant PTS2

protein in oleate-grown yeast cells. The study revealed a sequential assembly of the ternary PTS2 pre-import complex in the cytosol. Pex7p first recognizes the PTS2-containing cargo proteins in the cytosol, followed by binding of the co-receptor to Pex7p-cargo complex and this ternary pre-import complex is targeted to the peroxisomal membrane.

2.2 Docking at the Peroxisomal Membrane

After cargo recognition, the cargo-receptor complex is targeted to the peroxisomes by binding to the docking complex at the peroxisomal membrane. The docking complex comprises Pex14p, Pex13p and Pex17p in yeast. The N-terminal domain of Pex5p contains several di-aromatic pentapeptide motifs with the consensus sequence Wxxx(F/Y), which are also present in PEX7 co-receptors (reviewed in Schliebs and Kunau 2006). These WxxxF motifs bind to the N-terminal domain in Pex14p with high affinity, providing the basis for membrane docking of the cargo-receptor complex. Pex14p interacts with both the PTS1- and the PTS2-receptor and is the point of convergence of both import pathways (Albertini et al. 1997). Pex14 is an integral membrane protein but also partially released by carbonate treatment, which is typical for peripheral membrane proteins. Pex13p is an integral membrane protein of peroxisomes and contains a C-terminal Src Homology 3 (SH3) domain. This SH3-domain faces the cytosol and interacts with Pex14p, which provides a classical proline-rich SH3-binding motif (PxxP) for the interaction (Pires et al. 2003). Absence of docking complex components Pex14p or Pex13p abolishes import of both PTS1 and PTS2 proteins (Albertini et al. 1997; Girzalsky et al. 1999; Gould et al. 1996). The Pex13p-Pex14p interaction is important for the correct peroxisomal localization of Pex14p in different organisms (Girzalsky et al. 1999; Fransen et al. 2004; Brennand et al. 2012). Evidence has been provided that docking of the cargo-loaded receptors is first performed by binding to Pex14p (Urquhart et al. 2000). However, also Pex13p binds the import receptors. The SH3-domain of Pex13p interacts with N-terminal domain of Pex5p in a non-PxxP manner with one of the WxxxF motifs representing the binding site (Bottger et al. 2000; Douangamath et al. 2002; Pires et al. 2003). On the other hand, Pex7p can directly bind to the YG-rich N-terminal domain of Pex13p (Girzalsky et al. 1999). Trypanosomatids are the only organisms, which encode two isoforms of PEX13, PEX13.1 and PEX13.2. Although PEX13.2 lacks the SH3-domain, both isoforms are essential for glycosomal protein import (Brennand et al. 2012).

In addition to the analysis of the cytosolic PTS2 pre-import complexes (see Sect. 2.1.1), Grunau et al. (2009) also performed proteomic analysis of the membrane bound PTS2-import complexes. The study revealed a high-molecular-weight complex of Pex14p and PTS2 co-receptor Pex18p at the peroxisomal membrane, which lacks Pex7p and Fox3p. This indicates that the PTS2 pre-import complex seems to dissociate after docking.

Pex17p is an additional component of docking complex but so far has been identified only in yeast species. To characterize the function of Pex17p, Chan et al. (2016) isolated Protein-A tagged peroxisomal receptor-docking complexes from wild-type and *pex17* deletion strains followed by proteomic analysis of the complex composition. The analysis of this Pex14p complex revealed its composition, which included Pex14p, Pex17p as well as the dynein light chain protein Dyn2p as a core component of the complex. Pex17p and Dyn2p eluted with Pex14p in stoichiometric amounts. Previous studies had already implicated a role of Dyn2p in peroxisome biogenesis. First, yeast *dyn2* deletion cells displayed a reduced capacity to utilize oleic acid as sole carbon source indicative of defect in peroxisome function (Smith et al. 2006). Moreover, in *Yarrowia lipolytica*, Dyn2p was found to interact with the peroxisomal docking complex, particularly with Pex17p, and it turned out to be required for efficient peroxisomal protein import (Chang et al. 2013). The study by Chan et al. (2016) demonstrated that Dyn2p is associated with Pex14p and in a Pex17p-dependent manner. In particular, liquid chromatography-mass spectrometry (LC–MS) based protein profiling experiments indicated that Dyn2p fails to associate with Pex14p in the absence of Pex17p. Further, chemical cross-linking experiments combined with MS (XL-MS) revealed the binding interface of Dyn2p and Pex14p but also showed that Dyn2p also interacts with Pex17p. A high-molecular weight Pex14p-subcomplex that is formed in wild-type cells was absent in either *pex17* or *dyn2* deletion yeast strains.

Pex17p is present in yeasts but it is absent in filamentous fungi and higher eukaryotes. Bioinformatic analysis of PEX genes in fungal genomes indicated the presence of a gene, which encodes a protein with significant similarity to both Pex14p and 17p (Kiel et al. 2006). The N-terminal region of this protein exhibits sequence similarity with the highly conserved N-terminal domain of Pex14p, while the C-terminus shows a weak similarity to yeast Pex17p. Managadze et al. (2010) performed proteomic analysis of the docking complex components of filamentous fungus *N. crassa.* In this study, hexa-histidine tagged PEX14 (His_6-PEX14) was affinity-purified from digitonin-solubilized membranes, followed by further purification by using size-exclusion chromatography. The two-step purified PEX14 complex was then analyzed by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS), which revealed a novel component of the docking complex designated as PEX33. This protein shows sequence similarity with conserved PEX14 but only in short region of its N-terminal domain. PEX33 was shown to localize to glyoxysomes and to interact with PEX14, with the PTS1-receptor PEX5 as well as with itself. Biogenesis of glyoxysomes and Woronin bodies was defective in the single *pex14* or *pex33* deletion strains. The study showed that PEX33 plays an essential role in the import of PTS1 proteins and certain non-PTS proteins and the function of PEX33 is non-redundant with PEX14 (Managadze et al. 2010).

2.3 Peroxisomal Import Pores

Unlike mitochondria and chloroplast, peroxisomes can import folded, cofactor-bound or oligomerized proteins. How the transport of proteins across the peroxisomal membrane is achieved without affecting the permeability barrier was unknown for long time. The "transient import pore model" proposed that the cargo-loaded cycling import receptor associates with the peroxisomal membrane to form a translocation pore, and disassembles after the cargo is translocated across the membrane (Erdmann and Schliebs 2005). Transient assembly of the translocon on demand might explain how folded protein import occurs without damaging the integrity of peroxisomes. In this context, a minimal functional importomer was described for the peroxisomal import of Pex8p that only required the presence of the import receptors and Pex14p (Ma et al. 2009). To disclose the identity of the import pore, membrane-bound complexes of the import receptor Pex5p were isolated and tested for channel properties (Meinecke et al. 2010). To this end, higher molecular weight complexes containing Pex5p, PTS1 cargo protein, docking complex components and associated proteins were reconstituted into liposomes and electrophysiological characterization was performed using the planar lipid bilayer technique. The study revealed the ion-channel activity of complexes, which contain Pex5p and Pex14p as the principal components. The water-filled import pore can open up to 9 nm in a cargo-dependent manner, demonstrating the dynamic nature of the protein-conducting channel, which allows the translocation of the PTS1-cargo proteins across the membrane into the peroxisomal lumen.

The PTS1- and PTS2-dependent peroxisomal protein import pathways converge at Pex14p, which is also part of the PTS1 import pore, raising the question whether PTS2-proteins enter peroxisomes via same or distinct import pores. Pex7p is the receptor for PTS2 proteins but requires co-receptors for their peroxisomal targeting. Di-aromatic pentapeptide (WxxxF/Y) motifs in Pex5p that mediate the binding to Pex14p are also found in the N-terminal region of the PTS2 co-receptors (Schliebs and Kunau 2006). Pex20p, the PTS2 co-receptor in *Pichia pastoris* interacts with Pex14p via one of its WxxxF/Y motif, shown to be involved in the docking of PTS2 complex at peroxisomes (Léon et al. 2006). For the identification of the PTS2 import pores, membrane bound complexes of the PTS2 co-receptor Pex18p were reconstituted into liposomes, and tested for their channel properties using the single-channel planar lipid bilayer technique (Montilla-Martinez et al. 2015). The reconstituted complex, which contained Pex18p and the Pex14p/Pex17p heteromer as main constituents, showed pore-forming activity. The size of this pore ($\sim$4.7 nm) was large enough to accommodate import of folded PTS2-proteins. This demonstrated that PTS1- and PTS2-proteins are imported by distinct pores in yeast and that these pores can function independently. Whether PTS1- and PTS2-proteins are imported via distinct pores also in higher eukaryotes still needs to be investigated, as PEX5 is involved in both cases. PEX5 promotes import of proteins either as short isoform (PEX5S), which imports PTS1-proteins, or as a longer isoform (PEX5L), which in addition to its role in targeting PTS1 proteins also is required for

the topogenesis of PTS2 proteins. In particular, PEX5L harbors a binding site for the PTS2-receptor PEX7 and thus functions as a co-receptor for its peroxisomal targeting.

2.4 Cargo Release into the Peroxisomal Matrix

Although PTS1 and PTS2 import pores have been identified, it is still poorly understood how the cargo is translocated into the peroxisomal lumen. Evidence has been provided that Pex8p may play a role in cargo release in yeast (Wang et al. 2003). Pex8p is tightly associated with lumenal side of the peroxisome membrane and known to bind Pex5p (Rehling et al. 2000). Pex8p contains both PTS1 and PTS2 signals for peroxisomal targeting (Rehling et al. 2000; Zhang et al. 2006). Fluorescence Correlation Spectroscopy (FCS) based in vitro studies showed that Pex8p can dissociate PTS1 receptor/cargo complex, suggesting that Pex8p plays a similar role in vivo (Wang et al. 2003). Pex8p is present in all fungi but has not been identified in higher eukaryotes, implicating a different mechanism of cargo release. Interestingly, trypanosomatid parasite PEX13.1 contains a C-terminal PTS1-like signal TKL similar to Pex8p. The role of this putative PTS1 in cargo release into glycosomal matrix awaits investigation (Verplaetse et al. 2009). For mammals, a role in cargo release has been attributed to Pex14p (Freitas et al. 2011). Here, the interaction of the receptor PEX5 with cargo protein catalase is disrupted by PEX14, which might indicate that cargo release may take place early in the receptor cycle after docking of PEX5 to the N-terminal domain of PEX14. In *P. pastoris*, Pex5p dimerization and oligomerization with Pex8p controls binding and release of the cargo (Ma et al. 2013). Particularly, the redox sensitive amino acid cysteine 10 in *Pp*Pex5p plays a role in the regulation of cargo binding by Pex5p, cargo release and receptor recycling.

Some proteins are proteolytically processed during or upon release into the peroxisomal matrix in higher eukaryotes. Tysnd1 (trypsin domain containing 1) is an intraperoxisomal protease of mammalian cells, which cleaves the signal peptide from PTS2 precursor proteins and also processes subset PTS1 proteins (Kurochkin et al. 2007). Tysnd1 regulates the peroxisomal fatty acid β-oxidation pathway and the proteolytic activity of oligomeric Tysnd1 is controlled by self-cleavage. The cleaved products are degraded by peroxisomal Lon protease (PsLon) (Okumoto et al. 2011). In the early 1990s, presence of endopeptidase activity and a leucine-aminopeptidase in plant peroxisomal matrix was reported (Corpas et al. 1993). Seven endoprotease isoenzymes have been detected in plant peroxisomes (Distefano et al. 1997). A role for plant peroxisomal proteases during senescence, oxidative stress conditions, metal pollutant exposure has been suggested (Distefano et al. 1999; reviewed in Palma et al. 2002). Like mammalian Tysnd1, *Arabidopsis* DEG15 serine protease is involved in cleavage of the PTS2 signal from precursor proteins (Helm et al. 2007).

2.5 *Receptor Recycling*

2.5.1 Exportomer

Upon or after cargo translocation into the peroxisomal matrix, the import receptors are recycled back to the cytosol to facilitate further rounds of peroxisomal protein import. This receptor export machinery involves ubiquitinating enzymes and ATP-dependent extraction of receptors from the peroxisomal membrane. The exportomer comprises ubiquitin conjugating (E2) enzymes (Pex4p in yeasts, plants and trypanosomatids; Ubc4p in yeasts and UbcH5-family in mammals), docking proteins at the peroxisomal membrane (Pex22p), corresponding ubiquitin ligases (E3) (Pex2p, Pex10p, Pex12p) and the AAA-complex (Pex1p and Pex6p) as well as its membrane anchor (Pex15p in fungi, PEX26 in mammals, APEM9 in plants) (reviewed in Platta et al. 2014).

2.5.2 Receptor Ubiquitination

The import receptor Pex5p and the PTS2 co-receptor Pex18p is mono- or poly-ubiquitinated at its N-terminal region (Kragt et al. 2005; Hensel et al. 2011). Mono-ubiquitination of Pex5p involves the attachment of a single ubiquitin via a thioester linkage to a conserved cysteine reside, while poly-ubiquitination occurs at conserved lysine residues in the N-terminal region. When the receptor is non-functional, poly-ubiquitination is supposed to trigger its export and proteasomal degradation, serving as a quality control mechanism. On the other hand, mono-ubiquitination signals export of the receptor for further rounds of peroxisomal protein import. The activation of the ubiquitin and the export of ubiquitinated receptors are the only ATP-dependent steps in peroxisomal matrix protein import.

Mono-ubiquitination

The receptor ubiquitination machinery is well characterized. Pex4p was the first E2 enzyme identified to be essential for the peroxisome biogenesis and involved in both PTS1 and PTS2 import (Wiebel and Kunau 1992; van der Klei et al. 1998). Pex4p is anchored to the peroxisomal membrane by the integral membrane protein Pex22p (Koller et al. 1999). Association of Pex4p to the cytosolic soluble domain of Pex22p enables assembly of functional E2-complex (Williams et al. 2012). Pex4p and Pex22p are well conserved in yeast, plants and trypanosomatids where they mediate mono-ubiquitination of Pex5p. However, Pex4p and Pex22p orthologues apparently do not exist in mammals and their function is fulfilled by other proteins.

To identify mammalian PEX5 ubiquitin conjugating enzyme (E2) i.e. counterpart of yeast Pex4p, a proteomic approach was used (Grou et al. 2008). Authors utilized an in vitro system comprising highly purified rat liver peroxisomes, recombinant E1, E2s and GST-Ubiquitin. The substrate for ubiquitination in this assay is a truncated PEX5 (1-324), containing single conserved cysteine, which is

synthesized in vitro and radio-labelled to monitor ubiquitinated PEX5 by autoradiography. Supplementation of cytosol to the import reaction with purified peroxisomes resulted in appearance of thiol-sensitive ubiquitinated PEX5, indicating the presence of an E2 in the cytosol that ubiquitinates the cysteine of PEX5. The authors then fractionated the cytosol guided by peroxisome-dependent PEX5-ubiquitination activity to yield a fraction, which migrated as two protein bands in SDS-PAGE analysis. Mass spectrometry analysis of these bands identified E2-enzymes UbcH7, UbcH13 and three closely related UbcH5a-c. Using recombinant E2 enzymes, only the UbcH5 family members were found to mono-ubiquitinate PEX5 (Grou et al. 2008). Therefore, UbcH5 proteins are functional counterparts of yeast/plant Pex4p. But unlike peroxisome-specific Pex4p, the functions of the mammalian UbcH5 proteins are not restricted to peroxisomes as they are also involved in the ubiquitination of cellular targets that are evidently not related to peroxisomes (Brzovic and Klevit 2006).

The ubiquitination of Pex5p requires RING-domain containing ubiquitin-protein ligases (E3). Three conserved peroxins, Pex2p, Pex10p and Pex12p, are ubiquitin ligases that associate at the peroxisomal membrane to form the RING-finger complex (Platta et al. 2009). Pex12p facilitates Pex4p-dependent mono-ubiquitination of Pex5p, which may also involve Pex10p (Platta et al. 2009; El Magraoui et al. 2012). Like Pex5p, also the PTS2 co-receptors Pex18p (*S. cerevisiae*) and Pex20p (*P. pastoris*) are mono-ubiquitinated at a conserved cysteine residue (Hensel et al. 2011; Léon et al. 2006). Pex18p mono-ubiquitination is mediated by Pex4p as responsible E2 enzyme and the E3 ligases Pex10p and Pex12p (El Magraoui et al. 2013). Ubiquitin receptor proteins DSK2a and DSK2b interact with PEX12 in plants, although the role in receptor recycling is not clear (Kaur et al. 2013).

Poly-ubiquitination

Poly-ubiquitination of the PTS1-receptor serves as a quality control mechanism when the receptor is non-functional or when the normal mono-ubiquitination dependent recycling is defective (Platta et al. 2004, Kiel et al. 2005). This pathway has also been named “RADAR” pathway, standing for Receptor Accumulation and Degradation in Absence of Recycling (Léon and Subramani 2007). Here, Pex5p is poly-ubiquitinated by the attachment of K48-linked poly-ubiquitin chains to two conserved lysine residues in Pex5p, which primes the receptor for proteasomal degradation. E2 ubiquitin conjugating Ubc4p and E3 Ubiquitin ligases Pex10p and Pex2p are involved in the poly-ubiquitination of yeast Pex5 (Platta et al. 2004; Williams et al. 2008; Platta et al. 2009). PTS2 co-receptor Pex18p is also poly-ubiquitinated by Ubc4 and E3 enzymes Pex2p and Pex10p, leading to rapid turnover by proteasomal degradation (Hensel et al. 2011; El Magraoui et al. 2013). However, poly-ubiquitination of *P. pastoris* PTS2 co-receptor Pex20p is dependent on Pex4p and all three members of the RING-complex (Liu and Subramani 2013). In plants, Pex2p also interacts with ubiquitin-adapter DSK2 proteins (Kaur et al. 2013).

2.5.3 Membrane Dislocation of the Receptors

Pex1p and Pex6p are AAA-ATPases which function as dislocase for the export of Pex5p back to the cytosol (Platta et al. 2005). Pex1p and Pex6p form a hetero-hexameric AAA-complex (Ciniawsky et al. 2015), which is anchored to the peroxisomal membrane by Pex15p (yeast), PEX26 (human) or APEM9 (plants). Genetic mutations in PEX1, PEX6 or PEX26 abrogates peroxisomal protein import, which is the most common cause of Peroxisome Biogenesis Disorders (PBDs) in humans. Pex15p/PEX26 proteins show very low sequence conservation. To investigate the role of a putative PEX26 in *Neurospora crassa*, PEX26-complexes were isolated, and their composition was analyzed by mass-spectroscopy, which identified PEX6 and PEX1 as the prominent binding partners (Liu et al. 2011). This revealed that despite the low degree of sequence conservation, the *Nc* PEX26 is an orthologue of PEX26.

2.5.4 Receptor Deubiquitination

After or during cargo translocation, the PTS1 receptor is deubiquitinated. For mammalian cells, it was shown that the mono-ubiquitin, which is linked to the conserved cysteine via thioester-linkage, can be removed non-enzymatically by glutathione (GSH) in vitro (Grou et al. 2009). GSH also is a major nucleophile in the cytosol of mammalian cells, indicating that the non-enzymatic cleavage might also occur in vivo. However, numerous de-ubiquitinating enzymes (DUBs) are predicted in yeast and human genomes (Hutchins et al. 2013). DUBs acting on peroxisomal import receptors have been investigated in yeast (Debelyy et al. 2011) and mammalian cells (Grou et al. 2012).

In *S. cerevisiae*, the purified cytosolic complex of Pex1p-Pex6p (AAA-complex) was found to de-ubiquitinate membrane-bound monoUb-Pex5p (Debelyy et al. 2011). To identify the unknown associated factor or hydrolase, the authors isolated cytosolic AAA-complexes from yeast cells overexpressing His_6-Pex6p (Debelyy et al. 2011). Analysis of the complex revealed the presence of two dominant proteins, which were identified by mass spectrometry as Ubiquitin hydrolase Ubp15 and ubiquitin-adapter protein Ecm21p. In a complementary approach, mass-spectrometric analysis of isolated genomically tagged Pex1-complexes also revealed their association with Ubp15p. Finally, affinity purified cytosolic complexes of His_6-GST-Ubp15p also contained Pex1p and Pex6p. These studies identified Ubp15p as a novel component of the AAA-complex, which localizes to peroxisomes and is capable to cleave ubiquitin moieties from Pex5p. Accordingly, Ubp15p deficient yeast cells display a hydrogen peroxide stress-dependent PTS1 import defect (Debelyy et al. 2011).

In mammalian cells, biochemical studies indicate that mono-ubiquitinated PEX5 is the substrate of ubiquitin-specific protease 9X (USP9x) (Grou et al. 2012).

However, USP9x function is not restricted to peroxisomal protein import as it also has other cellular targets. Apart from yeast Ubp15p or mammalian USP9x, additional or redundant ubiquitin hydrolases may also be involved in Pex5p de-ubiquitination.

2.5.5 Connecting Docking and Export Complexes

Beside its role in cargo release (see Sect. 2.4), *S. cerevisiae* Pex8p plays a major role in connecting the docking complex or import pore with the export complex consisting of the receptor ubiquitination and export machinery (Agne et al. 2003). Proteomic approaches were applied to define the components of two purified complexes of the peroxisomal import machinery, the docking complex, isolated by affinity chromatography via genomically tagged Pex14p, and the RING finger complex isolated in a similar way via genomically tagged Pex2p (Agne et al. 2003). Mass-spectrometry analysis of isolated complexes revealed that Pex14p and Pex2p bind same set of peroxins. Proteomic analysis of the Pex8p complex revealed the presence of the docking as well as the export complex. However, In the absence of Pex8p the docking complex and the export complex could not be co-isolated. In the absence of individual RING-finger peroxins, Pex8p still associates with the docking complex. Taken together, the data indicate that the association of the docking complex and the export complex to form a larger complex requires the presence of the intra-peroxisomal Pex8p (Agne et al. 2003). However, in *P. pastoris*, this networking function is apparently performed by Pex3p (Hazra et al. 2002).

2.6 Peroxisomal Importomer—Stable Versus Transient Interactors

Peroxisomal importomer consists of the docking complex (Pex14p, Pex17p, Pex13p) linked to the RING finger complex (Pex2p, Pex10p, Pex12p) via intraperoxisomal Pex8p. A comprehensive analysis of the stable and transient interaction partners of Pex14p (the central component of importomer) was performed by Oeljeklaus et al. (2012). The quantitative proteomic study involved epitope tagged Pex14p combined with dual-track stable isotope labeling with amino acid in culture-mass spectrometry (SILAC-MS) analysis of the affinity-isolated Pex14p complexes. The study identified 9 core components and additional 12 transient components of the Pex14p interactome. Pex8p, Pex11p and Dyn2p were part of the core complex in addition to the known docking and RING complex proteins. The study also identified Pex25p, Hrr25p, Esl2p and prohibitin as novel transient interaction partners (Oeljeklaus et al. 2012).

3 Peroxisomal Membrane Protein Import

In contrast to the large set of peroxins required for matrix protein import, peroxisomal membrane protein (PMP) import involves only three peroxins, Pex19p, Pex3p and Pex16p. Defects in any one of these result in complete loss of peroxisomes, mislocalisation of matrix proteins to the cytosol and membrane protein mislocalisation to other cellular membranes. Genetic defects in these peroxins also causes severe peroxisome biogenesis disorders in humans.

Pex19p is the cytosolic receptor for peroxisomal targeting of newly synthesized PMPs and possesses chaperone activity to stabilize the PMPs in the cytosol (Jones et al. 2004). Most PMPs contain membrane peroxisome targeting signal (mPTS) comprised of a short α-helical segment with positively charged and hydrophobic residues, and additionally at least one transmembrane segment (Jones et al. 2001; Honsho and Fujiki 2001; Rottensteiner et al. 2004). The N-terminal domain of Pex19p contains binding sites for Pex3p and/or Pex14p, whereas mPTS-PMP binding site is located in the C-terminal domain of Pex19p (Sato et al. 2010; Neufeld et al. 2009). Pex19p proteins contain a C-terminal CaaX motif for farnesylation. This modification of Pex19p is important for its structural integrity and PMP recognition (Rucktäschel et al. 2009; Emmanouilidis et al. 2017). Interestingly, Trypanosomatid PEX19 proteins lack such a C-terminal CaaX motif.

Docking of the Pex19p-PMP receptor-cargo complex at the peroxisomal membrane is mediated by the integral membrane protein Pex3p (Fang et al. 2004). The transmembrane domain at the N-terminus of Pex3p anchors it to peroxisomal membrane, while its soluble domain facing the cytosol binds to Pex19p. To gain further insight into the Pex3p-Pex19p interaction, Hattula et al. (2014) used hydrogen exchange mass spectrometry (HXMS) to monitor conformational changes during Pex3p-Pex19p complex formation in vitro. The study disclosed that although Pex19p remains very flexible during interaction with Pex3p, the N- and C-terminus along with a short stretch in the middle (F64-L74) of Pex19p are shielded from hydrogen exchange upon interaction with Pex3p. The authors suggest that Pex19p stabilizes Pex3p within the cell by preventing its aggregation.

In mammalian cells, PEX3 can directly sort to peroxisomes, which is PEX19- as well as PEX16- dependent and PEX16 functions as membrane receptor for the PEX3-PEX19 complexes (Matsuzaki and Fujiki 2008). Colasante et al. (2013) performed proteomic analysis of purified glycosomal membranes from *Leishmania* parasites, which identified 464 proteins in the glycosomal membrane fraction including known PMPs and a protein with weak homology to PEX16. Further studies confirmed the putative protein as trypanosomal PEX16 involved in glycosome biogenesis in *Trypanosoma* parasites (Kalel et al. 2015). Although PEX16 is present in higher eukaryotes, plants, fungi and *Trypanosoma*, it has not yet been identified in *S. cerevisiae*. However, recently a novel peroxin (Pex36p) was identified in *Komagataella phaffii* (*P. pastoris*) (Farre et al. 2017). Remarkably, the growth defect observed in cells lacking Pex36p could be restored by human PEX16 or yeast Pex34p, opening the possibility of an analogous or orthologous

relationship of the three proteins. Interestingly, so far PEX3 has not been identified in trypanosomatid parasites.

Membrane biogenesis peroxins Pex19p, Pex3p and Pex16p are also involved in de novo biogenesis of peroxisomes from the endoplasmic reticulum (ER) (reviewed in Agrawal and Subramani 2016). Contribution of the ER to de novo peroxisome formation was proposed based on the observation that in *S. cerevisiae pex3* deletion cells, re-introduced YFP-tagged Pex3p first sorts to ER, concentrates as foci, and results in a subsequent Pex19p-dependent budding and maturation of fully functional peroxisomes (Hoepfner et al. 2005). However, recent studies show that also in absence of Pex3p, PMPs accumulate in pre-peroxisomal vesicles, which are morphologically distinct from the ER (Knoops et al. 2014). These vesicles contained Pex14p and to investigate the composition of these pre-peroxisomal structures, Wroblewska et al. (2017) performed the proteomic analysis of affinity-purified Pex14p complexes from *pex3* deletion yeast cells. These complexes contained Pex14p and docking components Pex13p, Pex17p as well as import receptors Pex5p, Pex7p and Pex4p/22p. However, the absence of Pex8p and the RING finger peroxins in this complex indicates that a functional importomer has not yet been formed in these prestructures. The study suggests that some PMPs are targeted to the pre-peroxisomal vesicles in a Pex3p-independent manner (Wroblewska et al. 2017).

4 Regulation of Peroxisomal Import Machineries

Reversible protein phosphorylation mediated by protein kinases and phosphatases is a common post-translational modification, regulating diverse cellular processes. Phosphorylation of the mitochondrial preprotein translocase proteins by cytosolic kinases regulate the mitochondrial protein import (Schmidt et al. 2011). There is increasing evidence that protein phosphorylation also plays an important role in peroxisome dynamics. Phosphorylation close to the PTS2-signal in Gpd1p is required for its dynamic localization to the peroxisomes (Jung et al. 2010). Analysis of the public protein databases demonstrates that numerous yeast and mammalian peroxisome biogenesis proteins are phosphorylated in vivo (Oeljeklaus et al. 2016). Currently, the exact role of these modifications in regulating the peroxisomal protein import is still lacking.

Recently, a strategy for a targeted analysis of peroxisomal phospho-proteome by affinity purification of epitope-tagged peroxisomal proteins using Phos-tag SDS-PAGE and high-resolution mass spectrometry has been described. Additionally, a protocol for MS-based in vitro kinase assay with recombinant peroxisomal proteins and specific kinase has been described, which allows the identification and localization of phospho-sites in peroxisomal proteins in vivo and identification of specific substrate-kinase relationships (Schummer et al. 2017).

These emerging proteomics-based technologies will contribute to a better understanding of the regulation of peroxisomal protein import and of the resulting molecular dynamics of these fascinating multi-purpose organelles.

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The Proteome of Fruit Peroxisomes: Sweet Pepper (*Capsicum annuum* L.) as a Model

José M. Palma, Paz Álvarez de Morales, Luis A. del Río and Francisco J. Corpas

Abstract Despite of their economical and nutritional interest, the biology of fruits is still little studied in comparison with reports of other plant organs such as leaves and roots. Accordingly, research at subcellular and molecular levels is necessary not only to understand the physiology of fruits, but also to improve crop qualities. Efforts addressed to gain knowledge of the peroxisome proteome and how it interacts with the overall metabolism of fruits will provide tools to be used in breeding strategies of agricultural species with added value. In this work, special attention will be paid to peroxisomal proteins involved in the metabolism of reactive oxygen species (ROS) due to the relevant role of these compounds at fruit ripening. The proteome of peroxisomes purified from sweet pepper (*Capsicum annuum* L.) fruit is reported, where an iron-superoxide dismutase (Fe-SOD) was localized in these organelles, besides other antioxidant enzymes such as catalase and a Mn-SOD, as well as enzymes involved in the metabolism of carbohydrates, malate, lipids and fatty acids, amino acids, the glyoxylate cycle and in the potential organelles' movements.

Keywords Catalase · Olive fruits · Pepper fruits · Reactive oxygen species (ROS) Ripening · Superoxide dismutase

Abbreviations

ACO	Aconitase
ALDH	Aldehyde dehydrogenase
FDH	Formate dehydrogenase
GDC	Glycine decarboxylase
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization-time of flight
NO	Nitric oxide
PGK	Phosphoglycerate kinase

J. M. Palma (✉) · P. Á. de Morales · L. A. del Río · F. J. Corpas
Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain
e-mail: josemanuel.palma@eez.csic.es

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RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase

1 Introduction

By definition, fruits are considered as mature ovaries developed by the taxon of Angiosperms (also known as Magnoliophyta; plants with flowers). The different parts of flowers evolve to the final components of fruits after several growing and differentiation steps (Mauseth 2003; Palma et al. 2011). Overall, fruits are peculiar structures whose main fate is to house the germ for the next generation of plant species. External appearances of fruits, including shape, texture, size, color, density, and firmness, among other features, are quite diverse and are influenced by genetic determination but also by environmental conditions. Thus, within the same family, it can be found fruits so visually different like tomato and pepper (Fam. Solanaceae), but they also differ in their intimate metabolism and the mechanisms which drive their ripening. In fact, whereas tomatoes are climacteric fruits whose ripening is characterized by a burst in the respiration rate and the production of the phytohormone ethylene at the onset of the process, pepper fruits are non-climacteric and, therefore, do not respond to stimuli (i.e., ethylene) as climacteric fruits do. Additionally, new data point out that molecules such as the free radical nitric oxide (NO) could be involved in the ripening process of pepper (Rodríguez-Ruiz et al. 2017a, b). In Fig. 1, examples of important crop species are shown, in which they are differentiated as climacteric and non-climacteric.

The diversity of fruits indicated above does not allow establishing a consensed metabolic pattern which can help understanding the key points to be investigated. Thus, despite of their economical and nutritional interest, the biology of fruits is still little studied in comparison with reports of other plant organs such as cotyledons, roots or leaves. Proteomes from fruits such as tomato, grape, peach, strawberry and many more have been reported in recent years (Palma et al. 2011 and references therein; Szymanski et al. 2017; Wang et al. 2017; Li et al. 2017). Many of these works report the changes underwent by fruits at ripening, as this is the physiological process by which fruits from crop species have gained attention since the Neolithic. Ripening is a developmental process occurring at the final phase of the fruit development, and implies noteworthy metabolic issues in the biochemistry, physiology and gene expression of fruits. During fruit ripening chlorophyll degradation, and pigment (carotenoids and anthocyanins) biosynthesis take place, but also the conversion of starch to simple sugars, accumulation of flavours and cell wall softening may happen, among other events (Schuch et al. 1989; Fray and Grierson 1993; Barsan et al. 2010, 2012; Palma et al. 2011). These, and other processes, can vary between species, what makes that essential specific research be conducted

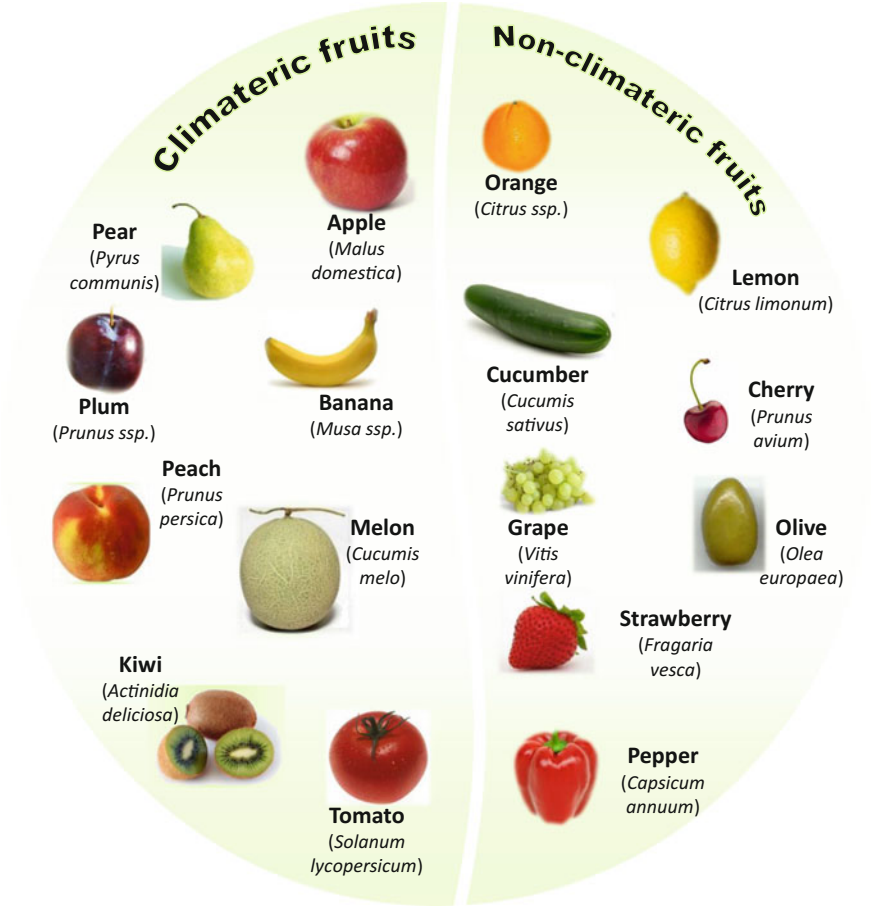

Fig. 1 Classification of the most worldwide consumed fruits as climacteric and non climacteric. Modified from Palma et al. (2011)

according to the fruit source and its characteristics. In most cases, and especially in crop species, during fruit ripening a shift takes place from green to colors such as red, yellow, purple, etc. This is the result of the conversion of functional chloroplasts into chromoplasts, where the chlorophyll, present in high content, is degraded and the synthesis of more and new carotenoids (carotenes plus xanthophylls) is triggered. This implies the transformation of chloroplasts into chromoplasts as it was already reported in sweet pepper (*Capsicum annuum* L) fruits (Fig. 2; Mateos et al. 2003) and, accordingly, relevant changes in their internal metabolism. As observed in Fig. 2, starch granules are accumulated in chromoplasts of ripe pepper fruits (red) as an indication of an advanced developmental stage. Recently, by carrying out morphological, enzymological and transcriptional studies in grape berry during fruit development and ripening, a function for starch was postulated that was relevant for

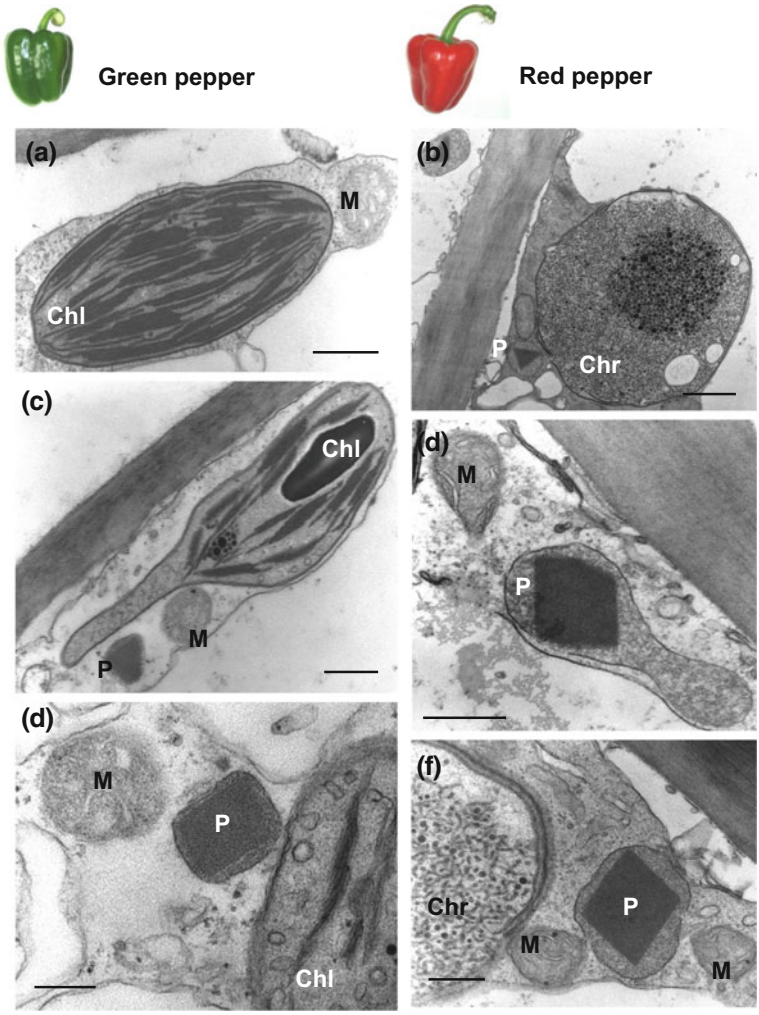

Fig. 2 Ultrastructure of green (**a**, **c**, **e**) and red (**b**, **d**, **f**) sweet pepper (*C. annuum* L.) fruits. Chloroplasts (Chl) were present in green fruits whereas chromoplasts (Chr) were visualized in the red ripe stage. Mitochondria (M) were decorated with crestae in both types of fruits, green and red. Peroxisomes (P) showed crystalline cores with different shapes and sizes in both green and red fruits. Bar, 1 μm. Modified from Mateos et al. (2003)

the berry quality (Zhu et al. 2017). It seems that phytochorme and their phytochrome interacting factors are involved in this physiological process (Rosado et al. 2016) and, in fact, in tomato the deficiency in the phytochrome chromophore phytochromobilin affects the sugar accumulation, as well as the chloroplast formation and the timing of fruit ripening (Bianchetti et al. 2017).

The biochemical reactions and metabolic pathways driven by enzymes in choroplasts have been throroughly investigated, and their protein components have been focus of research for many years. Thanks to advances in the proteomics field that allowed high throughput experiments on chloroplast samples, the proteome of these organelles has been accomplished (Ferro et al. 2010). Thus, chloroplast proteome databases have been issued in diverse platforms were proteins have been assigned to the distinct sub-compartments within these organelles (Kleffmann et al. 2004; Ferro et al. 2010; Bruley et al. 2012; Wang et al. 2016; http://www.plprot.ethz.ch/; https://omictools.com/chloroplast-proteins-category; http://ppdb.tc.cornell.edu/dbsearch/ subproteome.aspx, among others). Accordingly, in different crop species under distinct conditions the proteome of fruit plastids, either chloroplasts or chromoplasts, has been accomplished, and the protein components of these organelles during fruit development and ripening has been reported (Barsan et al. 2012; Marondedze et al. 2014; Suzuki et al. 2015; Du et al. 2016; Tamburino et al. 2017).

2 Sweet Pepper (*Capsicum annuum* L) as a Model to Isolate Fruit Organelles

During the last decade pepper fruits have been used as an excellent model to study at biochemical level the process of ripening under different environmental conditions, where molecules such as NADPH, glutathione (GSH), ascorbate, and reactive oxygen and nitrogen species (ROS and RNS, respectively) are clearly involved (Jiménez et al. 2003; Mateos et al. 2009, 2013; Pascual et al. 2010; Martí et al. 2009, 2011; Chaki et al. 2015; Rodríguez-Ruiz et al. 2017a, b; Corpas et al. 2018). However, this plant species has been also used as a model of non-climacteric fruit to isolate and analyze the main cellular organelles including peroxisomes, chloroplasts/chromoplasts and mitochondria by the combination of differential and sucrose density-gradient centrifugations (Mateos et al. 2003; Martí et al. 2009; Wang et al. 2013; Camejo et al. 2015; Barsan et al. 2017) allowing to get deeper information about its metabolism in comparison with climacteric fruits, mainly tomato (Barsan et al. 2010, 2012). Figure 3 outlines the protocol used to isolate peroxisomes from green and red sweet pepper fruits.

Unlike chloroplasts, mitochondria and peroxisomes do not seem to develop visible structural transformations during ripening of fruits. This absence of morphological changes was also observed in sweet pepper fruits at two different ripening stages (Fig. 2). The protein and enzymatic composition of mitochondria has been widely studied due to their important role in the energy metabolism of plant cells. Thus, the mitochondrial proteome was already referenced in early 2000s and reports on that subject have accumulated since then (Kruft et al. 2001; Millar et al. 2001, 2005; Heazlewood et al. 2004; Millar 2007; Huang et al. 2014; Wu et al. 2016). Likewise, the fruit mitochondrial proteome has been also reported in the last years, and its profile at different physiological stages and developmental

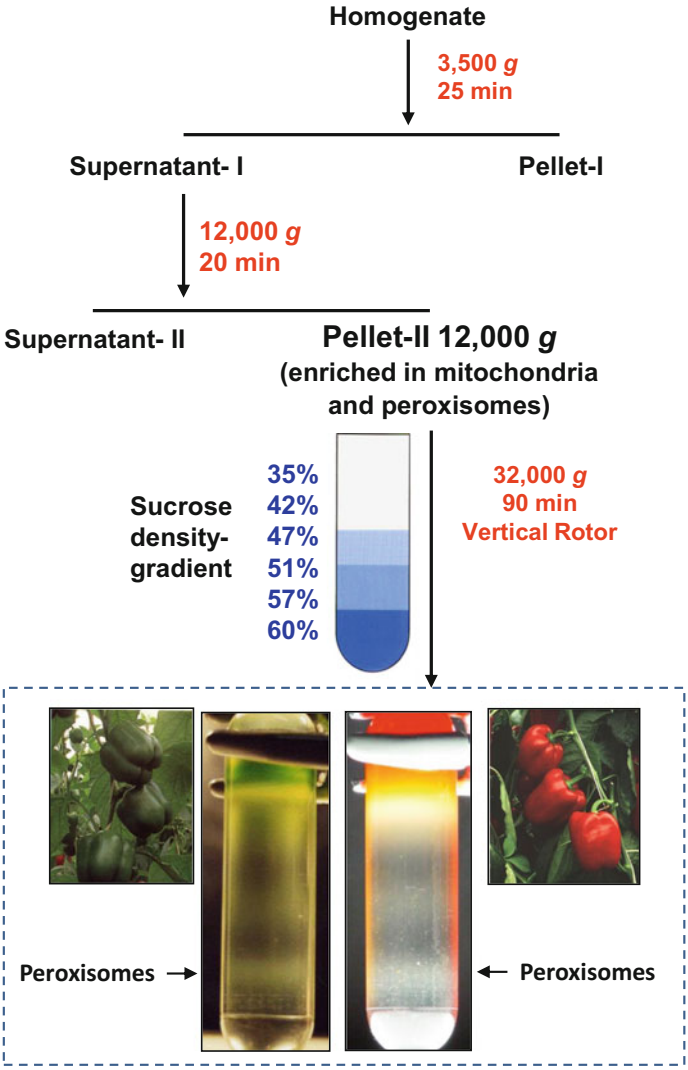

Fig. 3 Simplified scheme of the protocol used to isolate peroxisomes from green and red sweet pepper (*C. annuum* L.) fruits by the combination of differential and sucrose density-gradient centrifugations

conditions has been investigated in diverse species (Qin et al. 2009; Wu et al. 2016). The proteome of mitocondria from sweet pepper fruits was accomplished in our laboratory, where up to 44 typical mitochondrial proteins were identified, basically involved in the tricarboxylic acid cycle, electron transport of the respiratory chain, ion (H^+) and metabolites transport, ATP hydrolisis and synthesis coupled to proton transport, protein phosphorylation, protein folding, cysteine

biosynthetic process, oxidative metabolism, and response to diverse stress situations (Álvarez de Morales et al. 2011). More recently, the identification of carbonylated (oxidized) proteins of pepper fruits at two maturation stages by applying proteomic approaches has been reported (Camejo et al. 2015). Thus, the MALDI-TOF/TOF analysis of immature green and ripe red fruits from a California type sweet pepper variety displayed the presence of formate dehydrogenase, NAD-dependent isocitrate dehydrogenase, porin, and defensin as proteins involved in redox processes in both ripening stages. Conversely, other proteins were only found depending on the maturation stage. Thus, glycine dehydrogenase P subunit and phosphate transporter were only detected as carbonylated proteins in mitocondria from green fruits, whereas cytochrome *c* oxidase, ATPase β subunit, aconitase, prohibitin, and orfB protein were only identified as oxidized proteins in red fruits (Camejo et al. 2015).

Peroxisomes are still one of the less studied organelles in plant biology, and data on their proteome in fruits are actually absent. As seen in Fig. 2, in sweet pepper fruits peroxisomes are characterized by the presence of cristaline cores with distinct shapes and sizes which occupy the major part of the compartment. The structural analysis of these organelles during fruit ripening did not show differences (Fig. 2). Although abundant information is available on the enzymatic content of plant peroxisomes, mainly from leaves of the model plant *Arabidopsis thaliana* (Fukao et al. 2002; Eubel et al. 2008; Reumann et al. 2009; Reumann 2011; Kaur and Hu 2011; Quan et al. 2013; Bussell et al. 2013; Pan and Hu, in this volume), there are very few reports on both the proteome and the metabolism of fruit peroxisomes.

3 Proteome of Fruit Peroxisomes

To our knowledge, up until now no information on the proteome of peroxisomes from higher plant fruits has been reported. One of the first limitations to investigate the peroxisomal proteome from fruits is their nature. Due to the lability of peroxisomes and the diversity of fruits concerning structure, firmness, chemical composition (usually with high water content), and other anatomical and texture features, it is necessary to design appropriate protocols to isolate organelles of high purity to obtain their proteome. Similarly to other plant organs, the few references available on the isolation of peroxisomes from higher plant fruits have applied differential and density gradient centrifugations (Mateos et al. 2003; López-Huertas and del Río 2014). In a previous work carried out with peroxisomes purified from pepper fruits and further 2-D electrophoresis and MALDI-TOF/TOF analysis using MASCOT as the search engine, up to 39 polypeptides were subjected to spectrometer analysis, but only 13 could be identified with high score (Table 1; unpublished results; communicated by Rodríguez-Ruiz et al. 2016). In Table 1, a MASCOT protein score Confidence Interval (C.I.) above 99% indicates a high probability that the respective proteins have been appropriately identified. They have been also classified according to the functional groups they belong to and some of their molecular properties are given, such as theoretical molecular weight

Table 1 Identification of proteins from peroxisomes of sweet pepper (*C. annuum* L.) fruits through proteomic analysis combining 2-D and MALDI-TOF/TOF

Identified protein	Access number/ Uniprot	Protein score %CI (no. identified peptides)	MW/pI	Other subcellular localizations	Functional groups
Malate dehydrogenase	O81279	100 (6)	27545.4/ 5.56	Mitochondrion	Carbohydrate/ malate metabolism Tricarboxylic acid cycle
Superoxide dismutase (Fe-SOD)	Q6X1D0/ Q7YK44	100 (4) 100 (6)	27919/ 6.53 27893/6.6	Plastid	Oxidative metabolism
Superoxide dismutase (Mn-SOD)	O49066	100/8	25610.2/ 8.39	Mitochondrion	Oxidative metabolism
Catalase	Q9M5L6	100 (25)	56957.4/ 7.31		Oxidative metabolism
Phosphoglycerate kinase (PGK)	O81394	99.994 (9)	50594/ 7.68	Cytosol	Glycolisis
Aminomethyltransferase	O23936	99.916 (11)	44655.9/ 8.87	Mitochondrion	Glycine metabolism
Formate dehydrogenase (FDH)	Q07511	100 (16)	42094.6/ 6.64	Mitochondrion	Redox metabolism
Aconitase (ACO)	Q84TR4	99.993 (14)	98635/ 6.07	Mitochondrion	Tricarboxylic acid cycle Glyoxylate cycle
Kinesin-like heavy chain	Q9FKP4	99.986 (19)	140880.3/ 5.91	Microtubules Cytosol	Cytoskeleton Movement
Esterase/lipase thioesterase	Q2HUX9	99.723 (10)	42088.5/ 9.18		Lipid metabolism
Aldehyde dehydrogenase (ALDH)	Q8LST4	100 (9)	59520.9/ 6.65	Mitochondrion	Lipid metabolism
3-ketoacyl-CoA thiolase1	Q8LF48	99.483 (5)	49067.2/ 8.78		Lipid metabolism Oxylipines synthesis
Aspartate aminotransferase	Q2XTE6	99.512 (11)	50998.1/ 8.64	Mitochondrion Cytosol	Aminoacid metabolism

Concentrated peroxisomes from pepper fruits were subjected to 2-D electrophoresis. Isoelectric focusing was performed with precast IPG (immobilized pH gradient gels, pH 3–10), and gels were loaded with 100 μg of organelle proteins. The second dimension separation was carried out by glycine SDS-PAGE. The gels were stained with Sypro Ruby, scanned, and analysed with the Bio-Rad PDQuest software. Identified spots in the Sypro Ruby-stained gels were automatically picked using an Investigator ProPic Protein Picking Workstation equipment (Genomic Solutions). Then, they were destained and digested with trypsin using an Investigator ProGest Protein Digestion Station (Genomics Solutions). The identified spots were analysed by MALDI-TOF/TOF mass spectrometry after trypsin digestion. The MASCOT search engine was used to parse MS data to identify proteins from primary sequence databases. The closer value of Protein Score Confidence Interval (CI) to 100% indicates a strong likelihood that the protein is correctly matched. MW, molecular weight, pI, isoelectric point

(MW) and isoelectric point (pI), and other potential cellular localizations of each protein, as it has been reported before. Since catalase and superoxide dismutase are proteins related to the reactive oxygen species (ROS) metabolism, which deserves special attention in this chapter, they will be discussed in the next section. This is the first report of a proteome of fruit peroxisomes, obtained from organelles purified from sweet pepper (*C. annuum* L.) by the combination of differential and sucrose density-gradient centrifugation (Fig. 3).

Most of the proteins shown in Table 1 were previously present or associated to peroxisomes from other organs and/or species. Malate dehydrogenase catalyses the oxidation of (S)-malate to oxaloacetate using NAD^+ as electron acceptor, playing an essential role in the mitochondrial transport malate/aspartate and the tricarboxylic acid cycle. This enzyme has been reported in several cell compartments (Minarik et al. 2002; Ast et al. 2013), and it is also considered as a peroxisomal protein, sharing with the mitocondrial isozyme gene duplication events responsible for the dual localization (Ast et al. 2013). Due to the transport across membranes, this mechanism could function in the malate homeostasis among cell compartments.

Phosphoglycerate kinase (PGK) catalyses the first phosphorylation reaction at the glycolisis, promoting the synthesis of ATP from 1,3-phosphoglycerate and ADP, and it is also involved in the last step of CO_2 fixation in plants (Joao and Williams 1993; Bowler 2013). Although it has been basically localized in the cytosol, PGK was also described to be present in glycosomes from *Trypanosoma brucei* (Peterson et al. 1997; Blattner et al. 1998), which are specialized peroxisomes in protists of the taxon kinetoplastea (Haanstra et al. 2016; Bauer and Morris 2017). The presence of PGK in peroxisomes has been also reported in the fungi *Aspergillus nidulans* and *Uromyces madis* (Freitag et al. 2012; Ast et al. 2013). This implies new roles for peroxisomes in the metabolism of sweet pepper fruits unexplored thus far. Aconitase (ACO) is involved in the isomerization of citrate to isocitrate both in the mitochondrial Krebs cycle and the peroxisomal glyoxylate cycle. This enzyme was reported as a key step in the acid metabolism in citrus fruits (Degu et al. 2011), and in mitochocondria from red sweet pepper fruits ACO has been reported to be oxidized as the ripening processs goes further (Camejo et al. 2015). In *Arabidopsis* seedlings, the isoform ACO3 is more associated to the citrate catabolism at the glyoxylate cycle level than to the mitochondrial Krebs cycle (Hooks et al. 2014), what provides relevance to the peroxisomal isozyme in diverse plant organs.

The enzymes esterase/lipase thioesterase and 3-ketoacyl-CoA thiolase are involved in the lipid metabolism and their presence in peroxisomes has been referenced previously. Esterases are hydrolases which breakdown the ester bonds between alcohols and fatty acids, which then are degraded by lipid oxidation processes. The association of lipases with peroxisomes is a common event since in the lipid bodies these types of enzymes are necessary to initiate the β-oxidation in the organelles (Gerhardt 1992; Pistelli et al. 1996; Gerhardt et al. 2005). Recently, the localization of a triacylglycerol lipase (SPD1) in the peroxisomal membranes of *A. thaliana* seedlings has been demonstrated (Thazar-Poulot et al. 2015). On the other hand, 3-ketoacyl-CoA thiolase is the final step of the β-oxidation pathway of

plant peroxisomes, rendering acyl-CoA that is driven to the glyoxylate cycle within the organelle (Kleiter and Gerhardt 1998; Oeljeklaus et al. 2002; Baker et al. 2006). This enzyme is a typical peroxisomal protein that is synthesized harbouring a peroxisomal targeting signal type 2 (PTS2), that is degraded upon the protein entrance into the organelle (Johnson and Olsen 2003; Reumann 2004). As indicated in Table 1, the 3-ketoacyl-CoA thiolase has been also linked to lipid synthesis and oxilipins biosynthesis, and this suggests additional roles for fruit peroxisomes in pepper plants.

Kinesins are a family of motor proteins which mediate the intracelular transport of microtubules, as components of the cytoskeleton (Ganguly and Dixit 2013). They are distributed in all eukaryotes and play an important role during the mitosis, morphogenesis and signal transduction pathways (Li et al. 2012). Kinesins have been also located in peroxisomes (Hamada et al. 2013), where they could participate in the movement of these organelles (Kural et al. 2005; Rodríguez-Serrano et al. 2014). In pepper fruits, kinesins might perhaps drive the peroxisomes movement during ripening, as it has been proposed to occur in plant cells under stress conditions (Rodríguez-Serrano et al. 2016).

An aldehyde dehydrogenase was mapped in the proteome of peroxisomes from pepper fruits (Table 1). Aldehyde dehydrogenase (ALDH) are a group of enzymes that catalyse the oxidation of aldehydes to their specific carboxylic acids using NAD/NADH as coenzymes. These proteins modulate several cell functions, such as cell proliferation and differentiation and the response to oxidative stress, and their cell localization is diverse (Muzio et al. 2012). Distinct ALDH isoforms have been also reported in peroxisomes from *A. thaliana* (Missihoun et al. 2011) and barley (Fujiwara et al. 2008). The role of this enzyme in pepper fruit peroxisomes is still uncertain, although it could contribute to the internal acid metabolism, like aconitase, as it was indicated above.

Formate dehydrogenases (FDHs) carry out the formate oxidation to CO_2, using either NAD or cytochrome *c* as electron acceptors. This enzyme has been detected in mitocondria from *A. thaliana* leaves (Herman et al. 2002). In pepper, the isoform FDH1 from mitocondria has been characterized and is involved in the regulation of cell death and in the response against pathogenic bacteria (du Choi et al. 2014). The detection of this enzyme in plant peroxisomes is anomalous, although this event is not uncommon in yeast where there is an association between formate metabolism and these organelles (Yurimoto et al. 2004). The potential role of FDH in the metabolism of pepper fruit peroxisomes still has to be elucidated.

The biosynthesis of aspartate is facilitated by the aspartate aminotransferase which carries out the reversible transamination between glutamate and oxalacetate to provide aspartate and 2-oxoglutarate, mainly in the photorespiratoty process in C4 plants. The importance of aspartate in higher plants comes from its ability to be precursor of other essential aminoacids such as lysine, threonine, methionine and isoleucine (de la Torre et al. 2014). Aspartate aminotransferase is localized in the cytosol and mitocondria (Offermann et al. 2011), and the isoforms reported in peroxisomes are those displaying glyoxylate aminotransferase activity (Liepman

and Olsen 2001). This issue has to be investigated in pepper fruits since the photorespiratory pathway disappears after fruits ripe to final stages.

Finally, the enzyme aminomethyltransferase, also called subunit T, is one of the four proteins that are components of the complex glycine decarboxylase (GDC). The other proteins of this complex are protein L (dihydrolipoamide dehydrogenase), protein P (glycine dehydrogenase) and protein H which is responsible of the interaction among the other three proteins. In animal cells, this system is essential for the degradation of glycine since it is activated when the concentration of this aminoacid is high (Okamura-Ikeda et al. 2010). In plants, the GDC is an indispensable component of the photorespiration pathway and catalyzes the conversion of glycine into serine that then is transported to peroxisomes for further reactions of this pathway (Reumann 2002). However, all references available so far postulate a mitochondrial localization for GDC in plants, and the presence of this enzyme in peroxisomes from pepper fruits still needs additional confirmation.

As it has been described, most of the proteins detected in peroxisomes from pepper fruits after proteomic analysis have functional association with these organelles, and their presence in these compartments is not anomalous, since they have also been found in peroxisomes from different species. However, these data need further confirmation by alternative methods such as immunolocalization analyses using specific antibodies or recombinant proteins and either electron or fluorescence microscopy.

4 ROS Proteome of Fruit Peroxisomes

Some works have been carried out on the metabolism of peroxisomes in whole fruits. In fact, a rapid search in the Web of Science in November 2017 provided 64 references dealing with peroxisomes from fruits in plants, but only two of them referred to the peroxisomal proteome obtained from purified organelles (Palma et al. 2009, 2015). Pioneer works on the metabolism of peroxisomes isolated from pepper and olive fruits depicted different biochemical scenarios depending on the ripening stage of fruits and determined the involvement of the ROS metabolism of these organelles in the ripening process of both crops (Mateos et al. 2003; López-Huertas and del Río 2014). Thus, in pepper fruit it was found that the glyoxylate cycle was functional in peroxisomes from both immature green and ripe red fruits, whereas the photorespiratory pathway was only present in the green stage. The ROS metabolism was also investigated in those fruits and it was found that whereas the antioxidative enzymes catalase, manganese-containing superoxide dismutase (Mn-SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) decreased at ripening, the superoxide radical-generating system xanthine oxidase remained constant, what led us to propose fruit peroxisomes as relevant players in the ripening process (Fig. 4; Mateos et al. 2003; Palma et al. 2015). In those organelles, the presence of several NADPH-generating dehydrogenases (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase)

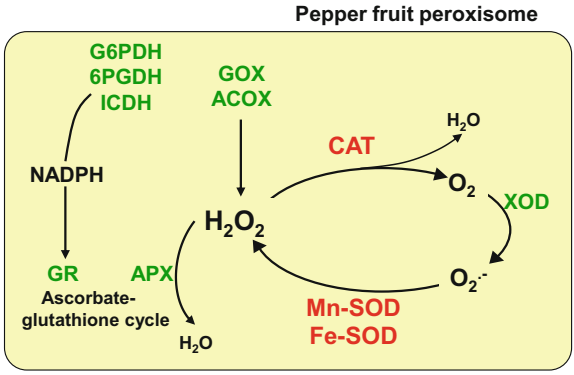

Fig. 4 Model of the metabolism of reactive oxygen species (ROS) in pepper fruit peroxisomes. Hydrogen peroxide (H_2O_2) generated by the photorespiratory enzyme glycolate oxidase (GOX) and the acyl-CoA oxidase (ACOX) from the fatty acid β-oxidation is removed by catalase (CAT) and the ascorbate peroxidase (APX) integrated in the peroxisomal ascorbate–glutathione cycle. The enzyme glutathione reductase (GR) of this cycle uses NADPH produced by glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and isocitrate dehydrogenase (ICDH) present in this organelle. On the other hand, the peroxisomal xanthine oxidase (XOD) activity generates superoxide radicals ($O_2^{\cdot -}$), which are the substrate of the peroxisomal Mn-SOD and Fe-SOD, and the enzyme product, H_2O_2, closes the oxygen cycle in the peroxisome. Those proteins related to the ROS metabolism, which have been identified by proteomic approaches, are marked in red. Modified from Palma et al. (2015)

that provide NADPH for the functioning of the ascorbate-glutathione cycle and other reactions was also proved (for a review see Corpas et al. 2017). However, in peroxisomes purified from olive fruits by sucrose density-gradient centrifugation only the presence of catalase, ascorbate peroxidase and glutathione reductase could be detected by activity and immunoblotting assays (López-Huertas and del Río 2014).

To date, from all the proteomic approaches applied to purified peroxisomes from plant fruits the only report of the presence of ROS-related proteins corresponds to pepper fruits (Palma et al. 2015). The typical peroxisomal marker catalase was identified in both green and red pepper fruits with 100% protein score (Table 1). Its subunit size and isoelectric point ranged those from other plant catalases reported so far. Catalase is one of the first antioxidant lines of defense to regulate cellular H_2O_2 content, which is exclusively located in peroxisomes and catalyzes the reaction: $2H_2O_2 \rightarrow 2H_2O + O_2$. By a nitro-protein analysis of sweet pepper fruit samples, catalase was identified as one of the main targets susceptible to undergo protein nitration and this eventuality increased during the ripening process (Chaki et al. 2015). Nitration is a post-translational modification occurring in some tyrosines of proteins which is promoted by peroxynitrite ($ONOO^-$), a reactive nitrogen species (RNS) that can be produced by the reaction of two radical molecules, nitric oxide (NO) and superoxide ($O_2^{\cdot -}$), both generated inside peroxisomes (Corpas et al. 2013; Corpas and Barroso 2014; del Río and López-Huertas 2016). In in vitro assays

carried out with pepper fruits, it was proved that catalase activity was reduced after incubation of samples with $ONOO^-$, what confirmed the potential nitration of catalase (Chaki et al. 2015). These data are in good agreement with the active metabolism of ROS/RNS in peroxisomes of leaves and roots from other plant species including pea (*Pisum sativum*) and *Arabidopsis* (for a review see Corpas et al. 2017), what suggests that peroxisomes have a dynamic nitro-oxidative metabolism not only under stress conditions but also under physiologal situations such as fruit ripening.

Two types of superoxide dismutases (SODs) were detected in purified peroxisomes from pepper fruits by proteomic analysis: one Mn-SOD and one iron-containing SOD (Fe-SOD) (Palma et al. 2015). The presence of the isozyme Mn-SOD in this subcellular compartment from pepper fruits was already reported by enzymatic analysis and immunoblotting assay using an antibody against the Mn-SOD from pea leaves (Mateos et al. 2003). Mn-SODs are isoyzmes which in plants are commonly located in mitochondria and peroxisomes, and their dual localization could be attributed to alternative splicing or alternative targeting during the transcription processes (Palma et al. 1998, 2013; del Río et al. 2003; Rodríguez-Serrano et al. 2007). Interestingly, the unequivocal localization of a Mn-SOD in fruit chromoplasts from a pepper cultivar has been demonstrated by using biochemical and immunocytochemical approaches (Martí et al. 2009). Regarding to Fe-SOD, this isozyme has been reported only in plant peroxisomes from carnation petals (Droillard and Paulin 1990) and pepper fruits (Palma et al. 2015). Fe-SODs are commonly present in chloroplasts together with CuZn-SODs (del Río et al. 2003) and the reason of this atypical localization of Fe-SOD in plant peroxisomes is still unknown, although considering all the data available, perhaps this situation could only take place in reproductive organs. In any case, the physiological reason of the presence of an Fe-SOD in peroxisomes from pepper fruits requires further research.

5 Conclusions

The biology of fruits is still little studied in comparison with other plant organs such as cotyledons, leaves and roots, perhaps due to their anatomical and metabolic diversity. But, the repercussion in human nutrition and economy makes this material prone to be investigated in more depth. Research at subcellular and molecular levels is necessary to understand the physiology of fruits in order to improve crop qualities. On this basis, proteomic studies from fruits are continuously growing, and the proteome of important crops such as tomato, grape, melon, peach, strawberry and others is being daily uploaded in public forums, either websites, specialized journals, books, etc. However, less attention is being paid to subproteomes including those from chloroplasts, mitochondria, and peroxisomes. Peroxisomes, and specially their metabolism of reactive oxygen species (ROS), have been proved to play an important role during ripening of pepper fruits, and this

model could be also operative in fruits from other crop species. Therefore, efforts addressed to gain knowledge of the peroxisome proteome and how it interacts with the overall metabolism of fruits can provide tools to be used in breeding strategies of agricultural species with added value. Thus far, these are the only proteome data reported from purified fruit peroxisomes. Several proteins with still not totally defined subcellular localization have been detected in peroxisomes from sweet pepper fruits, what implies that these peroxisomal proteins might have some relevance in the organelle metabolism and perhaps in the ripening process.

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Part IV
Peroxisomes in Relation to Other Subcellular Compartments

Peroxisomes and Their Central Role in Metabolic Interaction Networks in Humans

Ronald J. A. Wanders, Hans R. Waterham and Sacha Ferdinandusse

Abstract Peroxisomes catalyze a number of essential metabolic functions and impairments in any of these are usually associated with major clinical signs and symptoms. In contrast to mitochondria which are autonomous organelles that can catalyze the degradation of fatty acids, certain amino acids and other compounds all by themselves, peroxisomes are non-autonomous organelles which are highly dependent on the interaction with other organelles and compartments to fulfill their role in metabolism. This includes mitochondria, the endoplasmic reticulum, lysosomes, and the cytosol. In this paper we will discuss the central role of peroxisomes in different metabolic interaction networks in humans, including fatty acid oxidation, ether phospholipid biosynthesis, bile acid synthesis, fatty acid alpha-oxidation and glyoxylate metabolism.

Keywords Peroxisomes · Fatty acids · Ether phospholipids · Docosahexaenoic acid · Genetic diseases · Refsum disease · Phytanic acid · Tethering proteins Metabolic networks

Abbreviations

ACBP	Acyl-CoA binding protein
AGPS	Alkylglycerone-3-phosphate synthase
AMACR	2-Methylacyl-CoA racemase
ABCD	ATP-binding cassette protein, Family D
ACOT	Acyl-CoA thioesterase
BAAT	Bile acid-CoA: amino acid transferase
BSEP	Bile salt export pump
CACT	Carnitine acylcarnitine translocase
CRAT	Carnitine acetyltransferase
CROT	Carnitine octanoyltransferase

R. J. A. Wanders (✉) · H. R. Waterham · S. Ferdinandusse
Laboratory Genetic Metabolic Diseases, Departments Clinical Chemistry and Pediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
e-mail: r.j.wanders@amc.uva.nl

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DCA	Dicarboxylic acid
DHA	Docosahexaenoic acid
DHCA	Dihydroxycholestanoic acid
ELOVL	Elongation of very long-chain acids protein
EPL	Ether phospholipid
FA	Fatty acid
FAR1/2	Fatty acyl-CoA reductase (fatty alcohol forming) 1/2
FFAT	Phenylalanine Phenylalanine Acidic Tract
GR/HPR	Glyoxylate reductase/hydroxypyruvate reductase
GNPAT	Glycerone-3-phosphate acyltransferase
HAO1	Hydroxy acid oxidase 1 (glycolate oxidase)
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
THCA	Trihydroxycholestanoic acid
PexRAP	Peroxisomal reductase activating PPARgamma
VAP	Vesicle associated protein

1 Introduction

Peroxisomes are dynamic subcellular organelles, which play an essential role in a variety of different metabolic pathways, including the alpha- and beta-oxidation of a distinct series of fatty acids (FAs), the biosynthesis of bile acids and ether phospholipids (EPL) including plasmalogens, and the detoxification of glyoxylate (Wanders and Waterham 2006; Waterham et al. 2016; Van Veldhoven 2010). In order to fulfill their role in each of these metabolic pathways, peroxisomes need to interact with other subcellular organelles, because none of the metabolic pathways in which peroxisomes are involved, can be fully executed by peroxisomes on their own. A good example of the dependence of peroxisomes on other organelles concerns the degradation of FAs such as C26:0 in peroxisomes. For this metabolic pathway peroxisomes require the active interaction with the endoplasmic reticulum (ER), since the ER is the main site of synthesis of C26:0-CoA from shorter-chain FAs via the FAS-complex in the cytosol, followed by chain elongation to C26:0-CoA via the chain-elongation system in the ER. Very long-chain FAs (VLCFAs), like C26:0, are also derived from dietary sources albeit to a limited extent. In addition, VLCFAs such as C26:0 are also released from lysosomes, following the intra-lysosomal degradation of (complex) lipids like C26:0-containing ceramides (Tidhar and Futerman 2013). Whatever the source of C26:0-CoA, the C26:0-CoA needs to be transported to the peroxisomal membrane, followed by the transport of C26:0-CoA across this membrane by ABCD1. The products of beta-oxidation of C26:0-CoA in peroxisomes include acetyl-CoA, a medium-chain acyl-CoA and NADH, which all need to be shuttled to the

mitochondria for full oxidation to CO_2 and H_2O and reoxidation of NADH back to NAD^+, respectively. This example shows that with respect to fatty acid oxidation, peroxisomes are non-autonomous organelles in contrast to mitochondria which can catalyze the oxidation of long, medium and short chain fatty acids down to CO_2 and H_2O all by themselves.

In this paper we will present the current state of knowledge about the interaction between peroxisomes and other organelles with respect to the major metabolic pathways of peroxisomes, at least in humans, including the alpha- and beta-oxidation of FAs, the biosynthesis of EPL and bile acids and the detoxification of glyoxylate. For the role of peroxisomes in ROS/RNS metabolism the reader is referred to the Chapters by Fransen et al. and Corpas et al. in this issue.

2 Effective Peroxisomal Beta-Oxidation Requires a Highly Interconnected Metabolic Network Involving Mitochondria and the Endoplasmic Reticulum

Peroxisomes catalyze the beta-oxidation of a range of different substrates some of which are unique to peroxisomes since they cannot be degraded by mitochondria. These include: (1) saturated very long-chain fatty acids ($C \geq 22$ carbon atoms) including hexacosanoic acid (C26:0); (2) the branched-chain fatty acid pristanic acid (2,6,10,14-tetramethylpentadecanoic acid); (3) the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA), and (4) long-chain dicarboxylic acids (Wanders and Waterham 2006; Van Veldhoven 2010).

Oxidation of these fatty acids requires the interaction with other organelles, which differ for each of the different fatty acids as outlined below.

(a) *Hexacosanoic acid (C26:0)*: It was originally thought that VLCFAs like C26:0 would be derived from dietary products, but early studies, notably by Kishimoto and coworkers (Kishimoto et al. 1980) already established that the bulk of VLCFAs is not derived from exogenous sources but is synthesized from shorter-chain fatty acids like palmitic acid which itself may be synthesized endogenously via the FAS-complex from acetyl-CoA or can be derived from dietary sources (see Fig. 1). Chain elongation of fatty acids is performed by a multi-enzyme chain elongation complex localized in the ER-membrane with each of the four steps catalyzed by multiple enzymes (Kihara 2012). The first step in the elongation of fatty acids or rather of the corresponding acyl-CoAs, is brought about by seven different so-called ELOVLs, which catalyze the malonyl-CoA driven production of 3-ketoacyl-CoAs and differ in their tissue distribution and substrate specificities (see Kihara 2012; Naganuma and Kihara 2014 for review and Fig. 1). Work from our own laboratory has shown that ELOVL1 is the major enzyme responsible for the formation of C26:0-CoA (Ofman et al. 2010).

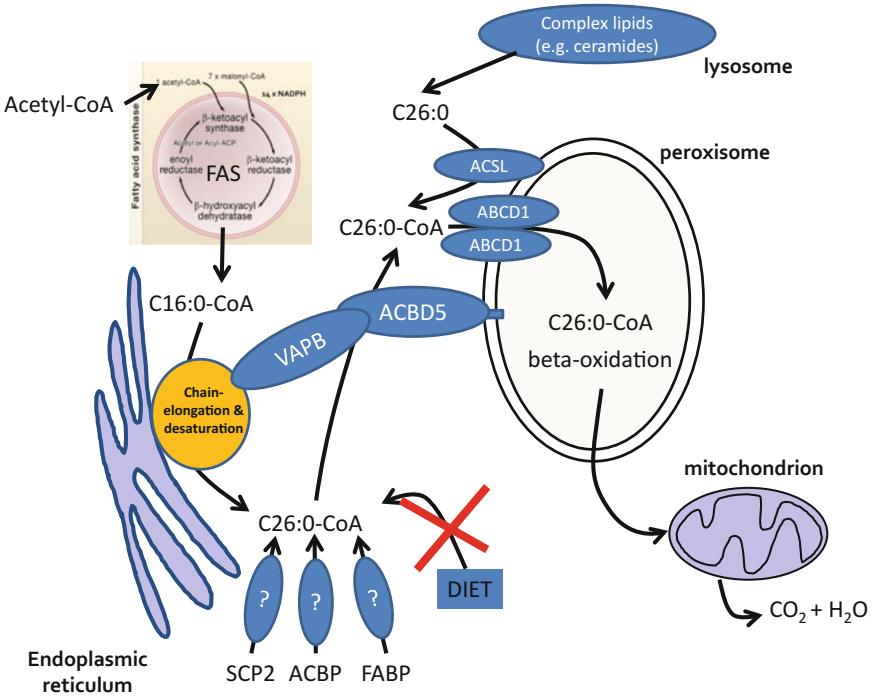

Fig. 1 Very long-chain fatty acid homeostasis in human cells and the complicated interplay between a variety of different subcellular compartments including peroxisomes, lysosomes, mitochondria, the cytosol, and the endoplasmic reticulum (ER). Very long-chain fatty acids like C26:0 start their life as acetyl-CoA in the cytosol followed by their conversion into C16:0-CoA by the Fatty Acid Synthase (FAS)-complex in the cytosol. Subsequently, C16:0-CoA undergoes chain-elongation at the ER membrane via repetitive cycles of chain elongation, after which the C26:0-CoA produced at the site of the ER is shuttled to the peroxisomal membrane, probably mediated by the VAPB-ACBD5 peroxisome-ER tethering complex. After transport of C26:0-CoA across the peroxisomal membrane via ABCD1, beta-oxidation occurs within the peroxisome, followed by full oxidation of the end products of peroxisomal beta-oxidation in mitochondria. Apart from de novo formation of VLCFAs via the concerted action of the FAS-complex and the chain elongation system at the ER, VLCFAs can also be generated within lysosomes following the degradation of VLCFA-containing (complex) lipids

(b) Once formed at the ER membrane, the C26:0-CoA needs to traverse the cytosol to reach the peroxisomal membrane. Spontaneous, non-assisted transfer of acyl-CoAs is very unlikely, especially since long-chain acyl-CoAs are only partially soluble in aqueous environments and are, in fact, predominantly membrane-bound (McGarry 2001; Schroeder et al. 2005). Eukaryotic cells express different members of at least three separate families of cytoplasmic acyl-CoA binding proteins. These include: (1) acyl-CoA binding proteins (ACBP) (Gossett et al. 1996; Faergeman and Knudsen 1997); (2) fatty acid binding proteins (FABP) (McArthur et al. 1999; Frolov et al. 1997), and

(3) sterol-carrier-protein-2 (SCP2) (Frolov et al. 1996; Gallegos et al. 2001). The affinity of C26:0-CoA for any of these proteins has never been tested experimentally and awaits further studies. Recently, however, it has been shown that ACBD5, which is a tail-anchored protein localized in the peroxisomal membrane and equipped with an acyl-CoA binding site, is involved in the transfer of C26:0-CoA from the ER to the peroxisome. The first indication supporting this notion came from studies on a patient with clinical signs and symptoms suggestive for a peroxisomal disorder, who had elevated VLCFAs which turned out to be caused by mutations in the *ACBD5* gene (Ferdinandusse et al. 2017). This report was soon followed by subsequent work, showing that ACBD5 interacts with the ER-resident VAMP-associated proteins A and B (VAPA and VAPB), and that this interaction is required to tether the two organelles together, thereby facilitating the exchange of metabolites which probably includes C26:0-CoA (Fig. 1). The two VAP proteins are also tail-anchored proteins, are both localized in the ER membrane and contain so-called MSP domains which recognize the FFAT-like motif [two phenylalanines (FF) in an acidic tract (AT)] as present in ACBD5 (see Fig. 1). When the interaction of the VAPs with ACBD5 is lost, peroxisome mobility is increased and lipid homeostasis disturbed (Costello et al. 2017; Hua et al. 2017). Once inside the peroxisome, C26:0-CoA undergoes beta-oxidation via acyl-CoA oxidase 1 (ACOX1), D-bifunctional protein, and the two thiolases ACAA1 and sterol carrier protein X (SCPx), respectively (see Fig. 2).

Although not rigorously established, the generally accepted notion holds that C26:0-CoA undergoes a number of cycles of beta-oxidation within peroxisomes until beta-oxidation stops at the level of a C6/C8-medium-chain acyl-CoA. Indeed, in 1978 Lazarow (1978) already showed that rat liver peroxisomes were only able to oxidize acyl-CoAs with a chain length of 8 carbon atoms or more. Later work has shown that rat as well as human ACOX1 has

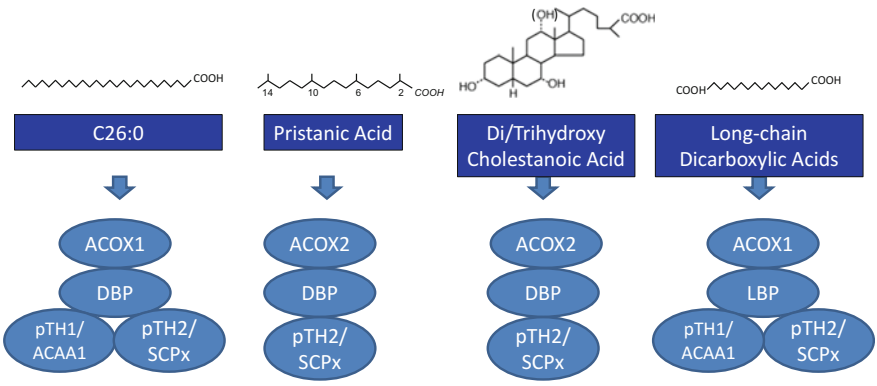

Fig. 2 Schematic diagram showing the identity of the different peroxisomal beta-oxidation enzymes involved in the oxidation of C26:0, pristanic acid, di-and trihydroxycholestanoic acid, and long-chain dicarboxylic acids

two different isoforms produced by the alternative use of two exons 3 which differ in substrate specificity. Rat Acox1-exon3I was found to be most reactive with C10:0-CoA whereas Acox1-exon3II was most reactive with C14:0-CoA. A somewhat different substrate specificity was found for human ACOX1-exon3I and ACOX1-exon3II with the 3I-isoform being most active with C8-C12 acyl-CoAs but not with acyl-CoAs $\geq$ C16 whereas the 3II-isoform was active with all acyl-CoAs with a chain length of 8 carbon atoms or more up to C24 with C12:0-CoA as best substrate. Both isoforms showed virtually no activity with hexanoyl-CoA (Ferdinandusse et al. 2007).

The CoA-esters generated by beta-oxidation in peroxisomes can be shuttled across the peroxisomal membrane to the cytosol by means of two different mechanisms including: (1) a carnitine-mediated pathway involving a carnitine acyl-transferase, and (2) a free-acid route. Whereas the carnitine-mediated export of acyl-CoAs out of the peroxisome is comparable between species, the situation is different for the free-acid route mediated by different acyl-CoA thioesterases. Indeed, in the mouse, four different peroxisomal acyl-CoA thioesterases (Acot3, Acot4, Acot5, and Acot6) were identified as part of a gene cluster spanning 120 kb on mouse chromosome 12D3 which consists of 6 thioesterase genes in total, whereas in humans a similar gene cluster was identified on chromosome 14q24.3 spanning 80 kb which contains only 4 thioesterase genes (Hunt et al. 2006). Remarkably, only one of these 4 genes turned out to code for a peroxisomal thioesterase (Hunt et al. 2006). The gene involved (*ACOT4*) showed the highest similarity to the mouse *Acot4* gene but surprisingly the kinetic parameters of the human enzyme were quite different from that of mouse Acot4. Both mouse Acot4 and human ACOT4 show highest activity with succinyl-CoA, but the human enzyme is also active with a range of long-chain acyl-CoAs of >8 carbon atoms and is also active with several unsaturated acyl-CoAs including C18:1-CoA, C18:2-CoA, and C20:4-CoA (Hunt et al. 2006) which is in marked contrast to the mouse enzyme (Westin et al. 2005). Since the pattern of activity of human ACOT4 mirrors the combined activity of mouse Acot3, Acot4 and Acot5, Hunt et al. (2006) concluded that the human peroxisomal ACOT4 can replace the function of the three distinct peroxisomal thioesterase enzymes in the mouse.

Interestingly, the expression of carnitine octanoyltransferase (Crot), carnitine acetyltransferase (Crat), and the different acyl-CoA thioesterases (Acot) was found to differ markedly between mouse tissues as shown by Westin et al. (2008). This was not only true for Crot and peroxisomal Crat, but also for the different Acots. In mice, Crot showed highest expression in the liver and proximal intestine followed by kidney and the distal intestine with low expression in all other tissues, whereas peroxisomal Crat showed low expression in all tissues including liver and the proximal intestine, but showed highest activities in brown adipose tissue (BAT) followed by white adipose tissue (WAT) and heart. Based on these findings, Westin et al. (2008) concluded that the fate of the medium-chain acyl-CoAs as well as short-chain acyl-CoAs,

including acetyl-CoA and propionyl-CoA, is different among tissues with: (1) acetate and propionate being the predominant products in liver, kidney and intestine; (2) acetyl- and propionylcarnitine in BAT, WAT, and the heart; (3) medium-chain FAs in brain and WAT, and (4) medium-chain acylcarnitines in liver, kidney and intestine (see Fig. 3). The underlying physiological relevance of this remarkable difference in expression of the various enzymes has remained unresolved so far (see Westin et al. 2008 for discussion). One logical option would be that the acylcarnitines produced in peroxisomes are primarily shuttled to mitochondria for full oxidation to CO_2 and H_2O as mediated by the mitochondrial carnitine acylcarnitine carrier (CACT; SLC25A20) whereas the short-chain and medium-chain FAs released from peroxisomes, may be shuttled to mitochondria, but can also move to the extracellular space. In line with this, it has long been established that acetate is the primary product of peroxisomal beta-oxidation in the liver as first shown by Leighton and coworkers (1989) and is readily released from the liver to be oxidized in other tissues.

(c) *Pristanic acid (2,4,10,14-tetramethylpentadecanoic acid)*: Pristanic acid is not synthesized endogenously but is solely derived from exogenous sources either directly or indirectly from phytanic acid after one round of alpha-oxidation. Since the alpha-oxidation machinery is strictly peroxisomal, activation of pristanic acid generated from phytanic acid is believed to occur within the

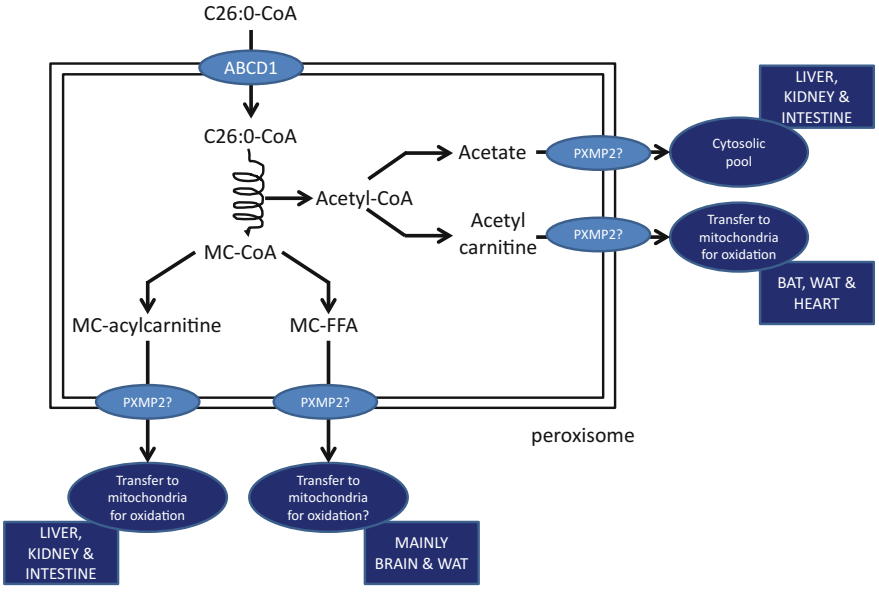

Fig. 3 Schematic diagram depicting the basic principle of C26:0-CoA beta-oxidation in peroxisomes and the transfer of the products of peroxisomal beta-oxidation of C26:0-CoA including acetyl-CoA and a medium-chain acyl-CoA to the cytosol, either as free acid or as carnitine ester and their different fates depending upon the type of tissue

peroxisome, whereas the pristanic acid derived from the diet is probably activated by extra-peroxisomal synthetase(s) after which pristanoyl-CoA enters the peroxisome via one of the half-ABC transporters, notably ABCD3 (PMP70) in order to be beta-oxidized. Although not proven definitively, activation of intra-peroxisomal pristanic acid, as generated from phytanic acid, is probably mediated by the enzyme very long-chain acyl-CoA synthetase encoded by *SLC27A2* which also codes for an isoform in the ER (Steinberg et al. 1999). The identity of the extra-peroxisomal pristanoyl-CoA synthetase has not been identified with certainty (see Fig. 4). Nevertheless, our own work has shown that peroxisomes as well as mitochondria and microsomes contain pristanoyl-CoA synthetase activity (Wanders et al. 1992).

Intraperoxisomal pristanoyl-CoA undergoes three cycles of beta-oxidation in the peroxisome after which the different products including 4,8-dimethylnonanoyl-CoA, propionyl-CoA and acetyl-CoA are exported out of the peroxisome via the carnitine-mediated route and/or the free-acid route. Indeed, making clever use of fibroblasts with a genetic deficiency of the mitochondrial carnitine acyl-carnitine carrier (CACT/SLC25A20), Verhoeven et al. (1998) discovered the accumulation of 4,8-dimethylnonanoyl-carnitine as main product of peroxisomal beta-oxidation next to acetyl- and propionylcarnitine. Subsequent work revealed that CROT is the enzyme which generates 4,8-dimethylnonanoyl-carnitine from the corresponding CoA-ester (Fig. 4) (Ferdinandusse et al. 1999). Furthermore, we established that peroxisomes also contain 4,8-dimethylnonanoyl-CoA thioesterase activity, at least in rat liver peroxisomes (Ofman et al. 2002). The identity of the thioesterase involved has been determined to be Acot8 (see Hunt and Alexson 2002). It should be noted that in vivo pristanic acid occurs in two different stereoisomers, including (2*R*,6*R*,10*R*,14)-tetramethylpentadecanoic acid and (2*S*,6*R*,10*R*,14)-tetramethylpentadecanoic acid. Since acyl-CoA oxidases in general only accept (2*S*)-branched-chain acyl-CoAs as substrate, conversion of (2*R*)- to (2*S*)-acyl-CoAs must occur which is mediated by the enzyme 2-methylacyl-CoA racemase (AMACR). The enzyme involved is a monomeric protein of 43 kDa present in peroxisomes and mitochondria which catalyzes the interconversion of a range of different 2-methyl-branched-chain acyl-CoAs. Interestingly, the peroxisomal and mitochondrial forms of AMACR are the products of a single gene which is targeted to the different organelles by virtue of an amino terminal mitochondrial targeting signal (MTS) and carboxy terminal PTS1 (-KASL in human AMACR).

(d) *Long-chain dicarboxylic acids*: Dicarboxylic acids (DCA) are generated from monocarboxylic acids by omega-oxidation via a 3-step mechanism which involves hydroxylation, followed by two subsequent steps of dehydrogenation. Hydroxylation of monocarboxylic acids is catalyzed by one of a variety of different cytochrome P450 enzymes localized at the ER membrane after which the omega-hydroxymonocarboxylic acids are converted into the corresponding dicarboxylic acid through the sequential action of cytosolic long-chain alcohol

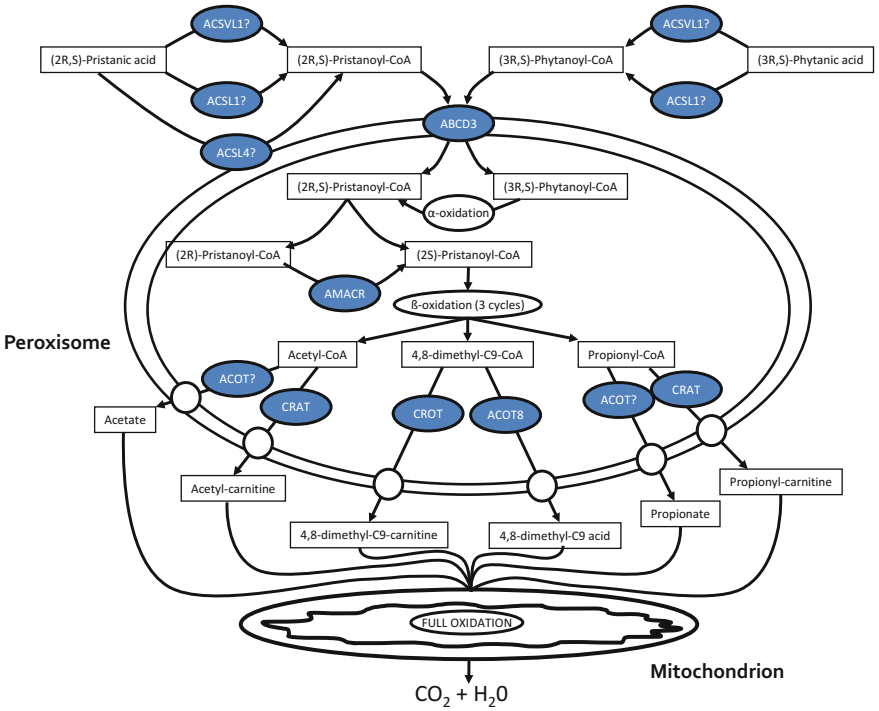

Fig. 4 Schematic diagram depicting the oxidation of pristanic acid in peroxisomes. Pristanic acid is derived from phytanic acid (3, 7, 11, 15)-tetramethylhexadecanoic acid, which occurs in two different isoforms including (3*R*, 7*R*, 11*R*, 15)-tetramethylhexadecanoic acid and (3*S*, 7*R*, 11*R*, 15)-tetramethylhexadecanoic acid. The two different isomers can be activated by one of a variety of different acyl-CoA synthetases. The two phytanoyl-CoA esters are then transported across the peroxisomal membrane by ABCD3. Once inside, (3*S*)- and (3*R*)-phytanoyl-CoA undergo alpha-oxidation to produce (3*S*, 6*R*, 10*R*, 14)-pentadecanoyl-CoA and (2*R*, 6*R*, 10*R*, 14)-tetramethyl pentadecanoyl-CoA which then undergo three rounds of beta-oxidation in peroxisomes to produce 4,8-dimethylnonanoyl-CoA (4,8-dimethyl C9-CoA), propionyl-CoA, and acetyl-CoA, which are then transported out of the peroxisome via two different routes including the free-acid route or the carnitine-mediated route. (2*R*, 6*R*, 10*R*,14)-pristanoyl-CoA can only undergo beta-oxidation if the (2*R*)-group is converted into the (2*S*)-group by means of the enzyme 2-methylacyl-CoA racemase (AMACR)

and aldehyde dehydrogenases. After activation by a specific dicarboxylyl-CoA synthetase, present in microsomes, at least in rat liver (Vamecq et al. 1985), the dicarboxylyl-CoA esters are shortened via beta-oxidation. Although in vitro studies with isolated organelles have shown that peroxisomes as well as mitochondria can catalyze the oxidation of dicarboxylic acids, studies in intact cells have shown that at least C16DCA is exclusively oxidized in peroxisomes (Ferdinandusse et al. 2004). Although not tested experimentally, the C16DCA probably undergoes a number of cycles of beta-oxidation in the peroxisome until a medium-chain dicarboxylyl-CoA is formed, which is then transported

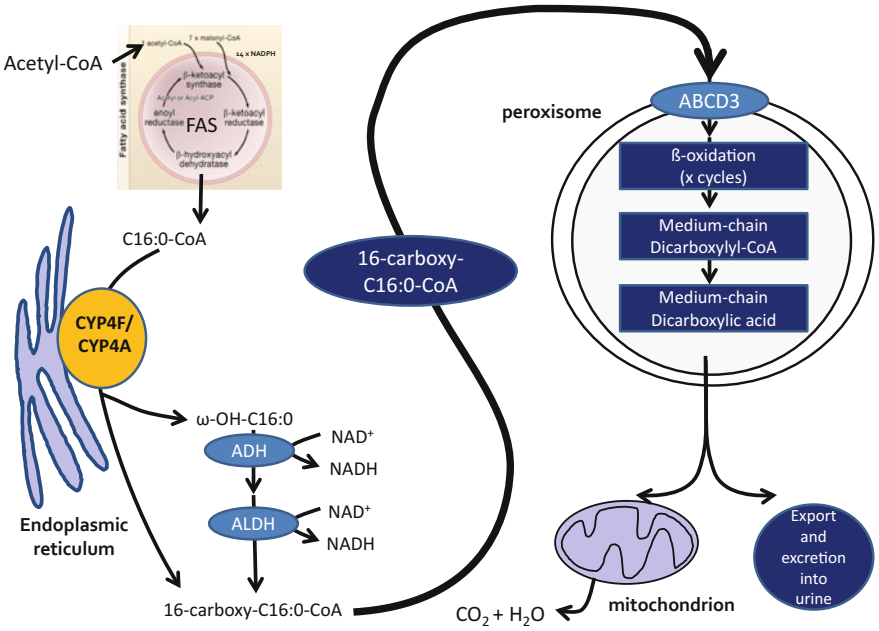

Fig. 5 Synthesis and degradation of long-chain dicarboxylic acids and the complicated interplay between different subcellular organelles. A typical long-chain dicarboxylic acid like hexadecadioic acid can either be formed de novo from acetyl-CoA as mediated by the FAS-complex or can be produced directly from palmitic acid via the omega-oxidation system in the endoplasmic reticulum (ER) with CYP4A and/or CYP4F as the main cytochrome P450 hydroxylases catalyzing the formation of omega-hydroxy-C16:0-CoA followed by the formation of the dicarboxylic acid via one of two different mechanisms involving CYP4A and/or CYP4F or an alternative route including an alcohol and aldehyde dehydrogenase. This is followed by activation of the dicarboxylic acid via an ER-bound acyl-CoA synthetase followed by transfer of the CoA-ester to the peroxisomal membrane, transport across the peroxisomal membrane by means of ABCD3 and beta-oxidation in the peroxisome. The products of dicarboxylic acid beta-oxidation in peroxisomes include acetyl-CoA and probably different medium-chain dicarboxylyl-CoAs whose identity has not been established definitively, followed by their transport out of the peroxisome most likely in their free-acid form

out of the peroxisome for further oxidation in mitochondria either via the carnitine- or free-acid route (see Fig. 5).

(e) *Docosahexaenoic acid synthesis (C22:6 n − 3)*: Docosahexaenoic acid (DHA) is one of the major n-3 polyunsaturated fatty acids (PUFAs) in adult mammalian brain and retina. A deficiency of DHA is associated with memory loss, learning disabilities and impaired vision acuity (Sun et al. 2017). DHA can be derived from dietary sources but can also be synthesized endogenously from linolenic acid (C18:3 n − 3) via a series of chain-elongation and desaturation reactions. Pioneering work by Sprecher and coworkers (1995) has revealed that this pathway cannot synthesize DHA directly, since this would require the active participation of a delta-4-desaturase, which turned out not to exist in

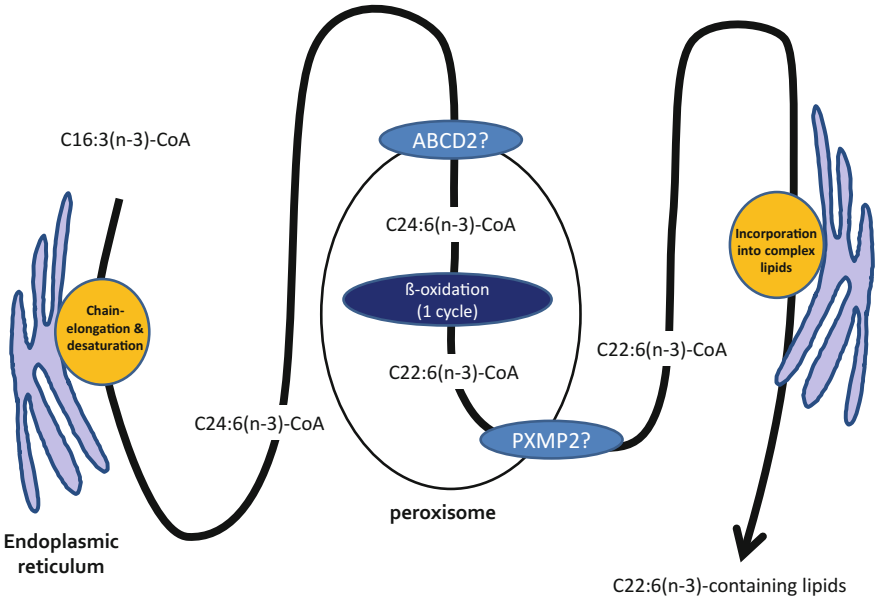

Fig. 6 Formation of docosahexaenoic acid (C22:6 n − 3) and its subsequent incorporation into complex lipids. Docosahexaenoic acid is produced from C16:3 (n − 3)-CoA which undergoes chain elongation and desaturation at the endoplasmic reticulum (ER) membrane to produce C24:6 (n − 3)-CoA, which is then transported to the peroxisomal membrane after which import of C24:6 (n − 3)-CoA occurs, possibly via ABCD2. C24:6 (n − 3)-CoA then undergoes one cycle of beta-oxidation, after which the C22:6 (n − 3)-CoA is converted into the free acid followed by its export from the peroxisome back to the ER for incorporation into complex lipids

mammalian cells. Instead, C18:3 n − 3 is first elongated and desaturated up to C24:6 n − 3, which then undergoes one cycle of beta-oxidation in the peroxisome followed by transfer back to the ER for incorporation in different lipid species (Fig. 6) (Ferdinandusse et al. 2001, 2003).

3 Effective Bile Acid Synthesis Requires a Highly Interconnected Metabolic Network Involving the Cytosol, Mitochondria, Endoplasmic Reticulum and Peroxisomes

Although bile acid synthesis is not strictly a “biosynthetic” process in the sense of an anabolic pathway but is in fact a catabolic pathway in which cholesterol, which contains 27 carbon atoms, is ultimately split into two parts, including the primary bile acids, cholic acid and chenodeoxycholic acid—which both contain 24 carbon

atoms—and propionic acid, we will discuss bile acid synthesis separately. Formation of cholic acid and chenodeoxycholic acid from cholesterol involves the complicated interaction between a large variety of enzymes localized in different compartments (Fig. 7), which can be separated into 5 distinct parts including: (1) the conversion of cholesterol to the CoA-esters of DHCA and THCA; (2) the transport of DHC-CoA and THC-CoA from the ER membrane to the peroxisome, followed by uptake into the peroxisome interior; (3) the cleavage of the side-chain of DHC-CoA and THC-CoA by beta-oxidation to produce chenodeoxycholoyl-CoA and choloyl-CoA; (4) the formation of the taurine or glycine esters, and (5) the export of tauro/glycocholate and tauro/

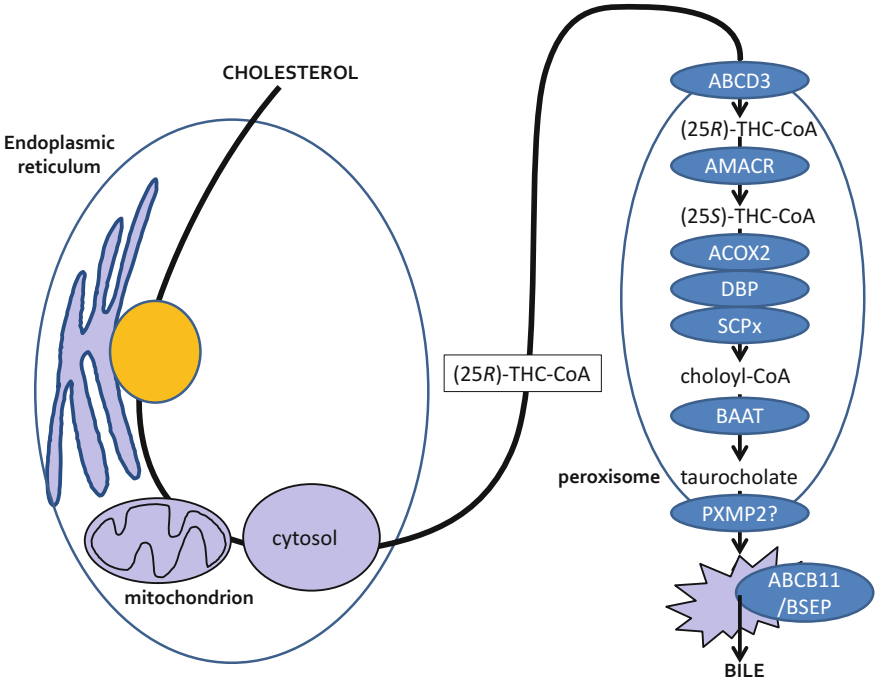

Fig. 7 Formation of the primary bile acids, cholic acid and chenodeoxycholic acid, from cholesterol and the complicated interplay between different subcellular compartments including the endoplasmic reticulum, mitochondria, cytosol and peroxisomes. Bile acids start their life as cholesterol which is converted into di- and trihydroxycholestanoic acid via the concerted action of a large number of different enzymes localized in the endoplasmic reticulum (ER), mitochondrion and the cytosol. Both DHCA and THCA are activated at the site of the ER membrane and are then transferred to the peroxisomal membrane, where uptake into the peroxisome takes place via ABCD3. After one round of beta-oxidation, chenodeoxycholoyl-CoA and choloyl-CoA are produced, which are then converted into the corresponding taurine and/or glycine esters via the enzyme bile acid-CoA: aminoacid transferase (BAAT), which is exclusively localized in peroxisomes. After export of tauro/glycocholate and tauro/glycochenodeoxycholate, the primary bile acids are extruded into bile mediated by the bile canalicular transporter ABCB11, also known as bile salt export protein (BSEP)

glycochenodeoxycholate out of the peroxisomes to the canalicular membrane followed by extrusion into bile.

Without going into full details about the bile acid synthesis pathway (see Russell 2009; Vaz and Ferdinandusse 2017 for reviews), it is important to emphasize that DHCA and THCA are produced from cholesterol via a series of different enzymes located in the ER, the cytosol and mitochondria and are then activated at the site of the ER membrane. Both Schepers et al. (1989) and Prydz et al. (1988) resolved that activation of DHCA and THCA is catalyzed by microsomes and not peroxisomes. The identity of the acyl-CoA synthetase involved has not been established definitively, although ACSVL1 has been claimed to be the responsible enzyme (Mihalik et al. 2002). However, this conclusion remains controversial, giving the normal levels of the bile acid intermediates in *Acsvl1*$^{-/-}$ mice (Heinzer et al. 2003). After activation at the ER membrane, DHC-CoA and THC-CoA have to move from the ER to the peroxisomal membrane. Whether this involves one of the acyl-CoA binding proteins like SCP2 has not been established and the same is true for the potential involvement of the VAPB-ACBD5-tether as discussed above for C26:0-CoA. What has been established is that DHC-CoA and THC-CoA enter the peroxisome via the half ABC transporter ABCD3 (PMP70) as recently concluded following the identification of a patient with a full deficiency of ABCD3 (PMP70) in whom there was marked accumulation of the bile acid intermediates combined with similar findings in an Abcd3 knockout mouse (Ferdinandusse et al. 2015). The actual beta-oxidation of DHC-CoA and THC-CoA proceeds via the branched-chain acyl-CoA oxidase (ACOX2), D-bifunctional protein, and SCPx as shown schematically in Fig. 2. Subsequent exchange of the CoA unit present in DHC-CoA and THC-CoA for taurine or glycine is mediated by the enzyme bile acid-CoA: amino acid transferase (BAAT), which was originally thought to be localized both in the cytosol and in peroxisomes, but has been shown to be fully peroxisomal, at least in rat hepatocytes (Pellicoro et al. 2007). After the export from the peroxisome, the taurine/glycine esters are shuttled to the bile canalicular membrane which contains a number of different transporters, including the Bile Salt Export Pump (BSEP; ABCB11) which catalyzes the ATP-driven export of tauro/glycocholate and tauro/glycochenodeoxycholate from the hepatocyte into bile.

4 Glyoxylate Metabolism Requires a Highly Interconnected Metabolic Network Involving Mitochondria, Peroxisomes and the Cytosol to Be Effective

Glyoxylate is a highly toxic substance by virtue of the fact that it may undergo rapid oxidation to oxalate which forms insoluble crystals with calcium. These calcium oxalate crystals precipitate in various organs, notably the kidneys, causing renal failure. This implies that glyoxylate needs to be detoxified rapidly since otherwise

glyoxylate will be converted into oxalate via the enzyme lactate dehydrogenase which is an ubiquitous enzyme expressed in all eukaryotic cells. Although not all aspects of glyoxylate metabolism have been resolved definitively, it is clear that glyoxylate is produced in different compartments, including the mitochondria (from 4-hydroxyproline, a degradation product of collagen), the cytosol, and in peroxisomes (see Fig. 8). The glyoxylate generated within mitochondria and the cytosol is rapidly converted into glycolate by the enzyme glyoxylate reductase (GR/HPR), which has a mitochondrial and cytosolic isoform. The high NADPH/$NADP^+$ ratios in both the mitochondrial and cytosolic space drive the GR/HPR reaction into the direction of glycolate formation, which ensures that glyoxylate will be rapidly detoxified at least under energized conditions [when the NADPH/$NADP^+$ ratio is high (>100)]. The peroxisome plays a unique role in the detoxification of glyoxylate since the only way to get rid of the glycolate generated in the mitochondrion and cytosol definitively is to convert glyoxylate into glycine within the peroxisome via the concerted action of the two peroxisomal enzymes glycolate oxidase (HAO1) and alanine glyoxylate aminotransferase (AGXT). The crucial role of GR/HPR and AGXT in the detoxification of glyoxylate as depicted in Fig. 8 is exemplified by the existence of two different forms of hyperoxaluria in man in which glyoxylate metabolism is disturbed due to mutations in the genes coding for *GR/HPR* and *AGXT* (Salido et al. 2012). The hyperoxaluria in these two genetic disorders is associated with usually very severe clinical signs and symptoms including the loss of kidney function, which in many cases can only be resolved by combined liver-kidney transplantation.

5 Effective Phytanic Alpha-Oxidation Requires a Highly Interconnected Metabolic Network Involving Peroxisomes and Mitochondria

Another unique function of peroxisomes involves the alpha-oxidation of 3-methyl branched-chain fatty acids. The latter FAs can only be degraded by beta-oxidation if the terminal carboxy group is first released by alpha-oxidation to generate the corresponding 2-methyl FA which can then be oxidized by beta-oxidation. Like all FAs, 3-methyl FAs can also undergo omega oxidation although this is usually regarded as a minor metabolic pathway (Wanders et al. 2011). Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is the most predominant 3-methyl FA present in our daily diet and as such it is taken up into cells either in its free acid form or in an esterified form, since phytanic acid is known to occur in multiple different lipid species, including lipoproteins (Wierzbicki et al. 1999).

After release of phytanic acid from these different lipid species, which (mainly) occurs in the lysosome, phytanic acid needs to be converted into phytanoyl-CoA via one of the many members of the ACS family. Although the identity of the enzymes involved has not been established with certainty, enzymatic studies have

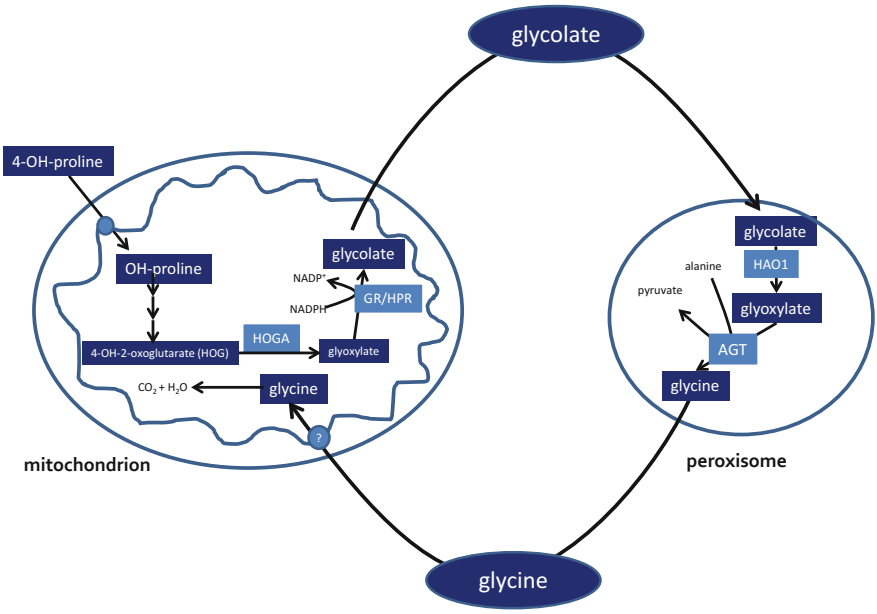

Fig. 8 Glyoxylate homeostasis in human hepatocytes. Glyoxylate is either produced in the cytosol or inside mitochondria from 4-hydroxyproline. In mitochondria glyoxylate is converted into glycolate via the enzyme glyoxylate reductase (GR/HPR) followed by export of glycolate to the cytosol and subsequent uptake into peroxisomes, probably via one of the peroxisomal porines including PXMP2. Once inside the peroxisome, glycolate undergoes oxidation to glyoxylate via the enzyme glycolate oxidase (HAO1) after which glyoxylate is converted into glycine via the enzyme alanine glyoxylate aminotransferase (AGXT). The glycine is then shuttled back to the mitochondrion where glycine is cleaved via the glycine cleavage system to CO_2 and H_2O

shown that activation of phytanic acid can occur in mitochondria, peroxisomes and the ER (see Wanders et al. 2011). After activation, phytanoyl-CoA has to be transported to the peroxisome, followed by transport into the organelle, probably mediated by ABCD3 (PMP70) (Ferdinandusse et al. 2015). Once inside, the alpha-oxidation machinery consisting of phytanoyl-CoA 2-hydroxylase, 2-hydroxyacyl-CoA lyase, pristanal dehydrogenase, and pristanoyl-CoA synthetase takes care of the oxidative decarboxylation of phytanoyl-CoA to produce pristanoyl-CoA which then undergoes beta-oxidation as described above. As described in detail in our 2011 review (Wanders et al. 2011), alpha-oxidation in peroxisomes heavily relies on the proper interaction with mitochondria for the provision of 2-oxoglutarate required in the phytanoyl-CoA hydroxylase reaction, the reoxidation of the NADH generated in the pristanal dehydrogenase step and the provision of ATP as required in the pristanoyl-CoA synthetase step (see Fig. 9). Although all these molecules are in principle water-soluble, future work has to reveal whether the close interaction between peroxisomes and mitochondria with respect to alpha-oxidation requires tethering of the organelles or not.

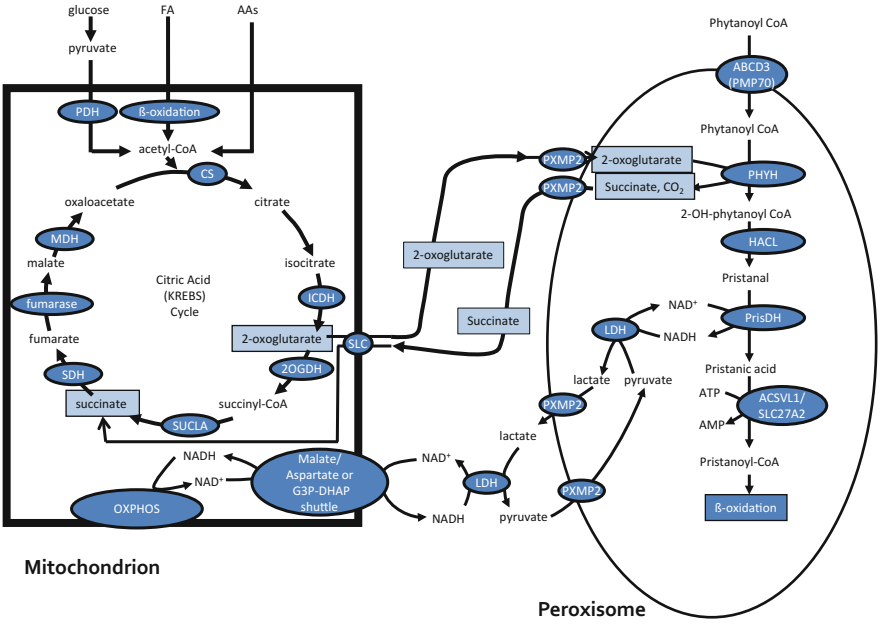

Fig. 9 Phytanic acid metabolism in human cells and the complicated interplay between peroxisomes and mitochondria. Phytanic acid first undergoes activation via one of several different acyl-CoA synthetases to produce phytanoyl-CoA, which then enters the peroxisome via ABCD3 (PMP70). The first step involved in phytanic acid alpha-oxidation is catalyzed by the enzyme phytanoyl-CoA 2-hydroxylase, which generates 2-hydroxyphytanoyl-CoA from phytanoyl-CoA. This enzyme reaction requires 2-oxoglutarate, which is derived from the mitochondrion in the citric acid (Krebs) cycle. The product of the phytanoyl-CoA hydroxylase reaction is succinate, which is then shuttled back to the mitochondrion to enter the citric acid cycle and produces 2-oxoglutarate, which can then undergo another cycle of phytanoyl-CoA 2-hydroxylase-mediated conversion of phytanoyl-CoA into 2-hydroxyphytanoyl-CoA. Furthermore, the NADH produced in the pristanal dehydrogenase step in peroxisomes is ultimately reoxidized by the oxidative phosphorylation system in mitochondria as mediated by the malate/aspartate- or glycerol-3-phosphate/dihydroxyacetone phosphate-shuttle (see text for more details)

6 Effective Ether Phospholipid Synthesis Requires a Highly Interconnected Metabolic Network Involving Peroxisomes and the Endoplasmic Reticulum

Another major function of peroxisomes concerns the biosynthesis of ether phospholipids of which at least the first few steps are unique to peroxisomes (Fig. 10). The underlying basis for the unique role of peroxisomes in EPL synthesis is the fact that higher eukaryotes only contain a single enzyme able to catalyze the formation of the characteristic ether-bond in ether phospholipids. This unique enzyme is alkylglycerone phosphate synthase (AGPS), which catalyzes the formation of

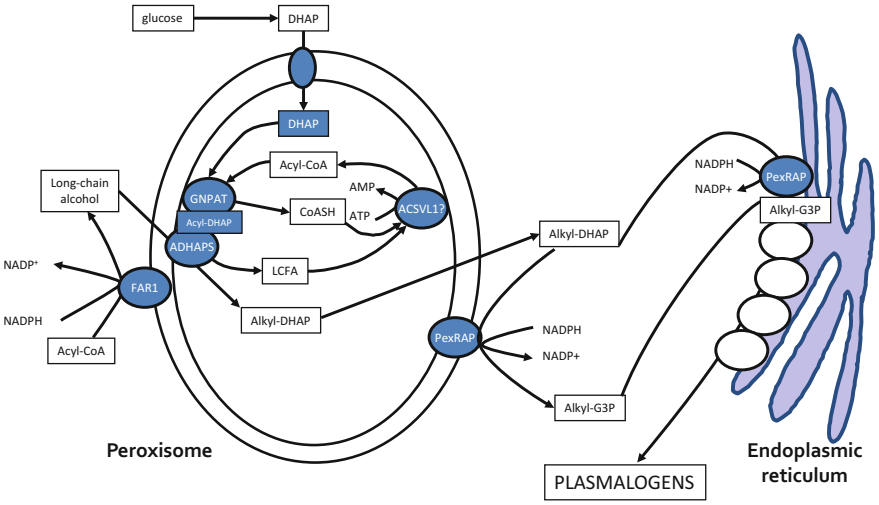

Fig. 10 Schematic diagram depicting the subcellular organization of the enzymatic machinery required to synthesize ether phospholipids. For details see main text

alkylglycerone phosphate from acylglycerone-3-phosphate. The acylglycerone-3-phosphate required in the AGPS reaction is provided by the enzyme glycerone-3-phosphate acyltransferase (GNPAT) from glycerone-3-phosphate and an acyl-CoA ester whereas the long-chain alcohol is provided by one of two different acyl-CoA reductases encoded by *FAR1* and *FAR2*. Interestingly, GNPAT and AGPS form a heterotrimeric enzyme complex attached to the peroxisomal membrane facing the peroxisome interior, whereas the acyl-CoA reductase is tail-anchored into the peroxisomal membrane facing the cytosol (Fig. 10). All three enzymes play an indispensable role in EPL biosynthesis as exemplified by the finding that EPL biosynthesis is fully deficient in patients with a deficiency of AGPS, GNPAT and FAR1 (see Wanders et al. 1992, 1994; Buchert et al. 2014). The product of the concerted action of AGPS, GNPAT and FAR1/2 is alkylglycerone-3-phosphate which is then converted into alkylglycerol-3-phosphate followed by a series of reactions not specified here but all localized in the ER (Malheiro et al. 2015; Braverman and Moser 1822; Dean and Lodhi 2017). The identity of the enzyme which catalyzes the reduction of alkylglycerone-3-phosphate into alkylglycerol-3-phosphate has long remained an enigma until recently (Lodhi et al. 2012), although the enzyme was already purified by LaBelle and Hajra in 1974 (LaBelle and Hajra 1974). The gene involved is *Dhrs7b* and the protein is named PexRAP (for peroxisomal reductase activating PPARgamma) (Lodhi et al. 2012). In contrast to the findings in the *Gnpat*$^{-/-}$ mouse, in which EPLs were found to be completely deficient (Rodemer et al. 2003), EPLs were only partially deficient (50–80%) in the *Dhrs7b*$^{-/-}$ mice. Apparently, PexRAP is not fully obligatory for EPL biosynthesis like FAR1, AGPS, and GNPAT, which may either point to the existence of a second reductase or an alternative pathway, which allows formation

of alkylglycerone-3-phosphate via some bypass. It has been suggested that this bypass involves dephosphorylation of alkylglycerone-3-phosphate to alkylglycerone, followed by its reduction to alkylglycerol and phosphorylation to produce alkylglycerol-3-phosphate (James et al. 1997). Whatever the mechanism, the alkylglycerone-3-phosphate needs to be shuttled from the peroxisome to the ER where all subsequent steps take place. It has been suggested that the VAPB-ACBD5 tether plays a role in this process (Hua et al. 2017).

7 Conclusions

Peroxisomes play a crucial role in cellular metabolism as exemplified by the existence of many different genetic diseases in which one or more peroxisomal functions are impaired. Most of these metabolic functions of peroxisomes are catabolic which implies the formation of degradation products. Ultimate degradation of these products to CO_2 and H_2O requires the active participation of mitochondria, since the citric acid cycle and oxidative phosphorylation system, which are required for this purpose, are solely confined to mitochondria. The interdependence between peroxisomes and mitochondria applies to all major functions of peroxisomes including fatty acid alpha- and beta-oxidation, glyoxylate detoxification but not ether phospholipid biosynthesis. Apart from the obligatory connection between peroxisomes and mitochondria, peroxisomes also require interaction with the ER for EPL synthesis, bile acid formation and fatty acid beta-oxidation, and the same is true for the interaction between peroxisomes and lysosomes, since several of the FAs degraded by alpha- and/or beta-oxidation in the peroxisomes are generated within lysosomes. At present, we are only at the very beginning of the understanding of the mechanisms behind these interactions and future studies will reveal the exact extent and nature of these inter-organellar interactions.

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Characterization of Peroxisomal Regulation Networks

Fred D. Mast and John D. Aitchison

Abstract Peroxisome proliferation involves signal recognition and computation by molecular networks that direct molecular events of gene expression, metabolism, membrane biogenesis, organelle proliferation, protein import, and organelle inheritance. Peroxisome biogenesis in yeast has served as a model system for exploring the regulatory networks controlling this process. Yeast is an outstanding model system to develop tools and approaches to study molecular networks and cellular responses and because the mechanisms of peroxisome biogenesis and key aspects of the transcriptional regulatory networks are remarkably conserved from yeast to humans. In this chapter, we focus on the complex regulatory networks that respond to environmental cues leading to peroxisome assembly and the molecular events of organelle assembly. Ultimately, understanding the mechanisms of the entire peroxisome biogenesis program holds promise for predictive modeling approaches and for guiding rational intervention strategies that could treat human conditions associated with peroxisome function.

Keywords Peroxisome biogenesis · EGRIN · Kinetic modeling
ASSURE motif · Systems cell biology · Multiscale modeling

Abbreviations

Adr1	Alcohol dehydrogenase II synthesis regulator
ASSURE	Asymmetric self-upregulated network motif
AOPY	Adr1, Oaf1, Pip2, Oaf3 regulatory motif
E12	Immunoglobulin enhancer-binding factor 12
EGRIN	Environment and gene regulatory inference network
GEO	Gene expression omnibus

F. D. Mast · J. D. Aitchison (✉)
Center for Infectious Disease Research, 307 Westlake Avenue North, Suite 500, Seattle, WA 98109-5219, USA
e-mail: john.aitchison@cidresearch.org

F. D. Mast · J. D. Aitchison
Institute for Systems Biology, 401 Terry Avenue North, Seattle, WA 98109-5219, USA

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GRIN	Gene regulatory inference network
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
Kss1	Kinase suppressor of Sst2 (supersensitive 2) mutations
LXRα	Liver X receptor alpha
MEME	Multiple expectation maximization for motif elicitation
MyoD	Myoblast determination protein 1
Oaf1	Oleate-activated transcription factor 1
Oaf3	Oleate-activated transcription factor 3
PBD	Peroxisome biogenesis disorder
PEX	Peroxisomal gene/protein (e.g., Pex3)
Pho85	Phosphate metabolism 85
Pip2	Peroxisome induction pathway 2
PPARα	Peroxisome proliferator activated receptor alpha
PTS1	Peroxisome targeting sequence type 1
PTS2	Peroxisome targeting sequence type 2
RAR	Retinoic acid receptor
Rtn1	Reticulon 1
ROS	Reactive oxygen species
RXR	Retinoid X receptor
UTR	Untranslated region
Yop1	YIP (YPT (yeast protein 2) interacting partner) one partner

1 Peroxisomes and Regulatory Networks

Peroxisomes are ubiquitous, dynamic organelles with regulated metabolic roles including β-oxidation of long chain fatty acids, bile acid and plasmalogen synthesis, and decomposition of H_2O_2 and ROS (van den Bosch et al. 1992; Lazarow and De Duve 1976; Lazarow and Fujiki 1985). Peroxisomes communicate with other cellular compartments and influence development, cell morphogenesis and differentiation (Titorenko and Rachubinski 2004). Dysfunctional peroxisome assembly results in inherited genetic disorders, known as peroxisome biogenesis disorders (PBDs) (Moser and Moser 1996; Steinberg et al. 2006; Gould and Valle 2000). The PBDs stem from mutations in *PEX* genes (PEroXisomal biogenesis factor) and are often fatal in infancy (Lazarow and Moser 1994). Symptoms include severe mental retardation, neuronal migration defects, neurodegeneration, renal and hepatic dysfunction (Wanders 1999; Lazarow and Moser 1994; Steinberg et al. 2006; Moser and Moser 1996). PBDs are generally estimated to occur at 1/25,000 to 1/50,000 live births; however, mutations in three *PEX* genes were recently found to underlie late-onset or slowly progressing neurological disorders (Matsui et al. 2013; Mignarri et al. 2012; Thoms and Gartner 2012; Ebberink et al. 2012), suggesting

higher incidence. Other peroxisomal disorders are caused by defects in fatty acid β-oxidation or transport (e.g. X-linked adrenoleukodystrophy). Peroxisomes are linked to several human health concerns including aging, cancer, heart disease, obesity, and diabetes (Wanders et al. 2003; Terlecky et al. 2006; Song 2002; Singh 1997; Schrader and Fahimi 2006; Périchon et al. 1998; Diano et al. 2011; Clarke et al. 1999). Furthermore, recent studies point to links between peroxisome dysfunction and α-synuclein and β-amyloid related pathogenesis (Yakunin et al. 2010; Santos et al. 2005) and as signaling platforms for antiviral innate immunity (Jefferson et al. 2014; Dixit et al. 2010).

To respond to environmental, developmental or other cues, cells mobilize sets of macromolecules, typically RNA or protein, through space and time, to coordinate and affect an appropriate response. Such regulation is typically complex, operating on multiple scales, including signal transduction cascades, chromatin dynamics, transcriptional control, mRNA processing, nuclear transport, translation, post-translation, and compartmentalization (Mast et al. 2014). The ensemble of macromolecules that mediate an effector response constitute a regulatory network and the number of components (nodes) and interactions between them (edges) define the complexity of the system.

In the case of peroxisome dynamics, regulatory networks have been modeled at the genome-scale level, revealing the players and their influence on peroxisome biogenesis, and at the level of mechanistic kinetic models, to elucidate mechanisms and dynamics of key transcriptional regulators of the process (Smith and Aitchison 2013; Ratushny et al. 2012; Danziger et al. 2014). Where appropriate throughout this chapter, we will highlight knowledge on how signaling components activate both gene regulatory and peroxisome assembly processes (Saleem et al. 2008, 2010a, b). However, whereas genome-wide transcriptional regulatory networks are commonly modeled, modeling of signaling networks on this scale is not widespread. Moreover, the integrative analysis of genome-scale communication between regulation at the level of signaling and transcriptional regulation has yet to be demonstrated. In this respect, regulatory network models offer a tantalizing promise but also provide a solemn challenge. When and where they are predictive, opportunity exists for the design of modulators through chemical- and synthetic-biology-based approaches with potential for therapeutic approaches to alleviate the debilitating consequences of peroxisome malformation and lack of function. However, this also represents a challenge as the scope under which such models are valid is oftentimes limited or too narrowly focused to be of broad utility. Whereas cells are complex, peroxisomes are less complex, and they provide opportunities for learning how to adequately model cellular complexity. We have had a glimpse into just how complex a process like peroxisome biogenesis is, and how extensively it is integrated into the broader cellular circuitry.

2 Biogenesis Is a Systems Process—The Complexity Factor

Our goal is an understanding of how to control peroxisome function and biogenesis, to have predictive insight into mechanisms leading to the formation of the organelle. This involves a systems-level view of the challenge, and demands that we understand the complex regulatory networks that respond to environmental cues leading to peroxisome induction and the molecular events that mediate peroxisome assembly. Systems cell biology approaches are best suited to addressing this challenge and start with a discovery component, in which high throughput, quantitative analyses reveal proteins involved in the regulation of the peroxisome response (Mast et al. 2014). Where possible, these initial exploratory datasets enable the response to be modeled as a network of interacting components inferred from various data integration strategies. Modeling of networks leads to new hypotheses, which are experimentally tested, the results of which lead to iterative model improvements. Technological advances are often critical to enabling the types of high throughput, quantitative analyses, required to initially seed and then refine these putative regulatory models. Refinement of these models towards predictive models necessitates incorporating multiple modeling and experimental based approaches. Here, we focus on uncovering regulatory networks that initiate oleate-induced organelle assembly in yeast because it is relatively well-studied and because transcriptional regulation is a principal mechanism for coordinated control of peroxisome biogenesis (Smith et al. 2002, 2006, 2007).

3 Yeast as a Model System

Peroxisome biogenesis and proliferation is well conserved through evolution; 13 of 14 human PBD genes were first identified in yeast (including transcriptionally regulated components of fission and import) and much mechanistic insight into peroxisome biogenesis and proliferation has been obtained from yeast (Steinberg et al. 2006). Recent characterization of a new complementation group centered on mutations in the peroxisome division protein Pex11, putatively extend this list to 14 of 15 (Thoms and Gartner 2012). The similarity between yeast and human also extends to the mechanistic underpinnings of the transcriptional response to peroxisome proliferators; peroxisome proliferation is controlled in metazoans by a heterodimer peroxisome proliferator-activated receptor alpha/retinoid X receptor (PPARα/RXR) (Issemann et al. 1993; Issemann and Green 1990; Devchand et al. 1996), which has a strikingly similar function, and ligand-mediated regulatory mechanism to the yeast heterodimer Oaf1/Pip2 (Phelps et al. 2006), which binds fatty acid and forms part of the core regulatory network responsible for induction of genes encoding peroxisomal proteins. Analysis of the interactions between these components has revealed a conserved network structure; Oaf1/Pip2 and PPAR/

RXR are components of ASymmetric Self-UpREgulated (ASSURE) network motifs that have properties of rapid and consistent induction in the presence of varying levels of stimuli (Ratushny et al. 2012; Issemann and Green 1990). Additionally, while required for cells grown on oleic acid as the sole carbon-source, peroxisomes are not required for growth on glucose or most other carbon-sources (Hohfeld et al. 1991). This permits the generation and exploitation of mutants that are specifically compromised in the presence of oleate, but not in glucose. This dynamic, controllable and dispensable peroxisome biogenesis regulatory network in yeast lends itself well to the study of a coordinated systems-level response that enables a dissection of how cells reorganize their metabolism, transcriptional networks, and build new organelles (Smith and Aitchison 2013).

4 A Genome-Scale Model of Yeast Responding to Oleate

Gene regulatory influence network (GRIN) models integrate high-throughput experiments with existing knowledge to fuel a virtuous cycle where the GRIN informs experimentation that yields a better GRIN model to inform further experimentation, and so on (Hecker et al. 2009). Large databases of high-throughput experiments abound; as of the beginning of 2018, the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) contained more than 2,338,000 expression datasets (Edgar et al. 2002). The availability of these data provides a starting point for informing an initial GRIN for virtually any biological system of interest. However, specific functional interactions are highly dependent on environment (St. Onge et al. 2007), thus it is necessary to include these influences—in Environment and Gene Regulatory Influence Networks (EGRIN) (Danziger et al. 2014; Bonneau et al. 2007), to extract predictive functional links between network approaches. Such an integrated approach was pioneered and developed to model the global transcriptional regulatory network of *H. salinarum NRC-1* (Bonneau et al. 2007), as it is found in its hypersaline environment.

EGRIN models are made possible by the integration of data from two primary algorithms: *cMonkey* (Reiss et al. 2006) and *Inferelator* (Bonneau et al. 2006). *cMonkey* identifies modules of conditionally co-regulated biclusters of genes. That is, for a given set of conditions, say a time course of yeast switched from the primary metabolism of glucose to the primary metabolism of oleic acid, *cMonkey* identifies sets of genes upregulated or downregulated, in response to the metabolic shift. Biclusters therefore contain genes that are co-expressed under a subset of conditions and they often share the same upstream sequence motif(s) in their promoter region. These motifs can be detected, de novo, using an algorithm for multiple expectation maximization for motif elicitation, or *MEME* (Bailey et al. 2009), that identifies motifs as position-dependent letter-probability matrices. Motifs are often functionally related and serve as shared and conserved sites for transcriptional regulation, for example, as transcription factor binding sites. Correspondingly the second primary EGRIN algorithm, *Inferelator* (Bonneau et al.

2006), identifies the most likely regulatory influences, including known and putative transcription factors but also putative upstream signaling molecules, chromatin modifiers, etc. *Inferelator* exhaustively proceeds through each gene and *cMonkey*-detected module in the dataset, based on measured mRNA expression levels. The results from these *cMonkey* and *Inferelator* algorithms are then integrated to construct *EGRIN* (Danziger et al. 2014; Bonneau et al. 2007), a model to predict global transcriptional responses to environmental and genetic perturbations.

The basic strategy for EGRIN generation is therefore twofold: First, coherently expressed genes are inferred to form coregulated modules. Modules are often associated with functional enrichment gene ontology (GO) terms, and can form the basis of an initial round of hypothesis generation and testing (Ashburner et al. 2000). Second, gene regulatory influences are correlated from temporally synchronized changes in mRNA expression data within modules and of putative transcription factors regulating each module (Bonneau et al. 2007). In a simple organism such as *H. salinarum NRC-1* an EGRIN model mapping the expression profiles of 72 transcription factors across 9 separate environmental conditions uncovered the regulatory and functional interrelationships of ~80% of the organism's genes (Bonneau et al. 2007).

The application of EGRIN modeling to the yeast response from the metabolism of glucose to that of oleic acid, utilized a compendium of 1516 expression studies, including 70 oleate-specific expression studies (Fig. 1) (Danziger et al. 2014). Validation of predicted transcription regulatory mechanisms were tested and confirmed experimentally in vivo using expression analysis of gene disruption strains and ChIP-Chip analyses to further refine the model. The outcome of this analysis identified a ranked list of regulators that control 6 coherent clusters enriched for peroxisome-related genes. Based on the gene lists in each cluster, transcriptional control of peroxisome biogenesis provides regulation for the differential expression of different classes of peroxisomal matrix enzymes, the genes required for peroxisomal matrix import, and positive and negative regulators of peroxisome numbers.

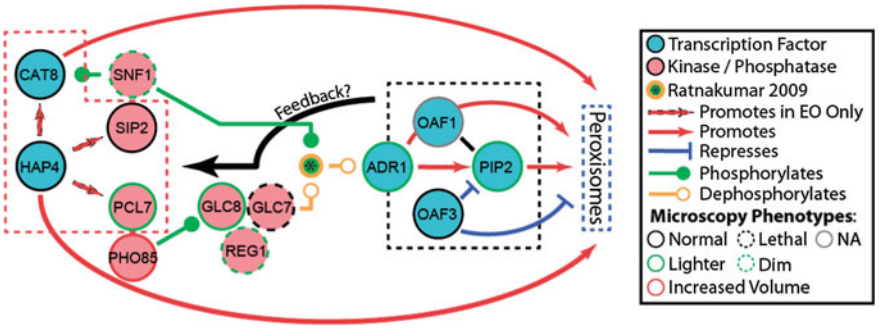

Fig. 1 An integrated gene-level regulatory model of transcriptional control for peroxisome biogenesis. The network representation combines data from Danziger et al. (2014) and Ratnakumar et al. (2009). See Danziger et al. (2014) for details

Separate biclusters for genes controlling peroxisome targeting sequence type 1 (PTS1) and PTS2 import suggest regulatory control for differential protein import into peroxisomes. Furthermore, the sharing of positive and negative regulators between the different biclusters implicates the ability of the transcriptional regulatory network to fine-tune peroxisome numbers in response to different stimuli. The EGRIN model also revealed details for altered regulatory mechanisms controlling peroxisome biogenesis under different metabolic conditions such as when yeast are present in low glucose containing medium, and temporal changes from "early" to "late" under exposure to oleic acid (Danziger et al. 2014).

However, there are limitations to EGRIN models because RNA abundance is a proxy for activities and they lack the granularity necessary to reveal detailed, direct mechanisms of regulatory network control. Global EGRIN models are important for identifying and prioritizing network perturbations leading to peroxisome dysfunction, and as a scaffold for providing the global network context to characterize remodeling mechanisms to restore functional peroxisomes. However, this coarse-grained network does not provide the fine detail necessary to make quantitative predictions that are sometimes necessary for fully understanding influences of network perturbations. For example, it is of limited utility for predicting effects of remodeling complex regulatory motifs (such as feed forward loops), and for comparing the relative magnitudes of various different influences on a set of targets (Brandman et al. 2005).

Therefore, to complement global models like EGRIN, it is advantageous to incorporate targeted kinetic modeling of critical subnetworks found in EGRIN models. As mentioned earlier, iterative approaches where models define experiments and data feedback to models are valuable to continually improve model accuracy and pose new hypotheses. Thus, EGRIN models are snapshots in time that continually evolve over iterations and become more accurate as complemented by additional mRNA expression data and mechanistic modeling efforts. EGRIN models provide statistically-robust, genome-scale regulatory networks important for understanding cellular processes at a systems level.

5 Mechanistic Kinetic Models of Peroxisome Transcriptional Control

In contrast to "systems-level network" models, kinetic models of smaller, critical molecular networks can interrogate and quantify the control logic of biological systems. They can reveal properties of network motifs, such as mechanisms of coordination and homeostasis under changing conditions (Ratushny et al. 2008, 2011a, 2012; Ramsey et al. 2006; Litvak et al. 2012; Barrett and Palsson 2006). Data-driven and mechanism-based modeling combines coarse- and fine-grained exploration of system parameters for understanding non-intuitive emergent properties in networks. This approach is indispensable for studying complexity in

regulatory network motifs and is complementary to genome-scale modeling efforts. Small gene regulatory networks can be modeled at the level of simplified biochemical kinetics using differential equations, which use parameters such as mRNA and protein synthesis and degradation rates to describe the cell population-average dynamics (Smith et al. 2006, 2011; Saleem et al. 2008; Bolouri and Davidson 2003; Ramsey et al. 2006). Critically, generating an initial model that can explore the properties of a dynamic system does not require detailed biochemical analysis of all parameters of a model. The potential influence of undetermined or imprecisely known (free) parameters can be systematically explored computationally, and then experimentally validated in a model-guided manner (Alon 2006; Ratushny et al. 2008, 2012; Ramsey et al. 2006; Orrell and Bolouri 2004; Barkai and Leibler 1997). This establishes an efficient process to understand the dynamics of a system and its components. To build a model, biochemical data are used to generate a network structure, and unknown parameters are projected through iterative model simulations and parameter optimizations. Like the large-scale EGRIN models, kinetic models lead to hypotheses, which can be experimentally tested to refine and expand the models. The utility of kinetic models is in how well they uncover many aspects of system behavior, including (i) temporal responses to a changing environment, (ii) steady-state properties including dose-response to an inducer, and (iii) stability of the steady-state to perturbation. The results may not be intuitive; for example, a single positive feedback loop can, in isolation, lead to signal amplification (Becskei et al. 2001), but two positive feedbacks loops with different time-scales can lead to multiple stable steady states, so-called multistability (Acar et al. 2005), or stabilize the "on" state of a molecular switch (Longabaugh and Bolouri 2006, Brandman et al. 2005).

The combination of experiment and modeling to study the overlapping feed forward network motif of the core peroxisome regulatory network showed that it imparts rapid and robust properties to the response (Ratushny et al. 2012). The topology of the network structure that was revealed in this analysis has properties that reduce variability in steady-state expression levels and in response to transiently varying ligand concentrations (Ratushny et al. 2008, 2011b). More broadly, the core ASSURE motif was demonstrated to be highly overrepresented in yeast, suggesting selective advantage and wide-spread utility of its robust properties (Ratushny et al. 2008). Other illustrative examples of the ASSURE network motif include PPARγ/RXRα (adipocyte differentiation), LXRα/RXRα (cholesterol homeostasis), RAR/RXR, (development and differentiation), MyoD/E12 (myogenesis) and IRF3/IRF7 (cellular antiviral defense) (Ratushny et al. 2012). ASSURE networks allow systems to respond quickly, leading to the rapid induction of target genes, while precisely tuning responses to constant levels. These features likely confer an evolutionary advantage as cells respond to concentration varying stimuli, and this might be the main reason for the prevalence of the motif across widely different biological systems.

The wide-spread prevalence of a core network motif throughout evolution demonstrates this model system is an excellent platform for discovering principles of regulatory network functions. More broadly, it suggests all aspects of our model (including motif properties, network structure, and our experimental and computational approaches) are applicable to diverse cellular processes in many organisms with wide-spread relevance.

Genome-wide and detailed kinetic models are good predictors of regulatory components and detailed mechanisms, respectively. However, large-scale GRINs suffer from a "hairball" problem where they are too complex to be readily interpretable at a mechanistic level, and kinetic models are often difficult to interpret due to a lack of global context. Integration of the two complementary approaches enables synergy and promises a more comprehensive, contextual and rigorous mechanistic understanding of network structure and function. Model integration addresses shortcomings of both approaches making the resulting models more reflective of how an organism senses and processes environmental change in order to readjust its physiology at a systems scale (Marbach et al. 2012).

6 Signaling Networks and Phosphoproteomics

Reversible phosphorylation is a common posttranslational modification. We have taken a multi-pronged approach to discover the signaling networks governing peroxisome biogenesis. First, we used a comprehensive genetic screen combined with morphological and expression data to reveal phosphatases and kinases required for peroxisome biogenesis (Saleem et al. 2008). This revealed that distinct signaling networks involving different functional modules, including glucose-mediated gene repression, derepression, oleate-mediated induction, and peroxisome formation promote stages of the peroxisome biogenesis pathway. The dynamics of peroxisomes and the complexity of phenotypes revealed in this analysis are suggestive of a tight spatial and temporal multiscale control of peroxisome assembly. This analysis was complemented by a global mass-spectrometry based "phosphoproteomic" methodology to identify and quantify proteins that are differentially phosphorylated upon transition from glucose to oleate (Saleem et al. 2010b). These data can be complemented with genome-wide genetics experiments to identify kinases and phosphatases, transcription factors and other regulators required for normal peroxisome biogenesis and reporter gene activation (Saleem et al. 2010a; Knijnenburg et al. 2011). Together, these analyses provide a comprehensive view of the networks specifically responsive to the exposure of cells to oleate. However, the experimental data generated for these studies were primarily limited to two steady states (cells growing in glucose or oleate-containing medium).

7 Regulatory Networks Functioning at the Level of the Peroxisome

Viewed at the level of the organelle, peroxisome biogenesis shows the potential for complex regulation and can be generalized as a multi-input nested feedforward loop with built-in partial redundancy and the potential for feedback regulation (Fig. 2) (Mast et al. 2015; Alon 2006). Vesicles carrying membranes and membrane proteins emerge from the ER and fuse with existing peroxisomes. These peroxisomes subsequently grow and can undergo division to form two or more resultant "daughter" peroxisomes that are each capable of receiving ER-derived preperoxisomal vesicles, giving rise to the nested topology of the feed forward regulatory loop controlling peroxisome proliferation (Smith and Aitchison 2013). In the absence of peroxisomes, the preperoxisomal vesicles can fuse with each other and form mature, functional organelles. Each step of the pathway offers points for positive and negative regulation. While much remains to be discovered, observations are consistent with our view of multiscale regulatory networks simultaneously operating at the level of production of effector molecules and at the level of the molecules themselves through posttranslational modification and dynamic subcellular localization (Smith et al. 2002; Saleem et al. 2008; Mast et al. 2016, 2018).

Pex11 is a positive regulator of peroxisome division and is itself, under multiple levels of regulatory control (Erdmann and Blobel 1995). At the transcriptional level, the *PEX11* gene and genes encoding many peroxisomal matrix proteins are controlled by a four-factor network composed of Adr1, Oaf1, Pip2 and Oaf3 (termed the AOPY). Oaf1/Pip2 bind to the oleate-response element (ORE) whereas Adr1 binds to its cognate site located in the 5′ UTR regions leading to upregulation when cells are grown in the presence of oleic acid (Smith et al. 2002; Knoblach and Rachubinski 2010). PEX11 is present in two of the six EGRIN biclusters (Danziger et al. 2014). This is consistent with a hypothesis that the regulation of peroxisome

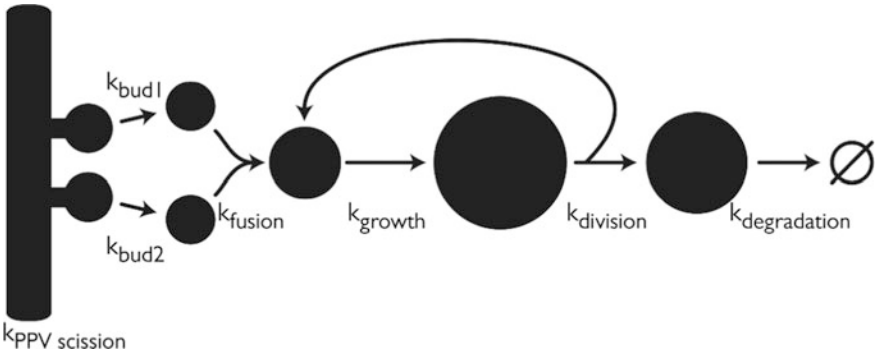

Fig. 2 A flux model for peroxisome proliferation dynamics reveals a nested feedforward loop with built-in partial redundancy. Each step of the model is denoted by the rate constant 'k' for that step

division is coupled to peroxisome matrix protein import at the transcriptional level. Pex11 is also phosphorylated at serine residues 165 and/or 167 by Pho85-initiated signal cascades, leading to enhanced peroxisome proliferation (Knoblach and Rachubinski 2010). The cyclin-dependent kinase Pho85 had been implicated as a positive-regulator of transcriptional control and has a complex role in the repression, induction and biogenesis of peroxisomes (Saleem et al. 2008; Danziger et al. 2014).

Multiscale regulation of negative regulators of peroxisome proliferation also exist. Pex29 is an ER-resident protein that functions to dampen preperoxisomal vesicle secretion from the ER (Mast et al. 2016). At the transcriptional level, it is found in biclusters controlled by the AOPY regulatory network along with genes involved in organelle autophagy, and separately with genes involved in peroxisome proliferation (Danziger et al. 2014). In the ER Pex29 is part of a reticulon-peroxin complex with the reticulon proteins Pex30, Rtn1 and Yop1 (Mast et al. 2016; David et al. 2013). There is in vitro evidence for the phosphorylation of Pex30 by Kss1 (Ptacek et al. 2005), which was demonstrated to have a role in the negative control of peroxisome proliferation (Saleem et al. 2008).

8 Understanding Pathogenic Mutations in a Network Context

Quantitative phenotyping of a comprehensive collection of yeast mutants induced to proliferate peroxisomes identified 211 genes required for peroxisome induction and function (Saleem et al. 2010a; Smith et al. 2006). The assays employed include growth in the presence of fatty acids (Smith et al. 2006), confocal imaging and flow cytometry through the induction process (Saleem et al. 2008, 2010a). The genes identified are required for robust signaling, transcription and normal peroxisomal biogenesis. The corresponding proteins are localized throughout the cell, and many have indirect connections to peroxisome function, highlighting the complex networks through which information flows during peroxisome biogenesis and activity. While there are hundreds of mutants in yeast that cause peroxisome dysfunction, there are only a couple dozen in humans (Smith and Aitchison 2013). It seems clear that this difference reflects our ability to quantitatively and rigorously screen for peroxisome function in yeast. Whereas, in humans, only those mutants that lead to recognizable and major disease phenotypes have been studied in sufficient depth to be linked to peroxisomal dysfunction. It is therefore critical to characterize these pathogenic mutations and their context within this multiscale regulatory network and this may even predict influences of candidate human genes involved in peroxisome dysfunction as 131 of the 211 genes required for peroxisome function have human homologs (Park et al. 2011).

9 Remodeling Networks to Overcome Effects of Genetic Perturbations—Looking Towards the Future

A systems view of medicine postulates that disease arises from disease-perturbed molecular networks (Hwang et al. 2009; Barabási et al. 2011). In disease, networks are perturbed by changes in relative activities (concentration changes) or by the architecture of the network (the connections). A grand challenge for systems biology is to understand the relationship between network behavior and phenotype (disease or health). A network understanding of biology, in principle, enables rational intervention to control the network function to yield a desired outcome or phenotype (Barabási et al. 2011). In the case of disease, systems approaches promise strategies to control a network toward health, potentially bypassing a missing node (protein) or stabilizing a key connection (interaction). This goal has not yet been achieved, likely because we are yet to be able to reliably link network behaviors to phenotypes and because the potential solution space is vast.

However, exploration of the peroxisomal regulatory network in yeast offers intermediate solutions where key network nodes identified by network analysis to destroy network function, can be exploited with the "awesome power of yeast genetics" (Botstein 2004) to identify ways in which the cells can correct the defect—so called "functional rescue". Mapping these corrections on the network, will inform and flesh out the network model, and will lay a foundation for understanding how we may use network biology for rational intervention and to correct diseased networks. Such future advances are envisioned to: (1) Identify general principles of network structure vulnerability and robustness leading to improved drug target identification. This is supported by a recent study showing relationships between network structure and controllability (Li et al. 2011). (2) Develop strategies to identify crosstalk signaling mechanisms to reveal and control off-target effects. Off-target effects are poorly understood, but account for a large majority of complications in current therapeutic strategies. (3) Establish the feasibility of using kinetic modeling and synthetic systems biology for drug development in humans. Importantly, these outcomes are not only applicable to classically defined PBDs and other diseases linked to peroxisomes [e.g. age-related neurodegeneration (Santos et al. 2005; Yakunin et al. 2010) and diet-induced obesity (Diano et al. 2011)] but are generally applicable to networks related to other diseases.

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Multi-localized Proteins: The Peroxisome-Mitochondria Connection

Joseph L. Costello, Josiah B. Passmore, Markus Islinger and Michael Schrader

Abstract Peroxisomes and mitochondria are dynamic, multifunctional organelles that play pivotal cooperative roles in the metabolism of cellular lipids and reactive oxygen species. Their functional interplay, the "peroxisome-mitochondria connection", also includes cooperation in anti-viral signalling and defence, as well as coordinated biogenesis by sharing key division proteins. In this review, we focus on multi-localised proteins which are shared by peroxisomes and mitochondria in mammals. We first outline the targeting and sharing of matrix proteins which are involved in metabolic cooperation. Next, we discuss shared components of peroxisomal and mitochondrial dynamics and division, and we present novel insights into the dual targeting of tail-anchored membrane proteins. Finally, we provide an overview of what is currently known about the role of shared membrane proteins in disease. What emerges is that sharing of proteins between these two organelles plays a key role in their cooperative functions which, based on new findings, may be more extensive than originally envisaged. Gaining a better insight into organelle interplay and the targeting of shared proteins is pivotal to understanding how organelle cooperation contributes to human health and disease.

Keywords Peroxisomes · Mitochondria · Tail-anchored membrane proteins Organelle fission · Protein targeting

Abbreviations

ACAD	Acyl-CoA dehydrogenase
ACAT	Acetoacetyl-CoA thiolase
ACBD5	Acyl-CoA binding domain containing protein 5
ACOX	Acyl-CoA oxidase

J. L. Costello · J. B. Passmore · M. Schrader (✉)
Biosciences, University of Exeter, Exeter EX4 4QD, UK
e-mail: M.Schrader@exeter.ac.uk

M. Islinger
Institute of Neuroanatomy, Center for Biomedicine & Medical Technology Mannheim, Medical Faculty Manheim, University of Heidelberg, 68167 Mannheim, Germany

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ATAD	ATPase Family, AAA Domain Containing
CoA	Coenzyme A
Drp1	Dynamin-related protein 1
EHHADH	Enoyl-CoA Hydratase and 3-Hydroxyacyl CoA Dehydrogenase
ER	Endoplasmic reticulum
Fis1	Fission 1
FALDH	Fatty aldehyde dehydrogenase
FFAT	Two phenylalanines (FF) in an acidic tract
GDAP1	Ganglioside-induced differentiation associated protein 1
GET	Guided Entry of Tail-anchored proteins
HD	Hydrophobic domain
L-PBE	Enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein
MAVS	Mitochondrial antiviral-signaling protein
Mff	Mitochondrial fission factor
MTS	Mitochondrial targeting signal
NAD	Nicotinamide adenine dinucleotide
PECI	Peroxisomal 3,2-trans-enoyl-CoA isomerase
PEX	Peroxin
PO	Peroxisome
PTS	Peroxisomal targeting signal
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCP	Sterol carrier protein
SGT	Small glutamine tetratricopeptide repeat-containing protein
SLS	Sjögren Larsson syndrome
SVM	Support vector machine
TA	Tail-anchored
TMD	Trans membrane domain
VAPB	Vesicle-associated membrane protein-associated protein B
VLCFA	Very long-chain fatty acids

1 The Peroxisome-Mitochondria Connection—an Overview

One of the hallmarks of eukaryotic cells is the presence of membrane-bound organelles, which create distinct, optimised environments to promote a variety of metabolic reactions required to sustain life. For the entire cell to function as a unit, coordination and cooperation between specialized organelles must take place. Peroxisomes are dynamic, multifunctional organelles that play pivotal, cooperative

roles in the metabolism of cellular lipids and reactive oxygen species (ROS) and are essential for human health and development (Wanders 2013). Peroxisomes interact and cooperate with many organelles involved in cellular lipid metabolism such as the endoplasmic reticulum (ER), mitochondria, lipid droplets and lysosomes (Schrader et al. 2015b; Valm et al. 2017). In mammals, peroxisomes contribute to important biochemical pathways with indispensable functions in the biosynthesis of ether-phospholipids (e.g. myelin sheath lipids), breakdown and detoxification of fatty acids (via fatty acid α- and β-oxidation), the synthesis of bile acids and docosahexaenoic acid, glyoxylate metabolism, amino acid catabolism, polyamine oxidation, and ROS/RNS metabolism. In addition to those metabolic functions, mammalian peroxisomes also serve as important signalling platforms modulating different physiological and pathological processes such as inflammation, innate immunity, and cell fate decision (Wang et al. 2014; Asare et al. 2017).

In recent years it has been demonstrated that mitochondria are functionally connected with peroxisomes (Fig. 1). This intimate interplay, the so called "peroxisome-mitochondria connection" (Camões et al. 2009; Schrader et al. 2013, 2015a) includes the metabolic cooperation of mitochondria and peroxisomes (e.g. in the β-oxidation of fatty acids, phytanic acid α-oxidation, synthesis of bile acids, and glyoxylate detoxification) (reviewed in Wanders et al. 2016), peroxisome-mitochondria redox interplay (reviewed in Lismont et al. 2015), cooperation in anti-viral signalling and defence (Dixit et al. 2010; Kagan 2012), as well as coordinated biogenesis by sharing of key proteins of their division machinery (Schrader et al. 2015a, 2016) (Figs. 1 and 2). The latter require organised targeting and recruitment of those proteins (Delille et al. 2009; Schrader et al. 2012; Costello et al. 2017a). Under certain experimental conditions mitochondria (and the ER) can also contribute to the biogenesis (de novo formation) of peroxisomes (Sugiura et al. 2017) (Fig. 1). Furthermore, mitochondria are defective in several peroxisomal diseases (Peeters et al. 2015; Schrader et al. 2015a; Shinde et al. 2017) underlining the physiological importance of the interplay between both organelles (Fig. 1; Table 1).

Although the mechanisms of communication between peroxisomes and mitochondria are not clear, they may involve physical contact sites and tethers, vesicular transport via mitochondria-derived vesicles and the release of biological messengers (Neuspiel et al. 2008; Horner et al. 2011; Antonenkov and Hiltunen 2012; Schrader et al. 2015b).

A well-known example for the metabolic cooperation of mitochondria and peroxisomes in mammals is their interplay in the β-oxidation of fatty acids to maintain lipid homeostasis (Fig. 1). Whereas fatty acid β-oxidation in yeast and plants is solely peroxisomal, in mammals both organelles possess their own β-oxidation pathway. Both pathways show substrate specificity (e.g. β-oxidation of VLCFAs is exclusively peroxisomal), and peroxisomal β-oxidation only leads to chain-shortened fatty acids, which have to be routed to mitochondria for complete β-oxidation. The interplay with mitochondria is thus important for further metabolism of the end-products of peroxisomal β-oxidation (e.g. NADH, acetyl-CoA, propionyl-CoA, and a variety of chain-shortened acyl-CoAs). Importantly,

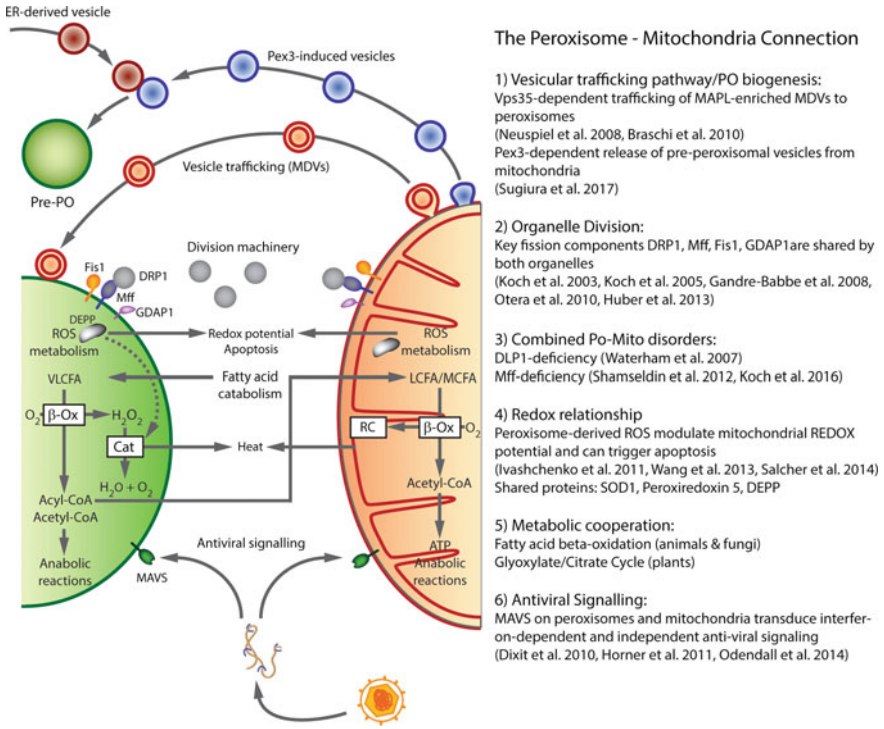

Fig. 1 Overview of the functional interplay between peroxisomes (**left**) and mitochondria (**right**) in mammals. β-Ox, fatty acid β-oxidation; Cat, peroxisomal catalase; DEPP, decidual protein induced by progesterone; DRP1/DLP1, dynamin-related/like protein 1; Fis1, fission protein 1; GDAP1, ganglioside-induced differentiation associated protein 1; MAPL, mitochondria-associated protein ligase; MAVS, mitochondrial antiviral-signaling protein; MDV, mitochondria-derived vesicle; Mff, mitochondrial fission factor; PO, peroxisome; RC, respiratory chain; ROS, reactive oxygen species; SOD, superoxide dismutase; VLCFA, LCFA, MCFA, very long-chain, long-chain and medium chain fatty acids; virus particle (yellow) at the bottom. Adapted from Islinger et al. (2012); Schrader et al. (2015a)

peroxisomal β-oxidation can only continue if the NADH formed in peroxisomes is reoxidized to NAD + , which can only be achieved in mitochondria (Wanders et al. 2016). Phylogenetic protein comparisons show that the β-oxidation pathways of both organelles co-evolved during eukaryotic evolution influencing each other's functions, and this likely also applies to other cooperative tasks.

In the following sections, we first focus on targeting and sharing of matrix proteins which are involved in metabolic cooperation of peroxisomes and mitochondria (see Sect. 2). Next, we discuss shared components and mechanisms involved in peroxisomal and mitochondrial division and division (see Sect. 3). We then present novel insights on the dual targeting of tail-anchored (TA) membrane

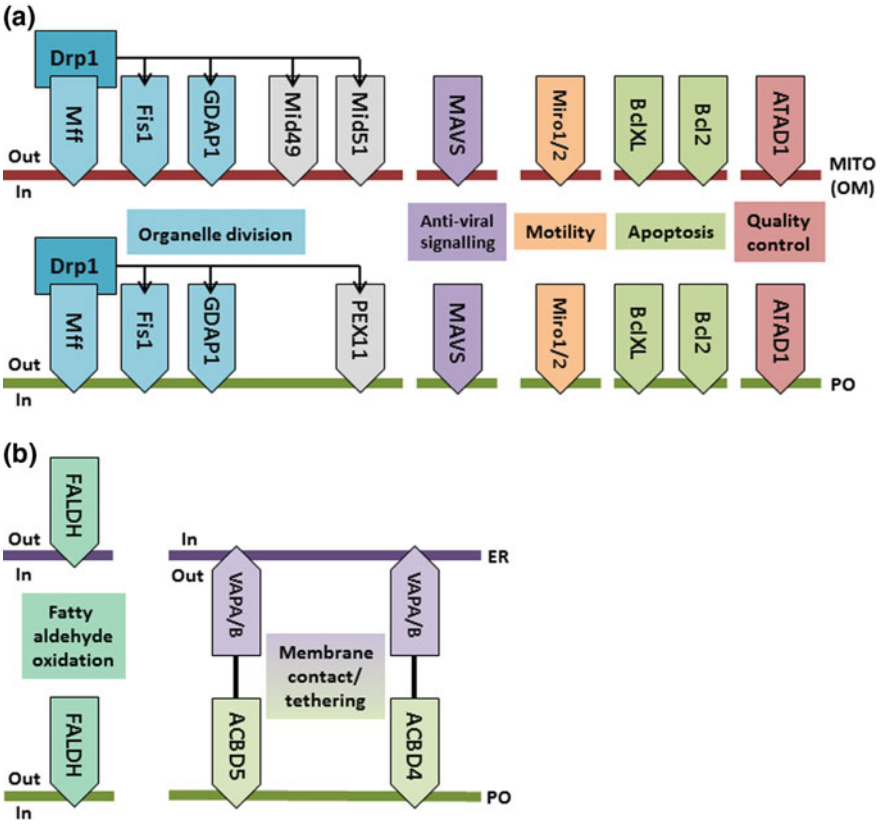

Fig. 2 **a** Peroxisomes (PO) and mitochondria (MITO) share components of their division machinery and other tail-anchored (TA) membrane proteins. **b** TA proteins at peroxisomes and the ER. For details see Sects. 3–5. Shared (color) and organelle-specific proteins (grey) are indicated. ATAD, ATPase family, AAA domain containing; ACBD, acyl-CoA binding domain containing protein; Bcl2, B-cell lymphoma 2 protein; BclXL, B-cell lymphoma extra-large protein; Drp1, dynamin-related protein 1; FALDH, fatty aldehyde dehydrogenase; Fis1, fission protein 1; GDAP1, ganglioside-induced differentiation associated protein 1; MAVS, mitochondrial antiviral-signalling protein; Mff, mitochondrial fission factor; Mid, mitochondrial dynamics protein; Miro, mitochondrial Rho GTPase; OM, mitochondrial outer membrane; PEX, peroxin; VAP, vesicle-associated membrane protein-associated protein

proteins to peroxisomes and mitochondria (see Sect. 4). Finally, we provide an overview of what is currently known about shared membrane proteins and their role in various disease conditions (see Sect. 5). We will mainly focus on findings obtained in mammalian cells.

Table 1 Disorders with defects in peroxisomal/mitochondrial dynamics and in tail-anchored membrane proteins

Gene	Function	Clinical features	Genotype	Organelle alterations	References
DNM1L	Dynamin 1-like protein (Drp1) Mediates mitochondrial and peroxisomal division	Microcephaly, abnormal brain development, optic atrophy, hypoplasia, lactic acidosis, slightly elevated VLCFA levels	c.1184C > A (p.A395D) *De novo* heterozygous	Defective fission of mitochondria and peroxisomes	Waterham et al. (2007)
		Infantile encephalopathy, lactic acidosis, poor feeding, global developmental delay, hypotonia and status epilepticus	c.1048G > A (p.G350R) or c.1135G > A (p.E379 K) heterozygous missense variants	(p.G350R): Increase in peroxisomal size and decreased number. Reduced mitochondrial size and defect in mitochondrial trafficking.* (p.E379 K): No notable alterations to peroxisomes or mitochondria*	Chao et al. (2016)
		Refractory epilepsy with prolonged survival	c.1085G > A (p.G362D) *De novo* heterozygous	Hyperfusion of the mitochondrial network	Vanstone et al. (2016)
		Postnatal microcephaly, developmental delay and pain insensitivity	c.1084G > A (p.G362S) *De novo* heterozygous	Decreased respiratory chain complex IV activity. Impaired fission with elongated mitochondrial structure	Sheffer et al. (2016)
		Epileptic encephalopathy	c.1207C > T (p.R403C) *De novo* heterozygous	Dominant-negative effect, with reduced Drp1 oligomerization and mitochondrial fission activity**	Fahrner et al. (2016)

(continued)

Table 1 (continued)

Gene	Function	Clinical features	Genotype	Organelle alterations	References
		Encephalopathy in infancy	c.1217T > C (p.L406S) De novo heterozygous	Elongation of peroxisomes and mitochondria	Zaha et al. (2016)
		Hypotonia and absent respiratory effort. Demyelination and reduction of the number of axons in the sural nerve of both siblings	c.261dup (p.W88Mfs*) + c.385_386del (p.E116Kfs*6)	Giant mitochondria, absence of Drp1	Yoon et al. (2016)
		Slowly progressive infantile encephalopathy	c.106A > G (p.S36G) + c.346_347delGA (p.E116Kfs*6)	Abnormally elongated mitochondria and aberrant peroxisomes	Nasca et al. (2016)
		Isolated dominant optic atrophy	c.5A > C (p.E2A) or c.575C > A (p.A192E) heterozygous	Dominant negative effect with elongated mitochondrial network	Gerber et al. (2017)
MFF	Mitochondrial fission factor Recruitment of Drp1 to mitochondria and peroxisomes	Developmental delay, abnormal intensity on brain MRI of *globus pallidus*, motor and speech deficits, mild hypertonia, borderline microcephaly and pale optic disc	c.190C > TQ64X Homozygous non-sense mutation Truncated protein lacking TMD	Elongated peroxisomes and mitochondria	Shamseldin et al. (2012)
		Developmental delay, peripheral neuropathy, optic atrophy, and Leigh-like encephalopathy	c.(184dup) (892C > T) p.(Leu62Profs*13) (Arg298*)	Elongation of peroxisomes and mitochondria, increased mitochondrial branching	Koch et al. (2016)
			c.(453_454del) (453_454del) p.(Glu153Alafs*5) (Glu153Alafs*5)		

(continued)

Table 1 (continued)

Gene	Function	Clinical features	Genotype	Organelle alterations	References
			c.(453_454del) (453_454del) p.(Glu153Alafs*5) (Glu153Alafs*5)		
GDAP1	Ganglioside-induced differentiation-associated protein 1 Glutathione-S transferase activity Involved in mitochondrial and peroxisomal fission	Charcot-Marie-Tooth disease; Hoarse voice and vocal cord paresis, onset at childhood with weakness and hand wasting leading to disability at the end of the first decade, sensory nerve action potential decreased or absent	Heteroallelic Frameshift mutation c.863insA T288fs290X Truncated protein	Inability to induce mitochondrial and peroxisomal fragmentation; Mislocalisation of protein to cytosol or degradation***	Cuesta et al. (2002), Niemann et al. (2005), Huber et al. (2013)
			Heteroallelic Nonsense mutation c.487T Q163X Truncated protein		
ACBD5	Acyl-CoA binding domain containing protein 5 Involved in peroxisome-ER membrane association, VLCFA transfer into peroxisomes	Cone-rod dystrophy, spastic paraparesis, white matter disease	c.1205 + 1G > A p.Gly402Aspfs5	Impaired β-oxidation of VLCFAs in peroxisomes	Abu-Safieh et al. (2013), Yagita et al. (2017)
		Retinal dystrophy, progressive leukodystrophy and microcephaly, ataxia, dysarthria, hypomyelination with diffuse T2 signal abnormality in deep white matter	homozygous deleterious c.626-689_937-234delins936 + 1075_c.936 + 1230inv mutation	Normal presence of import-competent peroxisomes. Increased VLCFA levels, reduced C26:0 β-oxidation, reduced plasmalogen biosynthesis	Ferdinandusse et al. (2017), Herzog et al. (2017)

(continued)

Table 1 (continued)

Gene	Function	Clinical features	Genotype	Organelle alterations	References
FALDH	Fatty aldehyde dehydrogenase Breakdown of fatty aldehydes, possible roles in protecting organelles and cells against oxidative stress associated with lipid peroxidation	Sjögren-Larsson syndrome; Erythrodermic skin lesions, "glistering spots" in parafoveal areas, photophobia, developmental delays, reduced myelinated white matter nerve fibers, spastic diplegia	Over 100 mutations, approximately 55% homozygous, primarily missense	Impaired oxidation of fatty aldehydes, accumulation of leukotriene B4	Reviewed in Fuijkschot et al. (2012), Wanders et al. (2016)

*Results from *Drosophila* cells expressing each mutation separately
**Results from MEFs expressing each mutation separately
***Results from COS7 cells expressing each mutation separately
Adapted from Schrader et al. (2014), Gerber et al. (2017)

2 Matrix Proteins Shared by Peroxisomes and Mitochondria in Animals

Generally, peroxisomal matrix protein import is mediated by two alternative targeting sequences: (i) the peroxisomal targeting signal 1 (PTS1) ending with the C-terminal consensus sequence—SKL, and (ii) the PTS2 with an R-(L/V/I/Q)-X-X-(L/V/I/H)-(L/S/G/A)-X-(H/Q)-(L/A) consensus sequence near to the N-terminus of the protein. While the PTS1 remains on the protein after import, the PTS2 sequence is subsequently removed by the protease TYSND1 (Kurochkin et al. 2007). Most soluble mitochondrial proteins possess an N-terminal presequence for recognition by the membrane-located import receptors Tom20 and Tom22. This presequence has no detectable consensus but forms an amphipathic helix with a stretch of positively charged amino acids binding negatively charged amino acid residues in TOM20 (Abe et al. 2000). After import, the targeting peptide sequence is cleaved from the proteins by the mitochondrial processing peptidase (MPP). Thus, while peroxisomal PTS2-mediated and mitochondrial protein import might show some mechanistic similarities, the amino acid compositions in the individual targeting sequences is different, such that there can be no competitive interaction of mitochondrial and peroxisomal import receptors at the same signal sequence. However, individual genes can contain both a peroxisomal and a mitochondrial targeting sequence (MTS), usually a MTS and a PTS1, suggesting a competitive recognition process between the individual targeting machineries. This process appears to include a hierarchical targeting system where individual receptor proteins scan a nascent protein in chronological order (Kunze and Berger 2015). Mitochondrial in contrast to peroxisomal matrix proteins are not folded in the cytosol, but have to be stabilized by interaction with hsp70 chaperones. Thus, a nascent protein can be detected by its N-terminal MTS as soon as the sequence emerges from the ribosome and consequently routed to the mitochondrial import pathway. A good example for such a hierarchical sorting is the human alanine-glyoxylate aminotransferase, which in humans is a peroxisomal, PTS1-containing enzyme. Specific mutations in the N-terminal sequence of the protein, however, create an MTS (Leiper and Danpure 1997). While a truly competitive targeting mechanism would suggest a mixed localization of the mutated proteins, the resulting localization is nearly exclusively mitochondrial inducing a knockout-like phenotype in the patients of Primary Hyperoxaluria Type I. However, recently a comparable N-terminal mutation in the peroxisomal L-bifunctional protein EHHADH was observed to lead to a bimodal distribution to both mitochondria and peroxisomes, implying that a weak, leaky MTS combined with a strong PTS allows for a mixed location to both organelles (Klootwijk et al. 2014).

Most proteins with a shared peroxisome-mitochondria distribution are targeted by isoforms of the same gene possessing alternative N-termini. These can be generated by alternate transcription initiation sites, alternative splicing events or read-through of the first translation initiation site and usually confers differential targeting by removal of the MTS (Kunze and Berger 2015). Examples of such

proteins include the mammalian enzymes enoyl-CoA delta isomerase 2 (PECI) (Zhang et al. 2002), carnitine O-acetyltransferase (Westin et al. 2008), malonyl-CoA decarboxylase (Joly et al. 2005), peroxiredoxin 5 (Knoops et al. 1999) as well as the rodent alanine-glyoxylate aminotransferase (Oda et al. 2000).

Alternatively, changes in splicing can uncover internal MTS as reported for the sterol-carrier protein SCP2/SCP-X (Pfeifer et al. 1993) and the acyl-CoA dehydrogenase ACAD11 (He et al. 2011). However, the potency of the MTS of SCP2 was recently questioned (Li et al. 2016), as both SCP2 and SCP-X were reported to be predominately in peroxisomes. While most of the matrix proteins shared by peroxisomes and mitochondria appear to be sorted via alternative RNA transcripts, there are some shared proteins which appear to exist only as a single isoform with an N-terminal MTS and a C-terminal PTS1 in a single peptide chain. Among those proteins are the glutathione S-transferase kappa (Morel et al. 2004), the hydroxymethylglutaryl-CoA lyase (Ashmarina et al. 1996) and the Δ(3,5)-Δ(2,4)-dienoyl-CoA isomerase ECH1 (Zhang et al. 2001). A recent study also reported that the long MTS and PTS carrying version of PECI is targeted to both peroxisomes and mitochondria (Fan et al. 2016). The authors claimed that parallel import of single PECI molecules into peroxisomes and mitochondria would act as a tethering site bringing both organelles into contact. However, as the study is only based on conventional confocal microscopy, additional high resolution imaging techniques are required to convincingly clarify this issue. Interestingly, in addition to the N-terminal insertion of an MTS discussed above, the dual localization of α-methylacyl-CoA racemase appears to be controlled by alternative splicing, resulting in a transcript lacking the PTS1 sequence at the C-terminus. Thus, the hierarchical principle of a dominant mitochondrial sorting pathway (Kunze and Berger 2015) may not generally account for peroxisomal proteins and some may possess such a potent PTS1, that they out-compete the hsp70 recognition by immediate binding to PEX5.

Based on subcellular separation and immuno-EM experiments, several publications reported a mitochondrial localization of the prominent peroxisomal marker enzyme catalase in heart and liver (Radi et al. 1991; Antunes et al. 2002; Salvi et al. 2007; Rindler et al. 2013). While the protein sequence of catalase bears a C-terminal PTS1, an MTS1 cannot be identified in any of the Ensembl-listed potential splice variants of rodent and human proteins using MitoFates or TargetP1.1 (Emanuelsson et al. 2000; Fukasawa et al. 2015). Likewise, cytosolic and not mitochondrial localization of catalase is observed in cells incompetent for peroxisomal import (Wanders et al. 1984; Tsukamoto et al. 1997; Kinoshita et al. 1998; Ito et al. 2000; Hashiguchi et al. 2002). In contrast, overexpressed catalase A from yeast was increasingly targeted to mitochondria, when the C-terminal PTS1 was removed (Petrova et al. 2004). Thus, according to the current data, further studies are required to decipher targeting mechanisms, which could ultimately prove the mitochondrial localization of catalase, the most abundant peroxisomal protein in mammalian cells.

Functionally, most of the dually localized proteins are associated with fatty acid and ROS metabolism, two tasks which are shared by both organelles (Schrader

et al. 2015a) (Fig. 1). Nevertheless, there are still proteins like the hydroxymethylglutaryl-CoA lyase, which have been characterized in mitochondria but where the peroxisomal function remains enigmatic. Mitochondrial acetoacetyl-CoA thiolase ACAT1 contributes to ketone body anabolism and isoleucine catabolism, whereas the cytosolic isoform ACAT2, which is encoded by a different gene, performs the first step in isoprene biosynthesis. The mitochondrial isoform possesses, in addition to an MTS, a PTS1-like sequence at its C-terminus (Olivier and Krisans 2000). Recently, ACAT1 in conjunction with further constituents of the presqualene segment were reported to be part of a peroxisomal cholesterol biosynthesis pathway (Kovacs et al. 2002). Indeed, most of the soluble enzymes of the pathway have similar C-terminal PTS-like sequences and in plants the pathway has been recently localized to peroxisomes (Sapir-Mir et al. 2008; Simkin et al. 2011; Thabet et al. 2011; Clastre et al. 2011). In mammals, the data remains still contradictory (Wanders and Waterham 2006; Faust and Kovacs 2014) and future research has to show, if ACAT1 is another enzyme shared by peroxisomes and mitochondria.

The human peroxisomal matrix proteome, as currently listed in PeroxisomeDB 2.0 constitutes of approx. 40 proteins (Schlüter et al. 2007). Since 7–8 of the dually localized proteins described in this article can be found in humans, about 6–7% of the human peroxisomal matrix proteins are shared with mitochondria. This appears to be a relatively limited amount of proteins, however, when we broaden our view to an evolutionary standpoint, the number of peroxisomal matrix proteins with a mitochondrial origin increases substantially. Gene duplication is a third mechanism, which can lead to protein isoforms localized to different organelles. However, the separation of one parental gene into two genes not only allows for the incorporation of individual targeting sequences but generally fosters mutational variation of the two daughter genes leading to increasing functional specializations. Three steps of the peroxisomal β-oxidation pathway are performed by enzymes with an α-proteobacterial origin (Bolte et al. 2014) and in total 17% of the peroxisomal proteome appears to have α-proteobacterial ancestors (Gabaldón et al. 2006). These findings imply that a significant amount of peroxisomal proteins were derived from mitochondrial precursor proteins after reshuffling the targeting sequence (Gabaldón et al. 2006). De novo peroxisome formation by reintroduction of PEX3 in PEX3-deficient mammalian cells has been recently reported to require an initiation step at the outer mitochondrial membrane (Sugiura et al. 2017). These findings, in combination with the above mentioned presence of the mitochondria-derived β-oxidation enzymes in peroxisomes, have led to the theory of a "mitochondria-first" scenario of peroxisome evolution (Speijer 2017), which argues that mitochondria-derived vesicles with a mitochondrial β-oxidation system merged with catalase-containing ER vesicles to form a proto-peroxisome. This might be a conceivable explanation for the overt presence of the significant amount of α-proteobacterial enzymes in peroxisomes. However, it should also be noted that some of the mitochondrial β-oxidation enzymes likely entered peroxisomes at later stages of the eukaryotic evolution; e.g. the closest relative of the α-subunit of the mitochondrial trifunctional enzyme is the peroxisomal L-PBE (Camões et al. 2015),

which is only found in animal species but not in fungi and plants, thus indicating a step-wise co-evolution of peroxisomes and mitochondria. Moreover, the acyl-CoA oxidase (ACOX) responsible for the first step in the peroxisomal β-oxidation pathway is not a protein with an α-proteobacterial ancestor (Gabaldón 2014a, b). Considering a mitochondria-independent evolution of peroxisomes from the ER would hence leave the ACOX precursors with an ancestral enzymatic function, which was initially not involved in β-oxidation (Gabaldón 2014a). However, ACOX are distantly related to the Acyl-CoA dehydrogenase of mitochondrial β-oxidation and all extant ACOX are merely involved in fatty acid chain shortening (which is also their function in the peroxisomal part of the pathway for PUFA synthesis). Since acyl-CoA dehydrogenases appear to also be ancient constituents of the peroxisomal proteome (Shen et al. 2009; Camões et al. 2015), it remains enigmatic how ACOX could efficiently replace ACAD in an already functional and complete pathway. In summary, without further data, these initial events in peroxisome evolution might never be convincingly resolved leaving the earliest steps in peroxisome-mitochondria cooperation as a classical "chicken and egg" scenario.

3 Shared Membrane Proteins in Peroxisomal and Mitochondrial Division

Peroxisomes and mitochondria are dynamic organelles which can multiply by division of pre-existing organelles (Westermann 2010; Schrader et al. 2016; Kraus and Ryan 2017). An important discovery in the field was that these organelles share multiple components of their membrane fission machinery (e.g. the mitochondrial fission protein Fis1 (Koch et al. 2005), mitochondrial fission factor Mff (Gandre-Babbe and van der Bliek 2008), the ganglioside-induced differentiation-associated protein GDAP1 (Huber et al. 2013), and the dynamin-related/like protein Drp1/DLP1 (Li and Gould 2003; Koch et al. 2003) (Fig. 2). A dual localisation of those and other peroxisomal and mitochondrial membrane proteins is in agreement with proteomics data (Wiese et al. 2007; Jadot et al. 2017).

Drp1 is a cytosolic, mechanochemical protein which belongs to the dynamin family of large GTPases. When recruited to organellar membranes, Drp1 can self-oligomerise into helical or ring-like structures and mediate membrane fission in a GTP hydrolysis-dependent manner (Antonny et al. 2016). Recruitment of Drp1 to peroxisomes or mitochondria depends on the shared membrane adaptor proteins Fis1, Mff, and GDAP1 (Fig. 2). In addition to the shared fission components, both organelles possess their own, specific division proteins such as peroxisomal PEX11β (Schrader et al. 2016), which is involved in peroxisomal membrane remodelling and acts as a GTPase-activating protein for Drp1 at peroxisomal division sites (Williams et al. 2015) (Fig. 2). Mitochondria possess additional Drp1 membrane adaptors such as Mid49 and Mid51 as well as components of the

membrane fusion machinery (e.g. the mitofusions Mfn1/2), which are not present at peroxisomes (Bonekamp and Schrader 2012) (Fig. 2). Overexpression, downregulation or loss of these adaptor proteins impacts on peroxisomal and mitochondrial morphology and dynamics, either promoting organelle division and fragmentation or the formation of highly elongated organelle membranes (Schrader et al. 2014) (Table 1). Loss of Drp1 or Mff function has been linked to a new group of disorders with defects in both peroxisomal and mitochondrial division (Waterham et al. 2007; Shamseldin et al. 2012; Koch et al. 2016) (see Sect. 5.1). Although the precise function and regulation of many of the adaptor proteins in organelle fission as well as the contribution of impaired peroxisomal and mitochondrial dynamics to the pathophysiology of above disorders remains to be determined, the importance of organelle dynamics for normal development and human health is generally accepted. Organelle division is important for other cellular processes, including organelle transport, distribution/cooperation, mitochondrial (mt)DNA inheritance, quality control, degradation/autophagy, and apoptosis/cell death (Chen and Chan 2009).

During the evolution of eukaryotic cells, mitochondria originated from the engulfment of an aerobic bacterium by a larger anaerobic archaebacterium. This has led to a close relationship between the host and the newly formed endosymbiont. The ancestral α-proteobacterial division machinery, which uses a dynamin-related protein to constrict the membrane at its inner face, was replaced with host-derived dynamin-related proteins to constrict the outer membrane from the cytosolic face. This has also led to the co-evolution and co-regulation of peroxisomal and mitochondrial division, and thus sharing of division components. Sharing these components is apparently an evolutionary conserved strategy among mammals, fungi and plants (Schrader and Fahimi 2006; Delille et al. 2009). As both organelles cooperate metabolically (see Sect. 1), co-regulation of peroxisomal and mitochondrial abundance and activity is likely beneficial under certain environmental conditions. Interestingly, the shared membrane adaptors Fis1, Mff and GDAP1 all belong to a specific class of membrane proteins, so called “tail-anchored” (TA) membrane proteins (Fig. 3). In the following, we will discuss their specific targeting and sorting mechanisms, as well as the prediction and identification of additional and new TA proteins at peroxisomes and mitochondria.

4 Sharing of Tail-Anchored Membrane Proteins

4.1 TA Protein Sharing Is Extensive and Regulated by Pex19 Interaction

One notable group of proteins increasingly recognised as having the potential to be localised to both mitochondria and peroxisomes are C-terminal, tail-anchored membrane proteins (Fig. 2). This class of proteins is characterised by the presence

of a transmembrane domain (TMD) close enough to the C-terminus to be recognised and processed post-translationally rather than co-translationally as is the case for the majority of membrane proteins (Kutay et al. 1993). This domain arrangement results in TA proteins being anchored in intracellular membranes, presenting the larger N-terminal domain to the cytosol whilst a shorter C-terminal tail remains inside the lumen of the target organelle (Fig. 3). TA proteins are found at all subcellular organelles, most commonly at the ER but also at the plasma membrane, Golgi, mitochondria and peroxisomes (Borgese and Fasana 2011). They are a large, diverse group of proteins estimated to make up approximately 3–5% of all transmembrane proteins in mammals (Kalbfleisch et al. 2007; Shigemitsu et al. 2016), which function in roles that require membrane anchorage. These include proteins involved in apoptosis, such as Bcl-2 family proteins (Westphal et al. 2011), formation of organelle contact sites, such as VAPB and ACBD5 (Stoica et al. 2014; Costello et al. 2017b) and protein translocation, such as Sec61β, Ramp4 and members of the TOM complex (Schröder et al. 1999; Johnston et al. 2002; Van den Berg et al. 2004).

The first TA proteins to be found to reside at both mitochondria and peroxisomes were known components of the mitochondrial division machinery, Fis1 (Koch et al. 2005) and Mff (Gandre-Babbe and van der Bliek 2008) (Fig. 2). As described in Sect. 3, Fis1 and Mff play a role as adaptors and activators of mitochondrial division, localising to and defining the sites where mitochondrial fission will occur and recruiting the dynamin-related protein Drp1 which then facilitates the fission step, ultimately separating the mitochondrial membrane (Smirnova et al. 2001; Ingerman et al. 2005). It has now become clear that the same set of factors is also involved in peroxisomal division and that Mff and Fis1, as well as Drp1, are recruited to peroxisomes and facilitate fission of the peroxisomal membrane (Koch et al. 2003; Kobayashi et al. 2007; Schrader et al. 2016). Consequently, mutations in Mff, Fis1 or Drp1 which reduce mitochondrial fission can also have an impact on peroxisomal fission (see Sect. 5.1) (Waterham et al. 2007; Shamseldin et al. 2012). An additional TA protein, GDAP1, involved in mitochondrial division was also found at peroxisomes and plays a role in peroxisomal division but its precise function remains elusive (Huber et al. 2013) (Fig. 2) (see Sect. 5.2). Finally MAVS, a TA protein involved in anti-viral defence, is also shared by peroxisomes and mitochondria, allowing both organelles to act together to coordinate the cells response to viral infection (Dixit et al. 2010) (Fig. 2). Whilst Mff, Fis1, GDAP1 and MAVS were initially characterised as mitochondrial proteins, their peroxisomal targeting was overlooked, presumably due to the majority of the protein being present at mitochondria which are also much larger and more easily discernible structures compared to the smaller, often punctate peroxisomes.

As peroxisomes and mitochondria are closely linked, also collaborating in fatty acid β-oxidation and working together to maintain ROS homeostasis (Schrader et al. 2015a) (Fig. 1), it is perhaps not surprising that they share protein components and that additional, unidentified shared components may also exist. Having already identified Fis1 and GDAP1 as shared peroxisomal/mitochondrial proteins, our group sought to identify additional shared proteins and to understand the targeting

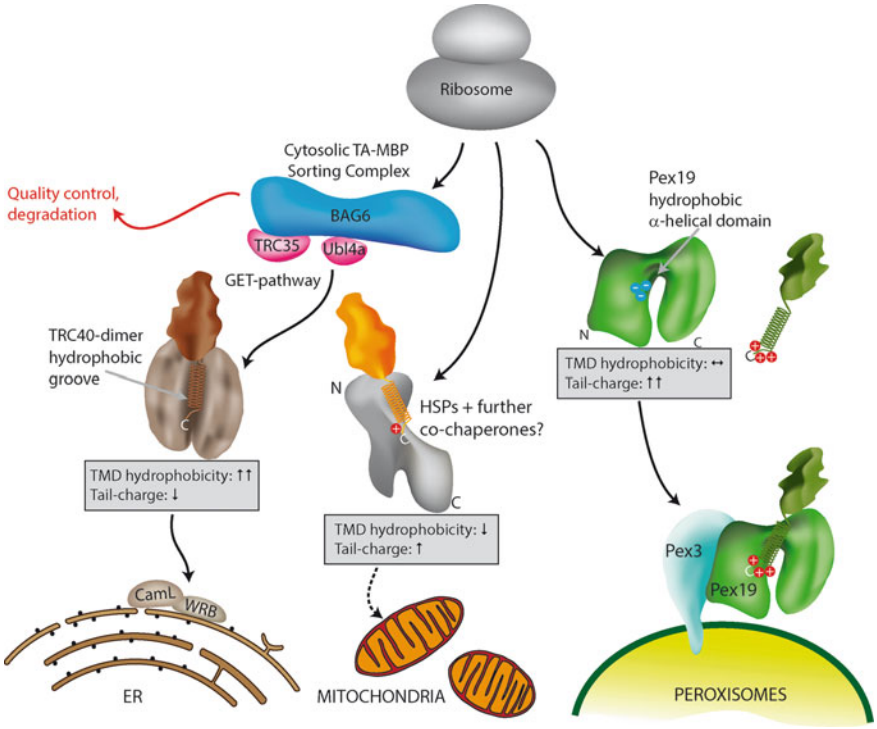

Fig. 3 Schematic view of tail-anchored protein targeting to subcellular organelles in mammalian cells. Specific delivery of TA proteins to ER, mitochondria and peroxisomes in mammalian cells is mediated by a combination of TMD hydrophobicity and tail charge. Targeting of TA proteins to the ER involves the GET (Guided Entry of Tail-anchored proteins) pathway. ER TA proteins interact with a cytosolic sorting complex (composed of BAG6, TRC35/GET4 and Ubl4a/GET5) and are delivered and inserted into the ER membrane by TRC40 (GET3) and WRB (GET1). A WRB/CAML dimeric membrane receptor (functional homolog to GET1/2) accepts the TA protein from TRC40 at the ER. ER targeting in mammals is supported by a hydrophobic TMD and low tail charge. Targeting of TA proteins to peroxisomes is mediated by the peroxisomal import receptor/chaperone PEX19 and the peroxisomal membrane protein PEX3. Peroxisomal TA proteins are characterized by a highly charged tail that promotes PEX19 interaction. TA proteins with a hydrophobic TMD require increased tail charge to be routed to peroxisomes. It is currently unknown if delivery and insertion of TA proteins into mitochondria involves specific targeting factors or is primarily unassisted. Mitochondrial TA proteins generally possess a less hydrophobic TMD than ER TA proteins and a less charged tail compared to peroxisomal TA proteins. This scheme is based on the steady state distribution of TA proteins. Note that other processes such as membrane extraction and TA protein degradation may also influence the subcellular localization. Note that the illustration of the GET pathway has been simplified. BAG6, BCL2-associated athanogene co-chaperone 6; HSP, heat shock protein; TRC, transmembrane domain recognition complex; Ubl4a, ubiquitin-like 4a; WRB, tryptophan-rich basic protein. This figure is taken from Costello et al., J Cell Sci 130:1675–1687. https://doi.org/10.1242/jcs.200204

signals involved in delivering TA proteins to particular organelles in the hope that this may allow us to predict subcellular localisations based on amino acid sequences (Costello et al. 2017a). Using ectopic expression of tagged proteins we discovered five additional proteins, Miro1, Miro2, BclXL, Bcl2 and OMP25 which are targeted to both mitochondria and peroxisomes (and in the case of Miro2 and Bcl2 also to the ER), expanding the repertoire of shared functions beyond division and anti-viral signalling to now include organelle motility (Miro1/2) (Wang and Schwarz 2009) and anti-apoptotic function (BclXL, Bcl2) (Billen et al. 2008) (Fig. 2). The physiological role of these proteins on peroxisomes awaits clarification. However, Miro1 was very recently identified as the missing adaptor for the recruitment of motor proteins in microtubule-dependent peroxisome motility (Castro et al. 2018; Okumoto et al. 2018), analogous to its role in mitochondrial movement. Miro1-mediated pulling forces were shown to contribute to peroxisome membrane elongation and proliferation in cellular models of peroxisome disease (Castro et al. 2018). In addition, a possible function for BclXL at peroxisomes in counteracting BAK-induced peroxisomal permeabilisation under conditions when mitochondrial localisation of BAK is lost due to mutation in VDAC2 was recently revealed (Hosoi et al. 2017).

To understand the targeting signals involved in TA protein distribution, and how so many proteins could be shared between organelles, we analysed the physicochemical properties of the TMD and tail region, which are known to consist of hydrophobic and charged regions which direct organelle targeting (Beilharz et al. 2003; Borgese et al. 2003). Previous studies had already extensively characterised the machinery involved in delivering TA proteins to the ER via the Guided Entry of TA protein (GET) pathway (Schuldiner et al. 2008) whilst the pathway for mitochondrial and peroxisomal targeting is less clear, but for peroxisomes clearly involves PEX19 and PEX3 which are also used for targeting of other peroxisomal membrane proteins (Yagita et al. 2013; Chen et al. 2014b) (Fig. 3). By analysing the C-terminal sequences of $\sim$50 human TA proteins we were able to classify ER, mitochondrial or peroxisomal proteins based on the hydrophobicity of the TMD and the net charge in the tail region. We used this data to train a SVM classifier allowing us to build a simple statistical model to predict the probability of a protein to be targeted to a particular organelle (Fig. 4). This analysis revealed a bi-partite signal for organelle targeting, consisting of the two key parameters, the hydrophobicity of the TMD and the overall net charge in the tail. Whilst high TMD hydrophobicity and low charge were characteristic of ER TA proteins, low hydrophobicity and moderate charge indicated mitochondrial TA proteins and high charge classified peroxisomal TA proteins, our data also suggested that the interplay between these two factors was an important factor. For example, TA proteins which, based on their TMD hydrophobicity, would be found at the ER could be found at peroxisomes if the charge in the tail was sufficiently high to counteract the hydrophobic signal from the TMD. This was best exemplified by fatty aldehyde dehydrogenase (FALDH) (see Sect. 5.4), which exists in two splice variants (Ashibe et al. 2007), one of which is found at the ER and the other at peroxisomes. Both variants share an identical TMD, which is highly hydrophobic, but differ in

the C-terminal tail with the ER variant containing a negatively charged tail whilst the peroxisomal variant contains a highly charged tail region. These observations are consistent with a recent study proposing that TA protein targeting to the ER is a process which is subject to selection filter checkpoints with handover from one sorting protein complex, or filter, to the next, dependent on the affinity of the TA protein for each protein complex (Rao et al. 2016). This three step model suggests that to reach the ER a TA protein would require the optimal physicochemical properties (i.e. low charge and/or high hydrophobicity) in order to pass each checkpoint. The first two steps are mediated by SGT2 and GET3 which form more stable complexes with highly hydrophobic TMDs whilst the final step, import into the ER membrane, is significantly less efficient for TA proteins with charged tail regions. In the case of the peroxisomal form of FALDH the highly hydrophobic TMD would presumably allow the protein to enter the GET pathway but it would then fail to enter the ER membrane due to the high charge in the tail. Release from a particular checkpoint complex would mean rejection from the ER pathway, and a protein could then either be degraded or would then be available to interact with the machinery for targeting to either mitochondria or peroxisomes (i.e. PEX19).

Whilst converting an ER TA protein into a peroxisomal TA protein may require a relatively large number of amino acid changes and therefore may be best achieved by alternative splicing as in the case of FALDH (Ashibe et al. 2007), the differences between mitochondrial and peroxisomal TA proteins can be very subtle with only a small alteration in tail charge sufficient to shift a protein from one organelle to the other (Fig. 3). For Miro1, different splice variants have recently been identified, which target mitochondria and/or peroxisomes (Okumoto et al. 2018). An amino acid insertion upstream of the TMD is supposed to promote peroxisomal targeting of Miro1 variants via interaction with PEX19. Interestingly, all peroxisomal membrane proteins tested show interaction with the peroxisomal membrane protein chaperone PEX19 (Sacksteder et al. 2000; Costello et al. 2017a). This interaction is dependent upon the charge in the tail with an increased charge leading to increased PEX19 binding (Yagita et al. 2013; Chen et al. 2014b; Costello et al. 2017a). This suggests that for a mitochondrial TA protein to also localise to peroxisomes simply requires affinity for PEX19 which can be achieved by addition of charged amino acids. Increasing charge further, resulting in high affinity for PEX19 can then eventually shift targeting in favour of peroxisomes (Fig. 3). Thus, as these two organelles co-evolved, binding to PEX19 may have been a selective force which allowed peroxisomal targeting and ultimately new peroxisomal functions in anti-viral signalling (MAVS), and potentially apoptosis (BclXL), and may also allow concerted organelle division (Mff, Fis1, GDAP1) and movement (Miro1/2) by utilising the same division and motility machinery (Fig. 2).

4.2 *Quality Control at Organelle Membranes*

Another factor which can play a role in regulating organelle location of TA proteins is a quality control system mediated by the AAA ATPAse Msp1 (ATAD1 in mammals) (Fig. 3). This pathway appears to have the capacity to identify mislocalised proteins on the mitochondrial membrane, extract them from the membrane and target them for degradation (Chen et al. 2014a; Okreglak and Walter 2014; Wohlever et al. 2017). Initial studies of this system have focused primarily on the clearance of the peroxisomal TA protein PEX15 (PEX26 in mammals) from mitochondria under conditions when the GET system was disrupted, when the TA proteins were over-expressed or when PEX15 was artificially targeted to mitochondria by alteration of its targeting signal. Under these conditions mitochondrial mis-localisation of PEX15 occurred which was further exacerbated in cells lacking Msp1 (Chen et al. 2014a; Okreglak and Walter 2014) Intriguingly, Msp1, which is itself an N-terminally tail-anchored (N-TA) membrane protein, was found not only at mitochondria but was also detected at peroxisomes. A recent report (Liu et al. 2016) showed that ATAD1 is also a shared mitochondrial/peroxisomal protein with peroxisomal targeting dependent on PEX19, further underscoring the importance of PEX19 interaction in influencing the sharing of TA proteins. ATAD1 is the only example so far of an N-TA protein which is shared by peroxisomes and mitochondria but others may exist which could rely on a similar sorting system to that used by C-TA proteins, based around affinity for PEX19.

The fact that Msp1 is shared by both organelles initially appeared to be problematic. How can the same quality control component distinguish between correctly targeted proteins at one organelle membrane (peroxisomes) and incorrectly targeted

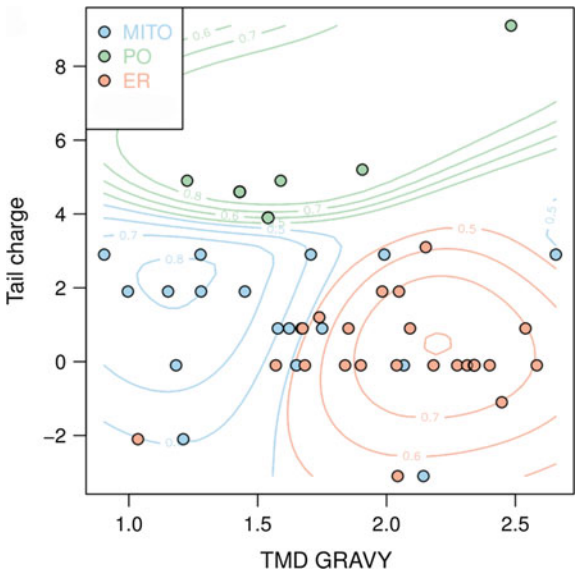

Fig. 4 Prediction map with probability contours showing clustering of TA membrane proteins to different organelle locations based on hydrophobicity (TMD GRAVY) and tail charge. Peroxisomes, green; mitochondria, blue; ER, red. This figure is taken from Costello et al., J Cell Sci 130:1675–1687. https://doi.org/10.1242/jcs.200204

proteins at another (mitochondria)? A more recent report has shed light on this process by demonstrating that Msp1 functions on peroxisomes to regulate PEX15 levels to prevent over-expression whilst at mitochondria Msp1 completely removes mis-targeted PEX15 (Weir et al. 2017). They hypothesised that additional protein binding partners found within the different organelles may shield proteins from Msp1 action, i.e. peroxisomal proteins interact with additional peroxisomal factors on peroxisomes which prevent Msp1 activity whereas a peroxisomal protein mis-targeted to mitochondria would not interact with such factors and so would be accessible to Msp1-mediated removal from the membrane. In an elegant set of experiments these authors provided supporting evidence for this concept, showing that PEX15 binding to the peroxisomal import factor PEX3 prevented Msp1 mediated removal of PEX15 from peroxisomes. The equivalent protein on mitochondria which protects mitochondrial proteins from removal by Msp1 remains to be identified. Whether or not this system applies to other proteins beyond PEX15, whether it also prevents targeting of other peroxisomal membrane proteins to mitochondria and if ATAD1 functions in an identical manner in mammalian cells also remains to be discovered.

This concept of quality control, essentially preventing peroxisomal proteins from aberrantly targeting mitochondria may be of particular relevance in light of recent data which suggests a mitochondrial origin for peroxisomes (Sugiura et al. 2017) (Fig. 1). This study is based on conditions where peroxisomes are absent and PEX3 is over-expressed. In these conditions PEX3 is initially targeted to mitochondria where it is able to generate pre-peroxisomal vesicles which then fuse with ER-derived vesicles containing PEX16 to produce import competent peroxisomes which can then mature to form new organelles. If PEX3 is the critical factor which protects peroxisomal TA proteins from Msp1-mediated degradation then targeting PEX3 to mitochondria could effectively override this process, allowing any peroxisomal protein to potentially associate with PEX3 at mitochondria and be protected from the Msp1-mediated quality control system. With this in mind it would be interesting to investigate the Msp1 system under conditions where peroxisomes are absent but PEX3 is still present or to artificially target PEX3 to mitochondria in normal cells and determine if the quality control abilities of Msp1 at mitochondria are now abolished.

5 The Role of Shared or New Tail-Anchored Organelle Membrane Proteins in Disease

5.1 Drp1 and Mff Deficiency

Similar to Fis1, the TA protein Mff acts as an adaptor protein for the fission GTPase Drp1 at mitochondria and peroxisomes (see Sect. 3) (Fig. 2). Loss of Drp1 or Mff function inhibits mitochondrial and peroxisomal division, resulting in highly

elongated organelles (Li and Gould 2003; Koch et al. 2003; Gandre-Babbe and van der Bliek 2008; Otera et al. 2010). Drp1 deficiency was the first disorder described based on a defect in both mitochondrial and peroxisomal fission (Waterham et al. 2007) (Table 1). The first reported patient died only a few weeks after birth, and combined clinical features of mitochondrial (e.g. autosomal dominant optic atrophy, neuropathy) and peroxisomal (dysmyelination, severity) disorders (Waterham et al. 2007). Genetic analysis revealed a heterozygous, dominant-negative missense mutation (Ala395Asp) in the middle domain of Drp1 (Waterham et al. 2007), which inhibits Drp1 oligomerization and thus function in membrane fission (Chang et al. 2010). Similar features, e.g. in synapse formation and brain development, have been described in Drp1 knockout mice (Wakabayashi et al. 2009; Ishihara et al. 2009), resulting in embryonic lethality. Additional Drp1 patients, who presented with developmental delay, refractory epilepsy or infantile encephalopathy, were recently described (Yoon et al. 2016; Chao et al. 2016; Sheffer et al. 2016; Vanstone et al. 2016; Fahrner et al. 2016; Nasca et al. 2016; Zaha et al. 2016) (Table 1). Genetic analysis also revealed missense variants in the Drp1 middle (oligomerisation) domain (Gly362Asp, G350R, E379 K) implying a dominant-negative mechanism, or recessive nonsense mutations leading to truncated unstable protein (Chao et al. 2016; Sheffer et al. 2016; Vanstone et al. 2016). Interestingly, the first dominantly inherited mutations in Drp1 affecting conserved amino acids within the Drp1 GTPase domain, were very recently identified (Gerber et al. 2017). Those Drp1 missense mutations have been linked to optic atrophy, a blinding disease due to the degeneration of the retinal ganglion cells, the axons of which form the optic nerves (Table 1). In most cases, dominant optic atrophy is caused by mutations in the mitochondrial large GTPase OPA1, which is involved in cristae remodelling and fusion of the inner mitochondrial membrane. Whereas Drp1 formed cytosolic aggregates and mitochondria were elongated in patient fibroblasts, peroxisome morphology appeared normal (Gerber et al. 2017). These findings suggest that mitochondrial division is also crucial for the maintenance of the optic nerve physiology and survival.

Whereas no patients with a defect in Fis1 are known, patients with loss-of-function mutations in Mff were recently reported (Shamseldin et al. 2012; Koch et al. 2016) (Table 1). They present with developmental delay, peripheral neuropathy, optic atrophy, and Leigh-like encephalopathy. Furthermore, mitochondria and peroxisomes are highly elongated in patient fibroblasts, likely due to a failure in Drp1 recruitment and organelle division. Interestingly, Mff was recently identified as a key effector of energy-sensing adenosine monophosphate (AMP)-activated protein kinase (AMPK)-mediated mitochondrial fission (Toyama et al. 2016). In contrast to the neurological features observed in Mff patients, mice deficient in Mff die at week 13 as a result of severe dilated cardiomyopathy leading to heart failure, which is likely due to mitochondrial defects (Chen et al. 2015). Like in patient fibroblasts, mitochondria and peroxisomes in Mff-deficient mouse embryonic fibroblasts were highly elongated. However, Mff-deficient mouse cardiomyocytes did not show a substantial change in mitochondrial or peroxisomal length. Instead, an increased heterogeneity in mitochondrial shape and abundance was observed (Chen et al. 2015).

It is currently not well understood, how a generalized defect of mitochondrial and peroxisomal division in patients leads to a specific neurological phenotype, and how peroxisomal and mitochondrial fission defects specifically contribute to the pathological features. It should be noted that biochemical parameters of organelle metabolism in Drp1 and Mff deficiency are often only slightly altered or remain unchanged. For example, lactic acidosis (indicative of defects in mitochondrial respiration) and (slightly) elevated levels of VLCFA (indicative of reduced peroxisomal β-oxidation) were not reported in all cases. This is in contrast to the classical peroxisome biogenesis disorders (e.g. Zellweger syndrome), where metabolic biomarkers such as VLCFA are largely altered. This can complicate the diagnosis of this novel group of peroxisome-mitochondria based disorders and underlines the importance of organelle morphology in diagnostics. It is likely that the pathological features of this group of disorders are related to impaired organelle dynamics and plasticity rather than metabolic dysfunction, e.g. altered organelle distribution within neurons. However, defects in organelle dynamics can impact on metabolic functions and metabolic cooperation of organelles.

5.2 GDAP1 and Charcot-Marie-Tooth Neuropathy

Ganglioside-induced differentiation associated protein 1 (GDAP1), a putative glutathione-S transferase, is another TA protein shared by mitochondria and peroxisomes (Niemann et al. 2005; Wagner et al. 2009; Huber et al. 2013) (see Sect. 3) (Fig. 2). GDAP1 is predominantly expressed in neural cells where it influences mitochondrial and peroxisomal dynamics and division (Huber et al. 2013). Mutations in GDAP1 are associated with the hereditary motor and sensory neuropathy Charcot-Marie-Tooth (CMT) disease (Suter and Scherer 2003; Niemann et al. 2006; Cassereau et al. 2011) (Table 1). Functional loss of GDAP1 results in elongated mitochondria and peroxisomes. However, peroxisome elongation is less prominent than observed in Drp1 or Mff deficiency. In contrast, overexpression of GDAP1 promotes mitochondrial and peroxisomal division dependent on Mff and Drp1. The observation that alterations in the C-terminal hydrophobic domain 1 (HD1) of GDAP1, or at the C-terminal tail affect both peroxisomal and mitochondrial fission, whereas N-terminal autosomal recessively inherited disease mutations still allow peroxisomal but not mitochondrial fission (Niemann et al. 2009; Huber et al. 2013) (Table 1), suggests that the pathophysiological alterations are presumably caused by changes in mitochondrial and not peroxisomal dynamics. However, peroxisomes may contribute to some degree to the clinical features of Charcot-Marie-Tooth disease. Recently, glutathione-S transferase activity for GDAP1 and its regulation via HD1 in an autoinhibitory manner was demonstrated (Huber et al. 2016). Moreover, the HD1 amphipathic pattern was required to induce membrane dynamics by GDAP1. Thus both, fission and glutathione-S transferase activities of GDAP1 are dependent on HD1, and GDAP1 may undergo a molecular switch, turning from a pro-fission active to an auto-inhibited inactive conformation (Huber et al. 2016).

5.3 ACBD5 Deficiency

ACBD5 (acyl-CoA binding domain containing protein 5) is a tail-anchored membrane protein with an exclusively peroxisomal localisation (Wiese et al. 2007; Islinger et al. 2007; Nazarko et al. 2014). Its role in PO-ER membrane contact formation was recently revealed (Costello et al. 2017b; Hua et al. 2017). ACBD5 interacts via a FFAT-like motif with ER-resident VAPB (vesicle-associated membrane protein-associated protein B), which is also a TA protein (Costello et al. 2017b; Hua et al. 2017) (Fig. 2). PO-ER contacts appear to be important for plasmalogen biosynthesis, which is mediated by peroxisome-ER cooperation. Interestingly, plasmalogen biosynthesis is impaired in ACBD5 deficient cells (Hua et al. 2017; Herzog et al. 2017). Patients with a defect in ACBD5 were first presented as having retinal dystrophy (Abu-Safieh et al. 2013), but further characterisation of another patient revealed a peroxisome-based disorder with progressive leukodystrophy, ataxia, progressive microcephaly, and retinal dystrophy (Ferdinandusse et al. 2017) (Table 1). Genetic analysis revealed a homozygous deleterious mutation deleting exons 7 and 8, causing a premature stop codon (Ferdinandusse et al. 2017). ACBD5 deficiency causes a defect in the β-oxidation of VLCFA, and it is suggested that ACBD5 facilitates transport of VLCFA into peroxisomes and subsequent β-oxidation (Yagita et al. 2017; Ferdinandusse et al. 2017; Herzog et al. 2017). ACBD5 is thought to be a human orthologue of yeast autophagy related protein ATG37 due to high region similarity (Nazarko et al. 2014). Using ACBD5 siRNA it was concluded that ACBD5 was also essential for pexophagy in human cells using a red-green fluorescent reporter assay to assess when peroxisomes are taken up by lysosomes. However, similar experiments with both ACBD5 patient and ACBD5 knock-out cells did not confirm a role in pexophagy (Ferdinandusse et al. 2017). Very recently, ACBD4, another acyl-CoA binding domain protein, was identified at peroxisomes (Costello et al. 2017c). ACBD4 is also a TA protein with a FFAT-like motif, which can interact with VAPB to mediate PO-ER membrane contacts (Fig. 2).

5.4 FALDH and Sjögren-Larsson Syndrome

Another disease-relevant TA protein is FALDH, which localises to the membranes of the ER and peroxisomes (see Sect. 4). FALDH-PO, the peroxisomal form, is a splice variant of FALDH which only differs from FALDH-ER in its C-terminal tail (see Sect. 4) (Ashibe et al. 2007). FALDH-PO was confirmed as a TA protein which exclusively targets peroxisomes (Costello et al. 2017a). Interestingly, alternative splicing of FALDH results in a C-terminal extension, which adds positive net charge to the C-terminal tail resulting in FALDH-PO. A highly charged tail now allows overcoming the highly hydrophobic TMD domain, which would mediate ER targeting, and interaction with the peroxisomal import receptor/chaperon PEX19

(Costello et al. 2017a). Mutations in FALDH cause Sjögren-Larsson Syndrome (SLS), which is characterised by mental retardation, spastic diplegia, and congenital ichthyosis following an autosomal recessive pattern (reviewed in Fuijkschot et al. 2012) (Table 1). FALDH deficiency results in the accumulation of fatty aldehydes and fatty alcohols in body tissues, as well as pro-inflammatory leukotriens, which are thought to contribute to the dermatological phenotype of SLS. The crystallographic structure of human FALDH was recently determined revealing a "gatekeeper helix" which controls access to the substrate cavity and supports orientation of the cavity towards the membrane surface for efficient substrate transit between membranes and the catalytic site (Keller et al. 2014). The membrane topology of FALDH was unclear, and we provided evidence that FALDH is a *bona fide* TA protein with its C-terminus anchored to the organelle membrane and the N-terminus with its catalytic domain exposed to the cytosol (Costello et al. 2017a) (Fig. 2). The majority of FALDH-activity appears to be associated with the ER, and the precise function of the peroxisomal variant is unclear. A role in the degradation of branched chain fatty acids via peroxisomal α-oxidation has been suggested (Ashibe et al. 2007), and peroxisomal aldehyde dehydrogenase activity for pristanal was detected (Jansen et al. 2001). However, FALDH-PO is a membrane-bound enzyme with its catalytic domain exposed to the cytosol, whereas the peroxisomal pristanal dehydrogenase activity is catalysed by a soluble matrix enzyme (Jansen et al. 2001). Furthermore, phytanic acid α-oxidation is normal in SLS patients and phytanic acid does not accumulate (Wanders et al. 2016). FALDH might also protect organelles and cells against oxidative stress associated with lipid peroxidation, which results in the formation of long-chain aliphatic aldehydes at cellular membranes. These aldehydes, which are also generated through lipid metabolism (e.g. metabolism of ether lipids, plasmalogens, sphingolipids) at membranes, are not accessible to cytosolic aldehyde dehydrogenases, and require membrane-associated enzymes.

6 Conclusions

As reviewed above, there is strong evidence that the intimate relationship between peroxisomes and mitochondria is the result of an organelle co-evolution originating in the early eukaryotic ancestors. The interplay between peroxisomes and mitochondria is reflected by an increasing number of cooperative functions, including fatty acid β-oxidation, peroxisome-mitochondria redox interplay, cooperation in anti-viral combat and coordinated biogenesis by sharing of the key division machinery (Fig. 1). There is overwhelming evidence from proteomics and molecular cell biology studies that both organelles share a significant number of proteins to fulfil the above mentioned functions and harbour an enzymatic inventory, which descended from common precursors. Multi-localised tail-anchored membrane proteins appear to play an important role as membrane-bound adaptors or enzymes, and their dual peroxisomal and mitochondrial localisation likely evolved through small alterations in their C-terminal physicochemical targeting properties. Further

studies on the phylogeny of the peroxisomal proteome may reveal their ancient protein inventory and contribute to the understanding of why mitochondria and peroxisomes gained such an intimate relationship. Although it is obvious that both organelles have to communicate in order to fulfil concerted actions in diverse cellular metabolic and signalling processes, the molecular mechanisms of these processes remain unclear. A first molecular tether linking peroxisomes to the ER has been identified in mammalian cells, which depends on the TA proteins ACBD5 or ACBD4 at peroxisomes, and VAP proteins (e.g. VAPB, VAPB) at the ER. It remains to be established if TA proteins are also involved in the association of peroxisomes and mitochondria, and thus in the exchange of co-factors, metabolites, and signalling molecules. A major challenge in the field is to reveal how peroxisome dysfunction impacts on mitochondria, if and how mitochondrial defects affect peroxisomal function, and how peroxisomal and mitochondrial interplay contributes to human health and disease. The ability to modulate the intricate relationship between peroxisomes and mitochondria may thus be beneficial for cell performance, e.g. in neurodegenerative disease, and may open new therapeutic avenues for combat of age-related disorders.

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Peroxisome Mitochondria Inter-relations in Plants

Jaideep Mathur, Aymen Shaikh and Neeta Mathur

Abstract A large amount of ultrastructural, biochemical and molecular analysis indicates that peroxisomes and mitochondria not only share the same subcellular space but also maintain considerable overlap in their proteins, responses and functions. Recent approaches using imaging of fluorescent proteins targeted to both organelles in living plant cells are beginning to show the dynamic nature of their interactivity. Based on the observations of living cells, mitochondria respond rapidly to stress by undergoing fission. Mitochondrial fission is suggested to release key membrane-interacting members of the FISSION1 and DYNAMIN RELATED PROTEIN3 families and appears to be followed by the formation of thin peroxisomal extensions called peroxules. In a model we present the peroxules as an intermediate state prior to the formation of tubular peroxisomes, which, in turn are acted upon by the constriction-related proteins released by mitochondria and undergo rapid constriction and fission to increase the number of peroxisomes in a cell. The fluorescent protein aided imaging of peroxisome-mitochondria interaction provides visual evidence for their cooperation in maintenance of cellular homeostasis in plants.

Keywords Peroxisomes · Mitochondria · Peroxules · Organelle-interactions Endoplasmic reticulum

Abbreviations

DRP3	DYNAMIN RELATED PROTEIN 3
ER	Endoplasmic reticulum

J. Mathur (✉) · A. Shaikh · N. Mathur
Laboratory of Plant Development and Interactions, Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road, Guelph, ON N1G2W1, Canada
e-mail: jmathur@uoguelph.ca

A. Shaikh
e-mail: ashaik03@uoguelph.ca

N. Mathur
e-mail: nmathur@uoguelph.ca

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FIS1	FISSION1
FP	Fluorescent protein
JEP	Juxtaposed elongated peroxisomes
MDVs	Mitochondria derived vesicles
MOPs	Mitochondria outer membrane derived protrusions
PMPs	Peroxisomal membrane proteins
ROS	Reactive Oxygen species
RNS	Reactive Nitrogen species
TPA	Tubular peroxisomal accumulations

1 Introduction

Maintaining cellular homeostasis in response to a continuously fluctuating environment is a fundamental property of all living cells. In plants, energy trapping during photosynthesis produces a large number of beneficial metabolites. Energy trapping also generates relatively harmful byproducts such as reactive oxygen species (ROS). All cellular membranes contribute to alterations in ROS levels (Bhattacharjee 2011) and at low cellular concentrations the different ROS, as well as reactive nitrogen species (RNS), act as signaling molecules. However, once their concentrations rise beyond a certain threshold they are able to interfere with and destroy cell-membrane architecture (Foyer and Noctor 2003; Apel and Hirt 2004; Gechev et al. 2010). An efficient cellular machinery is therefore required for maintaining a balance between the synthesis of biomolecules, their utilization and eventual breakdown. Two organelles, the mitochondria and the peroxisomes are intimately involved in this subcellular management (del Río et al. 2006; Schrader and Yoon 2007; Corpas et al. 2013). Although radically different views have been proposed to account for the presence of peroxisomes and mitochondria in the eukaryotic cell the two organelles share several common features that point to their intimate relationship with each other.

2 Conserved Features of Peroxisomes and Mitochondria in Plant Cells

In non-stressed plants both peroxisomes and mitochondria display a very similar morphology as small, spherical organelles that range in diameters from approximately 0.5 to 2 μm (Mathur et al. 2002; Logan and Leaver 2000). Interestingly, the first depiction of mitochondria, from observations made in plant cells, had shown them as having elongated forms (Meves 1904). These observations were reinforced by several subsequent light microscopy-based studies (Gunning and Steer 1986;

Lichtscheidl and Url 1990). Other investigations have also revealed that under certain conditions mitochondria become abnormally elongated and vermiform (Stickens and Verbelen 1996; Logan and Leaver 2000; Van Gestel and Verbelen 2002; Logan 2006; Seguí-Simarro et al. 2008). Observations made using fluorescently labelled mitochondria show that they become tubular and reach lengths of 8–10 μm in plant cells in response to sugar starvation and hypoxic conditions (Bereiter-Hahn and Vöth 1994; Logan 2006; Jaipargas et al. 2015).

A transient elongation response is also observed for peroxisomes, where they form 3 to 7 μm long tubules, in response to oxidative stress (Sinclair et al. 2009; Barton et al. 2014; Rodríguez-Serrano et al. 2016). Notably, in both organelles the tubulation progresses rapidly into a phase where the tubules are constricted. The final stage results in the fission of the tubular form into smaller sized organelles (Fig. 1). Interestingly, as for other eukaryotes, (detailed in Chapter "The Craft of Peroxisome Purification—A Technical Survey Through the Decades" SCHRADER) several molecular components of the fission machinery are common to both organelles in plants too. Amongst these are the mechano-chemical GTPases such as the Dynamin Related Protein (DRP3A and B/ADL2a and b), (Arimura and Tsutsumi 2002; Arimura et al. 2004a, b; Logan et al. 2004; Mano et al. 2004; Fujimoto et al. 2009; Zhang and Hu 2009; Aung et al. 2014). These proteins are well conserved in other organisms; Dlp1 in mammals (Pitts et al. 1999), Dnm1 in yeast (Bleazard et al. 1999) and form the major component responsible for tubule constrictions. Also, for both organelles a tail-anchored protein such as FISSION1 (Fis1/BIGYIN in plants: Scott et al. 2006; Zhang and Hu 2008; called hFis1 in mammals: Yoon et al. 2003; Stojanovski et al. 2004; Koch et al. 2005; Fis1p in yeast: Mozdy et al. 2000; Tieu and Nunnari 2000) serves to recruit and anchor the GTPases found in the cytosol. A FIS1A isoform has been shown to localize to the membranes of both organelles (Ruberti et al. 2014).

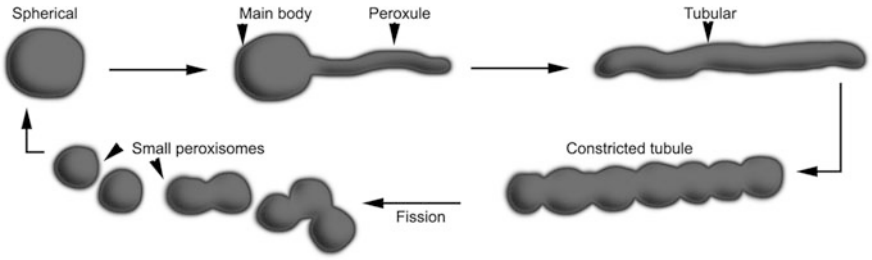

Fig. 1 Diagrammatic representation of peroxisome forms observed in living plant cells. Peroxisomes with one or more peroxules present a transient form that is intermediate between the spherical and the tubular shapes. The tubular form undergoes fission to create small sized peroxisomes that can become larger through import of peroxisomal matrix and membrane proteins

3 Fluorescent Protein Tools for Visualizing Peroxisomes and Mitochondria in Living Plant Cells

Observations on peroxisomes in plants have used a few dye-based approaches but have largely relied on the use of fluorescent proteins targeted to these organelles (Wang et al. 2014; Barton et al. 2014). Observations on mitochondria in living plant cells have been achieved using a variety of approaches where cationic, mitochondrial membrane-potential dependent dyes such as Rhodamine 123, tetra-methylrhodamine ethyl ester (TMRE), and tetra-methylrhodamine methyl ester (TMRM) and Mitotracker dyes of different colors (Invitrogen/Molecular probes; Chazotte 2011; Van Gestel et al. 2002; Sheahan et al. 2005) have been used. Presently a large number of fluorescent protein probes targeted to the two organelles are available (Table 1). In addition to the conventional fluorescent proteins that allow visualization in a single color the organelles have also been highlighted using photo-convertible fluorescent proteins like Kaede (Arimura et al. 2004a, b) and monomeric Eos (Sinclair et al. 2009; Mathur et al. 2010). Using the photo-convertible probes a sub-population of the targeted organelles can be differentially colored for understanding the interactions of similar organelles. This approach has been used to study mitochondrial fusion in plants (Arimura et al. 2004a, b; Schattat et al. 2012). Fluorescent protein probes aimed at understanding Ca^{2+} based sensing and signaling (Choi et al. 2012), H_2O_2 and redox perception and response have also been developed for both organelles (Schwarzlander et al. 2009; Costa et al. 2010; Loro et al. 2012).

Fluorescent protein aided observations on mitochondria and peroxisomes in living cells have also revealed a perplexing feature. Contrary to the situation in animal cell where the motility of both organelles appears to rely on the microtubule cytoskeleton and associated motor proteins (Wiemer et al. 1997; Schrader et al. 2003; Bowes and Gupta 2008), in plants they have been shown to move along actin filaments (Mathur et al. 2002; Van Gestel et al. 2002; Foissner 2004) in a myosin-mediated manner (Jedd and Chua 2002; Avisar et al. 2008; Sparkes and Gao 2014). Clearly the cytoskeleton-associated mechanisms related to the motility of these organelles require more investigation.

The recent development of double transgenic Arabidopsis plants that combine different coloured probes for the two organelles (Fig. 2) is beginning to facilitate investigations on the rapid behavior of the two organelles in response to a single stimulus and provides fresh cell biological insights on their interactions (Jaipargas et al. 2016).

Table 1 List of representative multi-coloured fluorescent protein (FP) probes targeted to mitochondria and peroxisomes in plants

Protein/targeting sequence mitochondria	FP	References
coxIV transit sequence of *Saccharomyces cerevisiae* cytochrome c oxidase IV	G	Kohler et al. (1997a, b)
Signal sequence mitochondrial chaperonin CPN-60 Mitochondrial-targeting sequence from *Nicotiana plumbaginifolia* β-ATPase	G G	Logan and Leaver (2000)
N 36 aa of the *Arabidopsis* mitochondrial ATPase δ-subunit	K	Arimura et al. (2004a, b)
First 29 aa of *Saccharomyces cerevisiae* cytochrome c oxidase IV	C	Nelson et al. (2007)
Mitochondrial-targeting sequence from *Nicotiana plumbaginifolia* β-ATPase	mEos	Mathur et al. (2010)
Mitochondrial-targeting sequence from *Nicotiana plumbaginifolia* β-ATPase	cpY	Schwarzländer et al. (2011)
pMt-RFP–N 78 aa γ subunit of the Arabidopsis mitochondrial F_1Fo ATPase	R	Uchida et al. (2011)
N′ 300 bp coding sequence of pentatricopeptide repeat protein ORGANELLE TRANSCRIPT PROCESSING (OTP)43	G	des Francs-Small et al. (2014)
Coding sequence-*Arabidopsis* Stomatin-like protein (SLP)1	Y	Gehl et al. (2014)
LATE EMBRYOGENESIS ABUNDANT (LEA) PROTEINS—LEA37, LEA38, LEA41	G	Candat et al. (2014)
Coding sequence of FISSION1A	Y	Ruberti et al. (2014)
Coding sequence of MITOCHONDRIAL CALCIUM UPTAKE (MICU)1	cpV	Wagner et al. (2015)
Coding sequence SUPPRESSOR OF (MOSAIC DEATH 1) (MOD1)3	G	Wu et al. (2015)
Peroxisomes		
Peroxisome targeting signal (PTS)1 appended to C′ of the fluorescent protein	Y G C C R mEos	Mathur et al. (2002) Jedd and Chua (2002) Nelson et al. (2007) Quan et al. (2010) Lin et al. (2004) Sinclair et al. (2009)
Peroxisome targeting signal (PTS)2 appended to N′ of the fluorescent protein	G R	Mano et al. (2002) Lin et al. (2004)
N′147 base pairs of Arabidopsis PEROXISOME DEFECTIVE1 thiolase containing peroxisome targeting signal (PTS)2	G	Woodward and Bartel (2005)
Arabidopsis PEROXIN10 (PEX)10	Y	Sparkes et al. (2003)
Arabidopsis PEROXIN2 (PEX)2	Y	Sparkes et al. (2005)
Arabidopsis PEROXIN 11 (PEX)11 family of proteins	C	Orth et al. (2007)

(continued)

Table 1 (continued)

Protein/targeting sequence mitochondria	FP	References
Coding sequence of FISSION1A	Y	Ruberti et al. (2014)
PEROXIN1 (PEX)1 PEROXIN6 (PEX)6	TagR	Goto et al. (2011)

cpY—circulary permutated Yellow FP; C-Cyan FP; mEos FP (photoconvertible); G-Green FP; K-Kaede (photoconvertible); R-Red FP; TagR-Tag Red FP; cpV-circularly permutated Venus FP; Y-Yellow FP

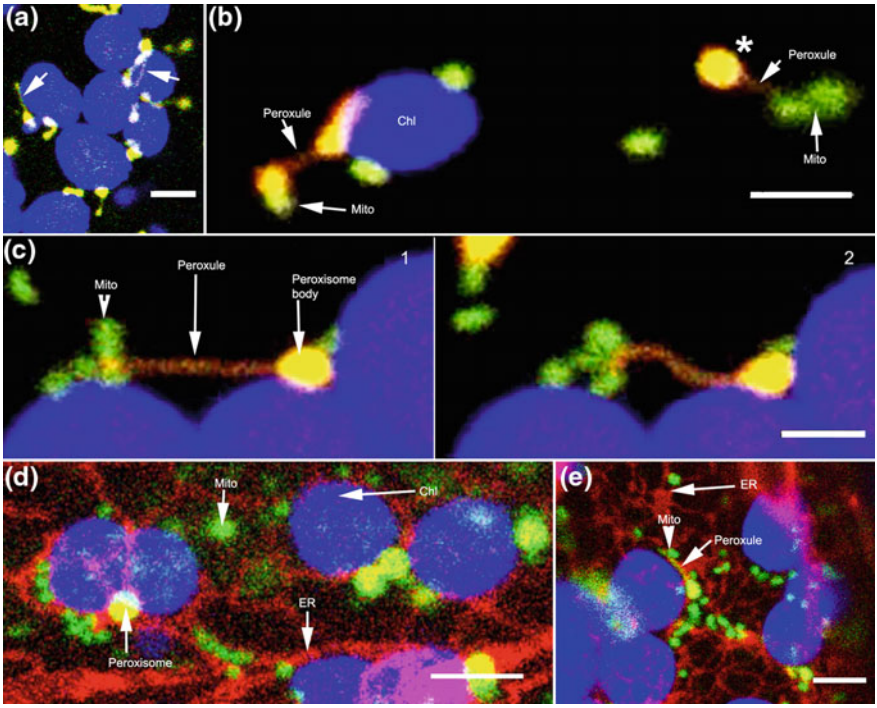

Fig. 2 Images from living cells in different transgenic Arabidopsis lines show peroxisomes in relation to chloroplasts (Chl), mitochondria (Mito), and the endoplasmic reticulum (ER) (based on Jaipargas et al. 2016). **a** Peroxisomes with peroxules (arrows) around mesophyll chloroplasts. **b** Following exposure to high intensity light for a few minutes a cluster of small mitochondria (mito), surround an extended peroxule while the peroxisome main body remains appressed to a chloroplast (Chl). A second independent peroxisome (*) also extends a peroxule that is surrounded by mitochondria. **c** Two sequential images (1, 2) from a time-lapse series show that a mitochondrial cluster is maintained around a peroxule even as it retracts towards the chloroplast-associated main peroxisome body. **d** A loose network of tubules and cisternae that make up the endoplasmic reticulum surrounds peroxisomes, mitochondria and chloroplasts. **e** Simultaneous visualization of chloroplasts, mitochondria, peroxisomes and the ER establishes the spatio-temporal relationship between the four organelles. Size bars = 5 μm

4 Imaging of Peroxisomes in Living Plant Cells Reveals Thin, Dynamic Peroxules

Imaging of living plant cells in which peroxisomes are highlighted using fluorescent protein probes of different colors has revealed that sporadically, the spherical peroxisome body extends and retracts thin tubules (Sinclair et al. 2009). These transient tubular extensions had been observed earlier by Cutler et al. (2000) and due to their morphological similarity to stromules extended from plastids (Köhler et al. 1997a, b) were named peroxules (Scott et al. 2007). It has been shown that mild, transient ROS stress increases the incidence of peroxule formation in a plant cell (Sinclair et al. 2009; Barton et al. 2013, 2014). However, the prolongation of oxidative stress, or higher stress intensity leads to a relatively long-lasting response where the entire peroxisome becomes tubular and the form is maintained until it undergoes fission (Sinclair et al. 2009; Fig. 1). Peroxule formation has also been shown to occur within 15 min as a relatively rapid plant response to the heavy metals Cadmium and Arsenic (Rodríguez-Serrano et al. 2016).

The conversion of nearly spherical peroxisomes into tubular forms has been demonstrated as a multistep process that involves the insertion of different peroxisomal membrane proteins (PMPs) into the existing peroxisomal membrane (Li and Gould 2003; Koch et al. 2004; Thoms and Erdmann 2005; Schrader 2006). Peroxin11 (PEX11) isoforms are especially important during the early phase of remodeling of the peroxisome membrane (Lingard and Trelease 2006; Kobayashi et al. 2007; Nito et al. 2007; Orth et al. 2007; Delille et al. 2010; Koch et al. 2010; Rodríguez-Serrano et al. 2016) and peroxule formation response is strongly inhibited in Arabidopsis *pex11a* RNAi lines (Rodríguez-Serrano et al. 2016). Interestingly the over-expression of PEX11 family proteins from yeast, plants and mammalian systems results in the formation of long tubules that have been called juxtaposed elongated peroxisomes (JEPs; Koch et al. 2010), and tubular peroxisomal accumulations (TPAs; Delille et al. 2010). Diagrammatic depictions of single peroxisomes within these clusters strongly suggest a resemblance to the peroxules. Notably the JEPs and TPAs that directly implicate PEX11 proteins in peroxisome tubulation and proliferation have usually been observed following over-expression of a specific PEX11 protein over several hours (Delille et al. 2010; Koch et al. 2010) whereas peroxules appear within seconds to a few minutes in normal plants as a rapid peroxisomal response to increased subcellular stress (Sinclair et al. 2009; Rodríguez-Serrano et al. 2009, 2016). The addition of PMPs into the peroxisomal membrane provides a very plausible and general mechanism for peroxisome elongation. While at present the rapidity with which peroxules are formed is not fully explained we consider these extensions as an early stage of membrane protein incorporation into the existing peroxisomal membrane (Fig. 1).

5 Peroxules Provide an Interaction Platform for ROS-Distressed Mitochondria

The imaging data collected from living plant cells clearly shows the phenomenon of peroxule formation while considerable literature points to the commonality in the fission machinery for peroxisomes and mitochondria. A question that became relevant was whether the proteins that are common for the fission of both organelles are recruited simultaneously to them or became available to each of them in a hierarchical manner. There is a basis for considering each possibility. The FISSION1 protein isoforms have been shown to exhibit non-conditional, dual localization to the membranes of both organelles (Zhang and Hu 2009; Ruberti et al. 2014). However, the DRP3 protein isoforms in plants exhibit a predominantly cytosolic localization (Zhang and Hu 2009). In general, the DRP3 proteins and their homologs have a tendency to bind to membranes through oligomerization and self-assembly based protein-protein interactions (Heymann and Hinshaw 2009). In yeast, it has been shown that following their recruitment and tethering by FIS1 proteins the DRP3 (and homologs) create spirals that are structurally tailored to fit mitochondria and promote fission (Ingerman et al. 2005; Mears et al. 2011). This raises the possibility that the cytosolic availability of DRP3 proteins with their recruitment factors already residing on peroxisome and mitochondrial membranes allows for simultaneous dual localization. However, there is also a basis for considering a hierarchical recruitment of the DRP3 proteins. Observations made in animal cells evoke scenarios of inter-organelle cooperation during ROS stress and suggest that metabolites and proteins may be transferred between the two organelles through mitochondria-derived vesicles (MDVs) (Neuspiel et al. 2008; Andrade-Navarro et al. 2009; Braschi et al. 2010; Mohanty and McBride 2013) and mitochondrial extensions known as matrixules (Scott et al. 2007; Schumann and Subramani 2008). Recent findings consider peroxisomes to be a hybrid organelle comprising mitochondrial and ER-derived pre-peroxisomal vesicles (Sugiura et al. 2017). Mitochondria in plants also respond rapidly to ROS (Yoshinaga et al. 2005) and vesicles similar to MDVs and mitochondria outer membrane-derived protrusions called MOPs have been described in Arabidopsis (Yamashita et al. 2016). It is quite likely that some proteins are exchanged between the two compartments through their close proximity.

The two possible scenarios have been investigated through simultaneous imaging of peroxisomes and mitochondria in stable double transgenic lines of Arabidopsis where the two organelles were highlighted by fluorescent proteins of two spectrally separable colors (Fig. 2). Under non-stressed conditions both mitochondria and peroxisomes in these plants displayed independent motility as part of the general cytoplasmic streaming. In the plants grown in the dark for a twelve-hour dark period the mitochondria appeared elongated with lengths ranging from 1.5 to 2.5 μm while almost none of the spherical peroxisomes exhibited peroxules (Jaipargas et al. 2016). However, exposing the dual-labelled plants to high intensity light for a few minutes resulted in rapid mitochondrial fission into

smaller and more diffuse-appearing structures. Also, within 10 min of the high-light treatment peroxules started being extended by a small subset of the peroxisome population. These observations were made in a more consistent manner in an Arabidopsis mutant, *anisotropy1* (*any1*) whose epidermal cells have a more curved, lens-like shape as compared to the wild-type plants (Fujita et al. 2013). A higher frequency of peroxule formation in *any1* in response to high-light was traced to increased ROS production in the mutant epidermal cells (Jaipargas et al. 2016).

Clusters of mitochondria were observed around small peroxules (Fig. 2) and maintained as the dynamic peroxules progressed into iso-diametric tubules that became increasingly constricted (Jaipargas et al. 2016). Although an actual demonstration of proteins being transferred from mitochondria to peroxisomes or vice versa, in the mitochondrial clusters around the peroxule is still missing these observations draw attention to yet another set of conditions that influence their behaviour.

6 The Chloroplasts and the ER as Interactors for Both Organelles

In plants the chloroplasts, peroxisomes and mitochondria are bound in very intimate biochemical associations (Noctor et al. 2007; Miller et al. 2010; Barton et al. 2017). In addition, it has been shown that each of the three organelles is enmeshed in the endoplasmic reticulum (ER) mesh (Schattat et al. 2011; Barton et al. 2014; Brocard 2014; Jaipargas et al. 2015). Moreover, the movement of plastids (Oikawa et al. 2008), peroxisomes (Mathur et al. 2002) and mitochondria (Van Gestel et al. 2002) has been shown to occur along actin filaments in a myosin mediated manner (Jedd and Chua 2002; Prokhnevsky et al. 2008; Avisar et al. 2008; Sparkes and Gao 2014). The membrane tubules, cisternae and vesicles making up the endoplasmic reticulum (ER) also track along actin filaments using myosin motor molecules (Ueda et al. 2010; Peremyslov et al. 2012). It has been observed that peroxisomes (Barton et al. 2013) and mitochondria (Jaipargas et al. 2015) closely align with the ER while peroxules extend along ER tubules (Sinclair et al. 2009; Mathur et al. 2012). While independent peroxisome—mitochondria interactions have been observed in living cells the incidence of such interactions increases several folds in plants that are exposed to light (Jaipargas et al. 2016). The rearrangement of the ER is one of the major changes that occurs in a cell in response to light (Griffing 2011) and in green tissues high light intensity often induces an aggregation of the ER around chloroplasts (Jaipargas et al. 2016). Under these conditions both mitochondria and peroxisomes are observed aggregating around the chloroplasts as part of the peri-plastid ER mesh (Jaipargas et al. 2016). Since the ER also responds quickly to changes in subcellular ROS (Margittai et al. 2008) and is a major compartment for the processing of proteins and lipids its actual role in bringing the two organelles closer to each other and in mediating their interactions requires more in-depth investigations.

7 A Model for Peroxisome-Mitochondria Interactions

A model that considers the interactions between mitochondria and peroxisomes to occur in a hierarchical manner has been put forward (Jaipargas et al. 2016). The model (Fig. 3) takes into account the general activity and localization of some of the plant proteins known to play a role in the fission of both mitochondria and peroxisomes (Mano et al. 2004; Fujimoto et al. 2009; Schrader 2006). However, a major assumption of the model is that the dynamin related proteins DRP3A and DRP3B in plants (Fujimoto et al. 2009; Zhang and Hu 2009) can bind and tubulate membranes in a stoichiometric, concentration dependent manner, as shown for dynamins and dynamin-like proteins in other organisms (Heymann and Hinshaw 2009; Roux et al. 2010). Thus, it is postulated that the fission of elongated, tubular mitochondria in response to increased subcellular ROS releases DRP3A and DRP3B (along with FIS1 collectively called constriction proteins in Fig. 3), and leads to an increase in their cytosolic concentration. The higher subcellular ROS levels also trigger the expression of different peroxisomal membrane proteins including Pex11a (Rodríguez-Serrano et al. 2016) whose insertion results in the extra membrane that constitutes the peroxule. The tubular peroxules might be pulled out by an ER mediated mechanism (Sinclair et al. 2009). As the peroxules

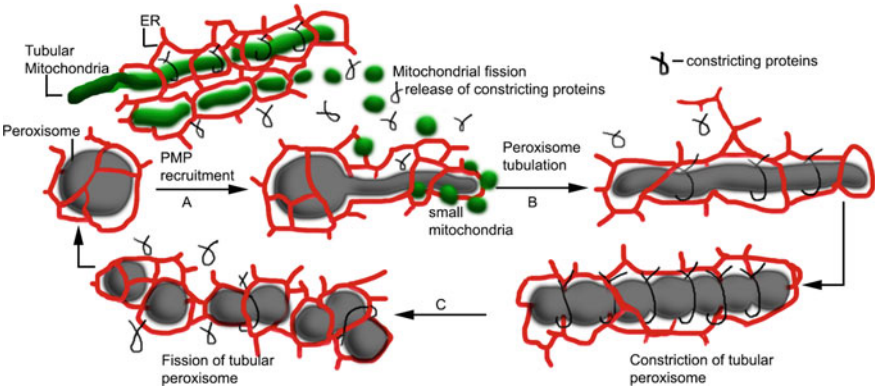

Fig. 3 A model depicting the idea that dynamin related proteins (DRP3A and DRP3B) and FISSION1 (collectively called constricting proteins), might be released by mitochondria as they undergo fission upon sensing an increase in subcellular ROS (A) followed by the recruitment of these proteins to tubular peroxules created through increased recruitment of peroxisomal membrane proteins. (B) Notably peroxules appear to have a diameter similar to elongated mitochondria. As part of their shared fission machinery a tail-anchored FIS1 protein serves to recruit the DRPs to both peroxisomes and mitochondria. The final steps depicted in (C) lead to progressive constriction of tubular peroxisomes by the DRP3 s and their fission in an ER mediated manner. While the present model uses FIS1 and DRPs as an example it also predicts that vesicle mediated trafficking as well as membrane contacts between the two organelles might result in many more proteins being shared by the two organelles to facilitate their shared function of maintaining cellular homeostasis

attain a diameter similar to that of elongated mitochondria their constriction is facilitated by the increased cytosolic availability of membrane tubulating and constricting proteins. The final fission of the constricted tubular peroxisome is brought about by through interactions with the contiguous ER (Sinclair et al. 2009; Mathur et al. 2012; Barton et al. 2013). This hypothetical model is very similar to that proposed for mitochondrial fission (Friedman et al. 2011) and does attribute an important role for the ER in the peroxisomal fission process.

8 Conclusions

The observed interactions between peroxisomes and the mitochondria highlight the fine tuning of biochemical and molecular processes for maintaining cellular homeostasis and also point to the conservation of cell biological mechanisms between diverse organisms. While the work presented here only considers a few of the elements common to both mitochondria and peroxisomes there are other proteins such as NETWORK MITOCHONDRIA/ELONGATED MITOCHONDRIA1 (NMT1/ELM1) considered a functional plant homolog of the yeast Mdv1/Caf4 proteins (Arimura et al. 2008; Logan 2010) whose roles need to be placed into context. In addition, new approaches towards understanding organelle interactions using optical tweezers (Gao et al. 2016) promise exciting discoveries. The interaction network of peroxisomes and mitochondria is bound to increase as we learn more about the interactions of these singular organelles with the neighbouring ER, plastid membranes, the cytoskeleton as well as metabolic accumulations like oil bodies and secretory products in plants.

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Peroxisomes and Cellular Oxidant/ Antioxidant Balance: Protein Redox Modifications and Impact on Inter-organelle Communication

Marc Fransen and Celien Lismont

Abstract Disturbances in cellular redox balance have been associated with pro-aging mechanisms and increased risk for various chronic disease states. Multiple lines of evidence indicate that peroxisomes are central players in cellular redox metabolism. Nevertheless, the potential role of this organelle as intracellular redox signaling platform has been largely overlooked for a long time. Fortunately, this situation is now changing. This review provides a snapshot of the current progress in the field, with an emphasis on the situation in mammals. We first briefly introduce the basics of redox biology and how reactive oxygen and nitrogen species can drive cellular signaling events. Next, we discuss current evidence linking peroxisome (dys)function to redox signaling, both in health and disease. We also highlight what is currently known about the downstream targets of peroxisome-derived oxidants. In addition, we present an extensive list of proteins that are involved in peroxisome functioning and have been identified as being responsive to oxidative stress in large scale redox proteomics studies. Finally, we address how changes in peroxisomal redox state may impact on functional mechanisms underlying inter-organelle communication. Gaining more insight into these mechanisms is key to our understanding of how peroxisomes are embedded in cellular signaling networks implicated in aging and diseases such as cancer, diabetes, and neurodegenerative disorders.

Keywords Peroxisomes · Catalase · Hydrogen peroxide · Redox balance
Oxidative modifications · Redox signaling · Gene expression · Mitochondria

Abbreviations

ACOX	Acyl-CoA oxidase
AKT	Ras-related C3 botulinum toxin substrate-alpha serine/threonine kinase

M. Fransen (✉) · C. Lismont
Department of Cellular and Molecular Medicine, KU Leuven,
Herestraat 49, Box 601, 3000 Louvain, Belgium
e-mail: marc.fransen@kuleuven.be

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ATM	Ataxia telangiectasia mutated
CAT	Catalase
CCS	Copper chaperone for SOD
CoA-SH	Free coenzyme A
CREB	cAMP-responsive element-binding protein
DAO	D-amino acid oxidase
EPXH	Epoxide hydrolase
FAD(H_2)	(Reduced) flavin adenine dinucleotide
FMN(H_2)	(Reduced) flavin mononucleotide
FOXO	Forkhead box O
GLRX	Glutaredoxin
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GSTK	Glutathione S-transferase kappa
GSTP	Glutathione S-transferase pi
IKBK	Inhibitor of NFKB
IMS	Mitochondrial intermembrane space
NAD(P)(H)	(Reduced) nicotinamide adenine dinucleotide (phosphate)
NFE2L	Nuclear factor, erythroid 2-like
NFKB	Nuclear factor kappa B
NFKB1	Subunit p50(p105) of NFKB
NOS	Nitric oxide synthase
ox-PTM	Oxidative posttranslational modification
PEX	Peroxin
PPARG	Peroxisome proliferator activated receptor gamma
PPARGC1A	PPARG coactivator 1 alpha
PRDX	Peroxiredoxin
PTS	Peroxisome targeting signal
RELA	Subunit p65 of NFKB
PTEN	Phosphatase and tensin homolog
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TXN	Thioredoxin
UOX	Urate oxidase
XDH	Xanthine dehydrogenase/oxidase

1 Introduction to the Basic Concepts of Oxidative Stress and Redox Signaling

The term "oxidative stress", coined in 1985 (Sies 1985), is widely used to describe a cell's state in which the production of reactive oxygen or nitrogen species (ROS and RNS, respectively) overwhelms the detoxifying ability of the antioxidant systems (Sies 2015). Major sources of ROS/RNS in living cells include the mitochondrial electron transport chain, the cytochrome P-450 and Ero1 enzymes in the endoplasmic reticulum, the flavin oxidases inside peroxisomes, and the NADPH oxidases and nitric oxide synthases (NOSs) that are located in different subcellular compartments (Nathan and Ding 2010). ROS/RNS species produced by these sources include the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and nitric oxide ($^{\cdot}NO$) radicals. These molecules can in turn readily react to form other ROS/RNS species, such as the hydroxyl radical ($^{\cdot}OH$), peroxynitrite ($ONOO^-$), and organic hydroperoxides (ROOH) (Sies et al. 2017). This process can occur spontaneously (e.g., in the case of $ONOO^-$) or via transition metal-catalyzed reactions (e.g., in the case of $^{\cdot}OH$) (Sies et al. 2017). To cope with excess ROS/RNS production, cells possess both enzymatic [e.g., superoxide dismutases (SODs), catalase (CAT), thioredoxins (TXNs), glutaredoxins (GLRXs), glutathione peroxidases (GPXs), and peroxiredoxins (PRDXs)] and non-enzymatic low-molecular weight [e.g., glutathione (GSH), ascorbic acid (vitamin C), and α-tocopherol (vitamin E)] antioxidant defense systems (Hanschmann et al. 2013; Marengo et al. 2016). For example, $O_2^{\cdot-}$ is rapidly detoxified by SODs to yield H_2O_2; H_2O_2 can be eliminated by CAT, GPXs, or PRDXs, and—if not detoxified—be converted to $^{\cdot}OH$ via the transition metal-catalyzed Fenton reaction; and $ONOO^-$ can be catalytically reduced to nitrite (NO_2^-) by PRDXs (Nordgren and Fransen 2014; Szabó et al. 2007).

Over the past decades, it has become increasingly clear that ROS/RNS do not only cause irreversible damage to cellular proteins, lipids, and nucleic acids, but also serve as important second messengers for a wide variety of normal cellular processes. Whether these reactive species cause oxidative damage or serve as signaling molecules, strongly depends on multiple variables, including their type, concentration, location, and kinetics of production and elimination (Auten and Davis 2009). For example, upon exposure to severe oxidative insults, many proteins undergo irreversible oxidative posttranslational modifications (ox-PTMs) such as lysine and arginine carbonylation, tyrosine and tryptophan nitration, dityrosine formation, cysteine sulfonylation (Cys-SO_3H), and protein–protein crosslinking (Ghezzi and Bonetto 2003). In general, these modifications have negative repercussion on protein folding, stability, and function. On the other hand, low levels of moderate oxidants (e.g., H_2O_2 and $^{\cdot}NO$) may lead to reversible oxidation of specific amino acid side chains, and especially cysteine thiol groups (Cys-SH) that tend to exist in thiolate form ($Cys\text{-}S^-$) due to their protein microenvironment are prime targets for redox modifications (Sies 2017). Examples of reversible oxidative

cysteine modifications include, among others, sulfenylation (Cys-S-OH), sulfinylation (Cys-SO_2H), S-nitrosylation (Cys-S-NO), S-glutathionylation (Cys-S-SG), and disulfide bond formation (–S–S–). Given that cysteine residues often play a crucial role in protein structure, localization, and function, these modifications frequently act as molecular redox switches that coordinate responses to oxidative changes in the surrounding environment. Importantly, this way of signal transduction is commonly used by distinct groups of functionally diverse proteins (e.g., transcription factors, kinases, protein phosphatases, and other regulatory proteins), not only under conditions of oxidative stress, but also under conditions of physiological oxidant production (often referred to as "oxidative eustress") (Berridge 2014; Sies 2017). To better deal with the latter aspect, the term "oxidative stress" was redefined as an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage (Jones 2006; Sies et al. 2017). Finally, it is important to note that disturbances in redox signaling have been identified as key contributors to the onset and progression of a broad spectrum of human diseases (Go and Jones 2017).

2 Redox Metabolism of Peroxisomes

As befits their name, peroxisomes play a critical role in cellular H_2O_2 metabolism. In general, they contain different sets of H_2O_2-producing enzymes as well as CAT, a H_2O_2-degrading enzyme. Depending on the organism, peroxisomes may also contain other ROS/RNS-producing and -degrading enzymes. Proteins and pathways involved in peroxisomal ROS/RNS metabolism and signaling are among the best studied in plants. However, as this review focuses on the situation in mammals, we refer the reader to other recent reviews for excellent overviews on this topic in plants (Sandalio and Romero-Puertas 2015; del Río and López-Huertas 2016; Corpas et al. 2017).

2.1 ROS/RNS Generation

Peroxisomes contain distinct sets of enzymes producing ROS (Table 1). The vast majority of these enzymes are the flavin adenine dinucleotide (FAD)- or flavin mononucleotide (FMN)-dependent oxidases, which are involved in a variety of biochemical processes such as the β-oxidation of fatty acids, the catabolism of amino acids and purines, and the oxidation of polyamines and 2-hydroxy acids (Van Veldhoven 2010; Wanders et al. 2016). Completion of their catalytic cycle is achieved by reoxidation of the reduced cofactor by molecular oxygen (O_2), a process yielding H_2O_2. Depending on the organism, tissue, and environmental conditions,

Table 1 ROS/RNS-producing enzymes in mammalian peroxisomes

Protein symbol	Localization	Substrates	Oxidant
ACOX1	PO	CoA-esters of straight-chain MCFAs, LCFAs, and VLCFAs (modifications at the w-end of the alkyl chain are tolerated)	H_2O_2
ACOX2	PO	2-Methyl-branched acyl-CoAs	H_2O_2
ACOX3	PO	Acyl-CoAs with or without a 2-methyl-branch (bulky or constrained groups at the w-end are not tolerated)	H_2O_2
ACOXL	PO	?	H_2O_2
DAO	PO	D-isoforms of neutral and polar amino acids	H_2O_2
DDO	PO	D-isoform of aspartate	H_2O_2
HAO1	PO	2-Hydroxy acids	H_2O_2
HAO2	PO	2-Hydroxy acids	H_2O_2
HAO3	PO	2-Hydroxy acids	H_2O_2
NOS2	C, PO, NU, MT	L-arginine	$^{\cdot}NO$, $O_2^{\cdot-}$
PAOX	PO	N_1-acetylated polyamines	H_2O_2
PIPOX	PO	Pipecolic acid and sarcosine	H_2O_2
UOX	PO	Uric acid	H_2O_2
XDH	C, PO, S	(Hypo)xanthine, NO_3^-, and NO_2^-	H_2O_2, $^{\cdot}NO$, $O_2^{\cdot-}$

Abbreviations: *ACOX* Acyl-CoA oxidase; *ACOXL* Acyl-CoA oxidase-like; *CoA* Coenzyme A; *C* Cytosol; *DAO* D-amino acid oxidase; *DDO* D-aspartate oxidase; *HAO* Hydroxyacid oxidase; *LCFA* Long-chain fatty acid; *MCFA* Medium-chain fatty acid; *MT* Mitochondria; *NOS* Nitric oxide synthase; *NU* Nucleus; *PAOX* Polyamine oxidase; *PIPOX* Pipecolic acid and sarcosine oxidase; *PO* Peroxisomes; *S* Secreted; *UOX* Urate oxidase; *VLCFA* Very long-chain fatty acid; *XDH* Xanthine dehydrogenase/oxidase

mammalian peroxisomes may also contain two potential physiological sources of $O_2^{\cdot-}$ and $^{\cdot}NO$ radicals: xanthine dehydrogenase/oxidase (XDH) (Angermüller et al. 1987) and the inducible form of nitric oxide synthase (NOS2) (Stolz et al. 2002). XDH is a key enzyme in purine degradation that catalyzes the oxidation of hypoxanthine to xanthine to uric acid, and—depending on the O_2 tension, pH, the purine concentration, and the NO_2^- and nitrate (NO_3^-) levels—the enzyme can produce $O_2^{\cdot-}$, H_2O_2, or $NO^{\cdot}$ as by- or end product (Battelli et al. 2016); and NOS2 is an enzyme that uses L-arginine as nitrogen donor for $^{\cdot}NO$ production (Nathan and Ding 2010). Interestingly, when L-arginine concentrations are limiting, NOS2 can also transfer electrons to O_2, thereby generating $O_2^{\cdot-}$ (Bronte and Zanovello 2005). Finally, the primary oxidant species generated by the enzymes listed above are also likely to react to form other ROS/RNS such as $ONOO^-$, $^{\cdot}OH$, and ROOH (see Sect. 1).

2.2 ROS/RNS Degradation

To maintain redox balance, peroxisomes also contain a battery of antioxidant defense mechanisms, including a set of antioxidant enzymes (Table 2) and low molecular weight antioxidants. The most abundant and best-characterized peroxisomal antioxidant enzyme is CAT, a heme-containing enzyme that can remove H_2O_2 in a catalatic (in case H_2O_2 functions as a hydrogen donor) or peroxidatic (in case ethanol, other short-chain aliphatic alcohols, nitrite, or formate act as hydrogen donor) manner (Zamocky et al. 2008). Another peroxisomal sink for H_2O_2 is PRDX5, an enzyme that can also detoxify $ONOO^-$ and ROOH (Knoops et al. 2016). Interestingly, while PRDX5 detoxifies H_2O_2 in the low micromolar range (Knoops et al. 2016), efficient decomposition of this molecule by CAT requires concentrations in the low millimolar range (Zamocky et al. 2008). On the other hand, the maximum rate of H_2O_2 conversion is orders of magnitude higher for CAT than for PRDX5 (Zamocky et al. 2008; Knoops et al. 2016). The physiological consequences of these differences in kinetic characteristics are discussed in Sect. 5.

In line with the view that mammalian peroxisomes can harbor potential sources of $O_2^{\cdot-}$ (see Sect. 2.1), it has been reported that these organelles also contain SOD activity (Wanders and Denis 1992; Dhaunsi et al. 1992). Later on, these findings were confirmed and extended by others, who showed that peroxisomes contain traces of Cu/Zn SOD (SOD1) (Moreno et al. 1997; Kira et al. 2002; Islinger et al. 2009). This enzyme lacks a typical peroxisomal targeting signal (PTS), but is imported into peroxisomes by piggyback transport using "copper chaperone for SOD" (CCS) as transport vehicle (Islinger et al. 2009). CCS is a PTS-containing protein that has been found in peroxisomes, the cytosol, and the mitochondrial intermembrane space (IMS) (Islinger et al. 2009). Interestingly, the localization and activity of SOD1 in the IMS do not only depend on CCS, but also on the redox state of the IMS (Suzuki et al. 2013).

Table 2 ROS/RNS-degrading enzymes in mammalian peroxisomes

Protein symbol	Localization	Catalytic activity
CAT	PO, C	$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ (catalatic activity) $H_2O_2 + AH_2 \rightarrow A + 2\ H_2O$ (peroxidatic activity)
EPHX2	PO, C	Epoxide + $H_2O \rightarrow$ diol
GSTK1	PO	RX + GSH $\rightarrow$ HX + R-S-SG
PRDX5	C, MT, PO, N	$PRDX5_{red} + ROOH \rightarrow PRDX5_{ox} + H_2O + ROH$ $PRDX5_{red} + ONOO^- \rightarrow PRDX5_{ox} + H_2O + NO_2^-$ $PRDX5_{red} + H_2O_2 \rightarrow PRDX5_{ox} + 2\ H_2O$
SOD1	C, MT, PO, N	$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow O_2 + H_2O_2$

Abbreviations: *AH_2* Hydrogen donor; *C* Cytosol; *CAT* Catalase; *EPHX* Epoxide hydrolase; *GSTK* Glutathione S-transferase kappa; *MT* Mitochondria; *N* Nucleus; *Ox* Oxidized; *PO* Peroxisomes; *PRDX* Peroxiredoxin; *Red* Reduced; *RX* Hydrophobic electrophile; *SOD* Superoxide dismutase

Mammalian peroxisomes also contain two other antioxidant enzymes, epoxide hydrolase 2 (EPXH2) and glutathione S-transferase kappa 1 (GSTK1), which are respectively involved in the detoxification of epoxides (Decker et al. 2009) and lipid peroxidation products (Wang et al. 2013). Direct experimental evidence that mammalian peroxisomes contain TXNs and/or GLRXs is currently lacking, even from large-scale proteomics studies (Kikuchi et al. 2004; Islinger et al. 2007; Wiese et al. 2007; Gronemeyer et al. 2013). However, given that (i) the reduction of oxidized PRDX5 normally involves the transfer of electrons from NADPH via a thiol-disulfide oxidoreductase such as TXN1 or TXN2 (Rhee and Kil 2017), and (ii) roGFP2, a ratiometric redox sensor that specifically equilibrates with the GSSG/GSH redox pair through the action of endogenous GLRXs (Meyer et al. 2007), quickly responds to redox changes in the peroxisomal matrix (Ivashchenko et al. 2011), the intraperoxisomal presence of such proteins or proteins with a similar activity can be expected. In this context, and based on other studies carried out with PRDX6 and GSTP1 (Manevich et al. 2004; Rhee and Kil 2017), it is tempting to speculate that GSTK1 may also be involved in the transfer of reducing equivalents from GSH to oxidized PRDX5. Finally, there is indirect evidence that also low molecular weight antioxidants such as GSH and vitamin C may play a role in the regulation of the peroxisomal redox state. However, many questions regarding their precise contribution and mechanism of action remain to be clarified (Lismont et al. 2015).

3 Peroxisomes, Thiol Redox State, and Oxidative Protein Modifications

To exert their activities, peroxisomes need to constantly communicate with their environment. A common strategy to convey information from one cellular compartment to the other is through the release of biological messengers such as ROS/RNS, lipids, or other metabolites (Fransen et al. 2017). Given that peroxisomes have the intrinsic ability to generate and scavenge ROS/RNS (see Sects. 2.1 and 2.2, respectively), it may not be surprising that these organelles are emerging as guardians and modulators of cellular redox balance and signaling (Fransen and Lismont 2018). In the following sections, we first highlight the role of peroxisomes in maintenance of the intracellular thiol redox equilibrium. Next, we discuss what is currently known about the cellular targets of peroxisome-derived ROS. Thereafter, we present an overview of proteins that are involved in peroxisome functioning and have been demonstrated to undergo oxidative modifications under redox-perturbing conditions.

3.1 Peroxisomes and Thiol Redox State

Given that (i) sulfur atoms in cysteine residues that exist as thiolate anions are highly susceptible to oxidation (Sies 2017), and (ii) such oxidative modifications often play a crucial role in protein structure, localization, and function (Stöcker et al. 2017), maintenance and regulation of the intracellular thiol redox balance is critically important for cell function. Up to now, only a few studies have investigated the direct relationship between peroxisomal redox metabolism and the thiol-disulfide equilibrium in mammalian cells. One such study reported that treatment of Chang liver cells with the CAT inhibitor 3-amino-1,2,4-triazole resulted in a 20% increase in protein disulfide content (Yang et al. 2007). In line with this finding, others have observed that transgenic overexpression of CAT in murine hearts diminished cysteine oxidation of numerous cardiac mitochondrial and contractile proteins (Yao et al. 2015). We recently found that the extent of protein cysteine oxidation in response to peroxisome-derived H_2O_2 depends on the subcellular localization of the target protein and is inversely correlated to CAT activity (Lismont et al. 2018). Finally, when discussing the link between peroxisomal redox metabolism and cellular thiol redox state, it is important to keep in mind that peroxisomal β-oxidation acts on acyl-CoAs, and not on free fatty acids (Van Veldhoven 2010). Given that (i) the intracellular levels of free coenzyme A (CoA-SH) are substantial (e.g., ~10–150 nmol/g tissue), and (ii) the GSSG/GSH balance can be influenced by the CoA-ester/CoA-SH ratio, this implies that variations in β-oxidizable peroxisomal substrate concentrations will not only affect peroxisomal H_2O_2 production, but also influence GSSG/GSH balance through CoA sequestration. For more details on this topic, we refer the reader to another review (Lismont et al. 2015; and references therein).

3.2 Molecular Targets of Peroxisome-Derived Oxidants

As already mentioned above (see Sect. 1), various oxidants are likely to have different biochemical targets. To date, two main strategies have been used to selectively generate different types of ROS inside peroxisomes. In one approach, a H_2O_2-producing peroxisomal oxidase (e.g., ACOX1, UOX, or DAO) was overexpressed in a mammalian cell line (e.g., CV-1 or COS-1 African green monkey kidney cells, HEK-293 human embryonic kidney cells, or MYP3 rat urothelial cells) and the cells were subsequently cultured in the presence of (precursor) substrates (e.g., linoleic acid, uric acid, or D-alanine) of the respective enzymes (Chu et al. 1995, 1996; Okamoto et al. 1997; Dadras et al. 1998; Li et al. 2000; Lismont et al. 2018). In the other approach, a peroxisome-targeted photosensitizer was used to generate $O_2^{\cdot-}$ in a light-controlled manner in murine fibroblast or

oligodendrocyte cell lines (Wang et al. 2013; Walbrecq et al. 2015). In summary, these studies demonstrated that (i) peroxisome-derived H_2O_2 can oxidize redox-sensitive cysteines in multiple endogenous proteins within (e.g., PRDX5) and outside (e.g., RELA/p65, NFKB1/p50(p105), PTEN, FOXO3, and PEX5) the peroxisomal compartment (Lismont et al. 2018), (ii) sustained intraperoxisomal H_2O_2 production leads to activation of NFKB (Li et al. 2000) and eventually causes cell transformation (Chu et al. 1995, 1996; Okamoto et al. 1997; Dadras et al. 1998), and (iii) excessive intraperoxisomal $O_2^{\cdot-}$ production results in the accumulation of lipid peroxides, a process that can be counteracted by overexpression of peroxisome-targeted SOD1, GSTK1 or PRDX5 (Wang et al. 2013; Walbrecq et al. 2015). Unfortunately, unbiased proteome-wide surveys of targets for peroxisome-derived oxidants have not yet been reported (see also Sect. 5).

3.3 *Redox Modifications of Proteins Involved in Peroxisome Functioning*

Oxidative protein modifications such as sulfenylation, sulfinylation, sulfonylation, nitrosylation, glutathionylation, and/or intra- and intermolecular disulfide bond formation are emerging as a common mechanism to rapidly and reversibly regulate protein function in living cells in response to changing redox state (see Sect. 1). Over the past decades, multiple (large-scale) studies have been carried out to identify proteins that are oxidatively modified under basal conditions or upon interventions that affect the overall redox state of cells [e.g., cardiac myocytes subjected to H_2O_2 treatment or metabolic inhibition (Brennan et al. 2004)] or tissues [e.g., overexpression of CAT in the mouse heart (Yao et al. 2015)]. A careful analysis of these studies revealed that also many proteins involved in peroxisome functioning are susceptible to oxidative modifications (Table 3). This finding points to the potential relevance of such modifications in the regulation of diverse peroxisomal processes (e.g., biogenesis, lipid metabolism, antioxidant defense, and inter-organelle communication) in response to local and environmental redox changes. Unfortunately, the biological relevance and functional significance of most of these oxidative modifications remain to be established. However, in this context, it is interesting to note that multiple large-scale redox proteomics studies have identified both human and mouse CAT as a target for S-nitrosylation (Table 3), and increased nitrosylation of CAT has been reported to be accompanied by reduced CAT activity (Ghosh et al. 2006; Visiedo et al. 2017). Note also that many (but not all) of these ox-PTMs have been catalogued in curated databases such as RedoxDB (https://biocomputer.bio.cuhk.edu.hk/RedoxDB), dbGSH (http://csb.cse.yzu.edu.tw/dbGSH), or dbSNO (http://140.138.144.145/~dbSNO): RedoxDB is a database of experimentally verified ox-PTMs, and—for most

Table 3 Redox features of human, mouse, and rat proteins involved in peroxisome functioning

Function	Protein symbol	UniProt accession number	Redox feature	Position	References
Biogenesis	PEX3 (*Hs*)	P56589	H_2O_2-sensitive	C65	Fu et al. (2017)
	PEX5 (*Hs*)	P50542	H_2O_2-sensitive	C11	Lismont et al. (2018)
	PEX6 (*Hs*)	Q13608	H_2O_2-sensitive	C309	Fu et al. (2017)
	PEX11A (*Hs*)	O75192	H_2O_2-sensitive	C85	Fu et al. (2017)
	PEX11B (*Hs*)	O96011	H_2O_2-sensitive	C25, C153	Fu et al. (2017)
	PEX19 (*Mm*)	Q8VCI5	S-NO	C229	Kohr et al. (2011)
Fission	DNM1L (*Hs*)	O00429	H_2O_2-sensitive	C367, C446,	Fu et al. (2017)
			S-NO	C644	Cho et al. (2009)
	DNM1L (*Mm*)	Q8K1M6	S-NO	C367	Kohr et al. (2011)
	DNM1L (*Rn*)	O35303	S-NO	C374	Paige et al. (2008)
	FIS1 (*Mm*)	Q9CQ92	S-NO	C41	Kohr et al. (2011), (2012)
	MFF	Q9GZY8	H_2O_2-sensitive	C209	Fu et al. (2017)
Lipid metabolism	ABCD3 (*Hs*)	P28288	H_2O_2-sensitive	C472, C477	Fu et al. (2017)
	ABCD3 (*Mm*)	P55096	S-NO	C24	Doulias et al. (2010)
	ACAA1 (*Hs*)	P09110	H_2O_2-sensitive	C123, C218, C381	Fu et al. (2017)
			S-NO	ND	López-Sánchez et al. (2008)
	ACAA1 (*Mm*)	Q921H8	H_2O_2-sensitive	C177	Yao et al. (2015)
	ACAD11 (*Hs*)	Q709F0	H_2O_2-sensitive	C636	Fu et al. (2017)
			S-NO	C547	Lee et al. (2014)
	ACOT2 (*Hs*)	P49753	H_2O_2-sensitive	C401	Fu et al. (2017)
	ACOX1 (*Hs*)	Q15067	H_2O_2-sensitive	C199	Fu et al. (2017)
	ACOX1 (*Mm*)	Q9R0H0	S-NO	C199, C392, C531, C559	Kohr et al. (2011), (2012)
	ACOX2 (*Hs*)	Q99424	H_2O_2-sensitive	C65, C412	Fu et al. (2017)

(continued)

Table 3 (continued)

Function	Protein symbol	UniProt accession number	Redox feature	Position	References
	ACSL1 (*Hs*)	P33121	H_2O_2-sensitive	C55, C108, C297, C336	Fu et al. (2017)
	ACSL1 (*Mm*)	P41216	H_2O_2-sensitive	C55, C109, C133, C275, C298, C626	Fu et al. (2008), Yao et al. (2015)
			TXN target	C109, C626	Fu et al. (2009)
			S-NO	C55, C109, C133, C221, C242, C275, C298, C510, C626	Kohr et al. (2012)
	ACSL1 (*Rn*)	P18163	S-S (inter)	ND	Brennan et al. (2004)
	ACSL4 (*Hs*)	O60488	H_2O_2-sensitive	C221, C420, C494, C495	Fu et al. 2017
	ACSL6 (*Mm*)	Q91WC3	S-NO	C91, C101, C158, C170, C181, C186	Kohr et al. (2011), (2012)
	ACSL6 (*Rn*)	P33124	S-S (inter)	ND	Brennan et al. (2004)
	AGPS (*Hs*)	O00116	H_2O_2-sensitive	C214, C226, C349, C413, C565	Fu et al. (2017)
	AMACR (*Hs*)	Q9UHK6	H_2O_2-sensitive	C117	Fu et al. (2017)
	DECR2	Q9NUI1	H_2O_2-sensitive	C22	Fu et al. (2017)
	ECH1 (*Hs*)	Q13011	S-NO	ND	Zhang et al. (2011)
	ECH1 (*Mm*)	O35459	TXN target	C186	Fu et al. (2009)
	EHHADH (*Hs*)	Q08426	H_2O_2-sensitive	C227	Fu et al. (2017)
	GNPAT (*Hs*)	O15228	H_2O_2-sensitive	C54, C66, C73, C668	Fu et al. (2017)
	GNPAT (*Mm*)	P98192	S-NO	C137	Kohr et al. (2011)
	HSD17B4 (*Hs*)	P51659	H_2O_2-sensitive	C189, C277	Fu et al. (2017)
	PECR (*Hs*)	Q9BY49	H_2O_2-sensitive	C191	Fu et al. (2017)
	PECR (*Mm*)	Q9BY49	S-NO	C43	Doulias et al. (2010)

(continued)

Table 3 (continued)

Function	Protein symbol	UniProt accession number	Redox feature	Position	References
Antioxidant	CAT (*Hs*)	P04040	H_2O_2-sensitive	C377, C460	Fu et al. (2017)
			S-NO	C232, C377, C460	Ghosh et al. (2006), Chen et al. (2014a, b), Lee et al. (2014)
	CAT (*Mm*)	P24270	H_2O_2-sensitive	C377	Yao et al. (2015)
			S-NO	C232, C377, C393, C425, C460	Doulias et al. (2010), Kohr et al. (2012)
	EPHX (*Mm*)	P34914	S-NO	ND	Benhar et al. (2010)
			TXN target	ND	Benhar et al. (2010)
	GSTK1 (*Hs*)	Q9Y2Q3	S-NO	C176	Kohr et al. (2011), (2012)
	PRDX5 (*Hs*)	P30044-2	H_2O_2-sensitive	C48	Fu et al. (2017)
			S-OH	C48	Declercq et al. (2001)
			S-NO	C48	Liu et al. (2010), Lam et al. (2010)
			S-S (intra)	C48 & C152	Seo et al. (2000)
	PRDX5 (*Mm*)	P99029-2	H_2O_2-sensitive	C48	Fu et al. (2008)
			S-NO	C48, C200	Kohr et al. (2011), (2012)
			S-S (intra)	C48 & C152	Fu et al. (2009)
			TXN target	C48	Fu et al. (2009)
	PRDX5 (*Rn*)	Q9R063-2	S-OH	ND	Tyther et al. (2010)
	SOD1 (*Hs*)	P00441	H_2O_2-sensitive	C58, C112	Fu et al. (2017)
			S-SG	C112	Wilcox et al. (2009), Redler et al. (2011)
			S-O_2H	C112	Fujiwara et al. (2007)
			S-O_3H	C112	Fujiwara et al. (2007)
			S-NO	C7	Chen et al. (2014a, b), Lee et al. (2014)
			S-S (intra)	C58 & C147	Tiwari and Hayward (2003)
	SOD1 (*Mm*)	P08228	H_2O_2-sensitive	ND	Fu et al. (2008)
			S-NO	C7	Zaręba-Kozioł et al. (2014)
	SOD1 (*Rn*)	P07632	S-OH	ND	Tyther et al. (2010)
			S-S (inter)	ND	Brennan et al. (2004)

(continued)

Table 3 (continued)

Function	Protein symbol	UniProt accession number	Redox feature	Position	References
Protein/AA metabolism	IDE (*Hs*)	P14735	S-NO	C110, C178, C789, C812, C819, C966	Cordes et al. (2009), Ralat et al. (2009)
			S-OH	C178	Ralat et al. (2009)
			Y-NO_2	Y831	Ralat et al. (2009)
	LONP2 (*Hs*)	P14735	H_2O_2-sensitive	C137	Fu et al. (2017)
	PIPOX (*Hs*)	Q9P0Z9	H_2O_2-sensitive	C119	Fu et al. (2017)
PO-ER contact sites	ACBD5 (*Hs*)	Q5T8D3	H_2O_2-sensitive	C83	Fu et al. (2017)
	VAPB (*Hs*)	O95292	H_2O_2-sensitive	C173	Fu et al. (2017)
	VAPB (*Mm*)	Q9QY76	S-NO	C41, C121	Chen et al. (2015)
Other	ATAD1 (*Hs*)	Q8NBU5	H_2O_2-sensitive	C137, C359	Fu et al. (2017)
			S-NO	C137	Kohr et al. (2011)
	ATM (*Hs*)	Q13315	H_2O_2-sensitive	C532, C790, C1177, C1736, C2246, C2323, C2991	Fu et al. (2017)
	HAO2 (*Rn*)	Q07523	S-OH	ND	Tyther et al. (2010)
			S-SG	ND	Eaton et al. (2003)
	NOS2 (*Mm*)	P29477	ND	C592	Yang et al. (2012)
	IDH1 (*Hs*)	O75874	S-NO	C73	Lee et al. (2014)
	NUDT12 (*Hs*)	Q9BQG2	H_2O_2-sensitive	C117	Fu et al. (2017)

Abbreviations: *AA* Amino acid; *ACAA* Acetyl-CoA acyltransferase; *ACOT* Acyl-CoA thioesterase; *ABCD* ATP-binding cassette subfamily D member; *ACBD* Acyl-CoA binding domain-containing; *ACOX* Acyl-CoA oxidase; *ACSL* Acyl-CoA synthetase long-chain family member; *AGPS* Alkylglycerone-phosphate synthase; *AMACR* Alpha-methylacyl-CoA racemase; *ATAD* ATPase family AAA domain-containing protein; *ATM* Ataxia telangiectasia mutated; *CAT* Catalase; *DECR* 2,4-dienoyl-CoA reductase; *DNM1L* Dynamin 1 like protein; *ECH* Enoyl-CoA hydratase; *EHHADH* Enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase; *EPHX* Epoxide hydrolase; *ER* Endoplasmic reticulum; *FA* Fatty acid; *GNPAT* Glycerone-phosphate O-acyltransferase; *FIS* Mitochondrial fission protein; *GSTK* Glutathione S-transferase kappa; *HAO* Hydroxyacid oxidase; *Hs homo sapiens*; *IDE* Insulin-degrading enzyme; *inter* Intermolecular; *intra* Intramolecular; *HSD17B* Hydroxylsteroid 17-beta dehydrogenase; *IDH* Isocitrate dehydrogenase; *LONP* Lon peptidase; *Mm Mus musculus*; *Mff* Mitochondrial fission factor; *ND* Not determined; *NOS* Nitric oxide synthase; *NUDT* Nucleoside diphosphate-linked moiety X-type motif; *PECR* Peroxisomal trans-2-enoyl-CoA reductase; *PEX* Peroxin; *PIPOX* Pipecolic acid oxidase; *PO* Peroxisome; *PRDX* Peroxiredoxin; *Rn Rattus norvegicus*; *S-NO* S-nitrosylation; *SOD* Superoxide dismutase; *S-OH* Sulfenylation; *S-O_3H* Sulfonic acid; *S–S* Disulfide bond; *S-SG* S-glutathionylation; *TXN* Thioredoxin; *VAPB* Vesicle-associated membrane protein-associated protein; *Y-NO_2* Tyrosine nitration

modified cysteines—the exact position, modification type, and flanking sequences are provided (Sun et al. 2012); and dbGSH (Chen et al. 2014a, b) and dbSNO (Chen et al. 2015) are informative resources for experimentally verified Cys-S-SG and Cys-S-NO peptides, respectively, along with their structural or functional characteristics.

4 Peroxisomes and Inter-compartmental Redox Communication

Redox communication involves at least three critical components: a redox-signal generator, a redox signal, and a redox signal sensor (Jones 2006). Given that (i) peroxisomes contain different sets of enzymes involved in the metabolism of

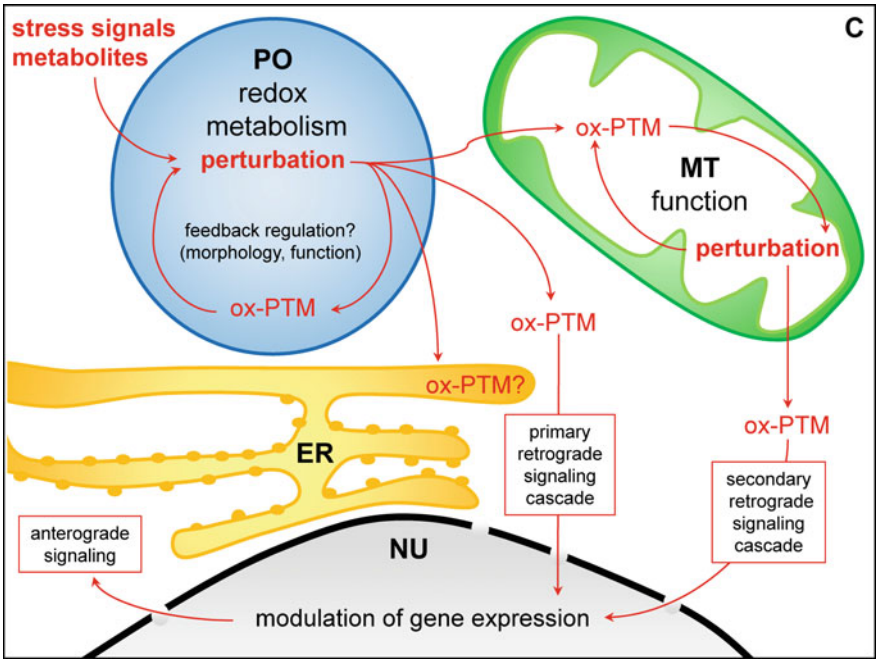

Fig. 1 Peroxisomes as intracellular redox signaling nodesChanges in the intracellular environment affecting peroxisomal redox metabolism are likely to modulate basal ox-PTM levels within and outside the peroxisomal compartment. These PTMs may serve distinct functions, ranging from feedback regulation to control of gene expression. Given that disturbances in peroxisomal redox balance can cause impaired mitochondrial fitness, peroxisomal retrograde signaling may also occur through mitochondria-mediated events. See text for more details. C, cytosol; ER, endoplasmic reticulum; MT, mitochondrion; NU, nucleus; ox-PTM, oxidative posttranslational modification; PO, peroxisome

H_2O_2 and $^{\cdot}NO$ (see Sects. 2.1 and 2.2), (ii) H_2O_2 and $^{\cdot}NO$ are important physiological second messengers in a wide range of physiological and pathological processes (Sies 2015, 2017), and (iii) many proteins involved in peroxisome functioning are apparently able to sense ROS/RNS (see Table 3), peroxisomes are likely to function as intracellular redox communication platforms (Fig. 1). In the following sections, we first focus on the redox communication between peroxisomes and their environment. Next, we briefly highlight the importance of such communication in overall cell physiology and health.

4.1 Peroxisomes as Mediators and Modulators of Redox Signaling

Alterations in peroxisomal redox metabolism have been reported to modulate diverse molecular and cellular processes, ranging from transcription factor activation (Itoh et al. 2010) and mTORC1 activity (Zhang et al. 2013) to neuronal activity (Diano et al. 2011) and cell fate decisions (Carter et al. 2004; Elsner et al. 2011; Wang et al. 2013). These findings, among others, clearly demonstrate that peroxisomes have the capability to exchange redox-active molecules with their environment. Currently, there is reasonable evidence to support the ideas that (i) small molecules such as H_2O_2, GSH, and ascorbic acid can freely permeate across the peroxisomal membrane through PXMP2, a non-selective pore-forming protein with an upper molecular size limit of 300–600 Da (Rokka et al. 2009), and (ii) the redox equivalents of NADH, generated by peroxisomal β-oxidation, are transferred across the peroxisomal membrane via lactate/pyruvate- and malate/aspartate-based redox shuttles (Baumgart et al. 1996; Hofhuis et al. 2016). However, the exact mechanisms of how these substances are precisely transported across the peroxisomal membrane remains to be elucidated. A related problem is how peroxisomes regulate their GSH/GSSG and NAD^+/NADH pools to maintain redox homeostasis and keep peroxisomal β-oxidation going.

Previous studies have shown that changes in peroxisomal H_2O_2 metabolism can modulate the activities and/or expression levels of various proteins displaying a cytoplasmic/nuclear distribution pattern. Specific examples include (i) the transcription factors CREB1, FOXO, NFKB, NFE2L2, PPARG, and PPARGC1A, (ii) the antioxidant enzymes GPX4, PRDX1, and PRDX5, and (iii) the kinases ATM and AKT1 (Fransen and Lismont 2018; and references therein). These observations clearly indicate that alterations in peroxisomal H_2O_2 metabolism have the potential to activate retrograde signaling pathways that may adaptively modify gene expression according to the metabolic needs.

Peroxisomes also have the ability to communicate with mitochondria through redox-driven processes. For example, it has been shown that (i) peroxisome-derived

oxidative imbalances rapidly perturb mitochondrial redox state (e.g., GSG/GSSG balance) and function (e.g., membrane potential, aconitase activity) (Ivashchenko et al. 2011; Walton and Pizzitelli 2012), (ii) the production of excessive amounts of $O_2^{\cdot-}$ or H_2O_2 inside peroxisomes shifts the intramitochondrial redox balance to a more oxidative state (Wang et al. 2013; Lismont et al. 2018), ultimately resulting in the activation of mitochondrial cell death pathways (Wang et al. 2013), and (iii) peroxisomally-located PRDX5 protects cells from mitochondria-derived superoxide (Walbrecq et al. 2015). Although these findings clearly demonstrate that peroxisomal and mitochondrial redox metabolism are closely intertwined, the molecular mechanism underlying this redox interplay remain to be fully established. Importantly, depending on the type and amount of oxidants involved, different mechanisms may be active.

Finally, despite the fact that it is well-known that functional peroxisomes are necessary to prevent chronic ER stress (Faust and Kovacs 2014), there are currently —to the best of our knowledge—no studies that specifically address how alterations in peroxisomal redox metabolism influence ER redox state and/or function.

4.2 Peroxisomes as Targets of Redox Regulation

A growing body of evidence indicates that alterations in the intracellular redox state may also influence peroxisome morphology, number, and activity. For example, it has been reported that (i) treatment of cells with UV light or micromolar levels of H_2O_2 promote peroxisome tubulation (Schrader et al. 1999), (ii) conditions of mild chronic oxidative stress elevate peroxisome number (Legakis et al. 2002; Walton and Pizzitelli 2012), and (iii) conditions of acute oxidative stress promote peroxisome degradation (Zhang et al. 2015). With respect to the changes in peroxisome morphology, it is interesting to note that human PEX11α and PEX11β, two proteins that have been shown to exhibit membrane curvature activity (Opaliński et al. 2011), also appear to contain redox-sensitive cysteines (Table 3). In addition, in plants, PEX11α has been reported to mediate the formation of dynamic peroxisomal membrane extensions in response to ROS (Rodríguez-Serrano et al. 2016). These extensions, termed “peroxules” (Sinclair et al. 2009), are thought to play a regulatory role in ROS accumulation and ROS-mediated signaling networks (Rodríguez-Serrano et al. 2016). Until now, peroxule formation has not yet been described in mammals. However, it is tempting to speculate that a similar mechanism also exists in mammalian cells to regulate redox communication between peroxisomes and other cell organelles.

With respect to the changes in peroxisome number and activity, increased oxidant levels may—depending on the circumstances—stimulate peroxisome biogenesis or interfere with the degradation of these organelles. Whether these changes in organelle abundance are regulated at the transcriptional (e.g., through modulation

of PPAR activity), post-transcriptional (e.g., through non-coding RNAs), or post-translational (e.g., through ox-PTMs) level (Farr et al. 2016), remains to be investigated. However, in this context, it is important to note that also human PEX5 has been identified as a redox-sensitive protein (Apanasets et al. 2014). PEX5 functions as the shuttling import receptor for peroxisomal matrix proteins containing a C-terminal peroxisomal targeting signal (PTS1), and—to free the receptor from the peroxisomal membrane for another round of import—the protein requires monoubiquitination at Cys-11 (Francisco et al. 2017). This process appears to be less efficient under conditions of oxidative stress (Apanasets et al. 2014; Lismont et al. 2018), thereby resulting in the accumulation of PEX5 at the peroxisomal membrane (Legakis et al. 2002) and a decrease in the import efficiency of PTS proteins, and in particular of those containing a weak targeting signal such as CAT (Legakis et al. 2002; Walton et al. 2017). Given that cytosolic CAT has recently been shown to protect cells against exogenous H_2O_2-induced redox changes in a manner that peroxisomally targeted CAT does not, this may constitute a cellular defense mechanism to cope with cytosolic redox imbalance (Hosoi et al. 2017; Walton et al. 2017) (see also Sect. 5). Finally, given that (i) monomeric ubiquitin is sufficient to target peroxisomes for degradation by autophagy (Kim et al. 2008), (ii) export-deficient Cys-11-monoubiquitinated PEX5 can function as a trigger for peroxisome removal, at least under certain conditions (Nordgren et al. 2015), and (iii) monoubiquitination of PEX5 at Cys-11 is impaired under conditions of mild oxidative stress (Apanasets et al. 2014; Lismont et al. 2018), it may not surprise that a chronic increase in cellular redox balance (e.g., during cellular aging) may lead to an increase in peroxisome number (Legakis et al. 2002). On the other hand, the disappearance of peroxisomes under conditions of acute oxidative stress may be explained by the fact that such conditions trigger ATM-mediated phosphorylation of PEX5 at Ser-141, an event that subsequently promotes PEX5 monoubiquitylation at Lys-209 (Zhang et al. 2015). Together, these findings provide strong evidence that peroxisomes have the capacity to actively sense and respond to changes in their redox environment.

4.3 Peroxisomal Redox Dysfunction in Cell Physiology and Disease

Over the past two decades, it has become apparent that physiological oxidant production is a key factor in the regulation of many fundamental biological processes, such as stem cell self-renewal, cell proliferation and differentiation, wound healing, healthy immune responses, and longevity (Sies 2017). As such, dysregulation of oxidant levels (e.g., caused by enhanced ROS/RNS production or reduced antioxidant capacity) has been recognized as a potential contributor to the

pathophysiology of many diseases and aging (Go and Jones 2017). Given that fluctuations in peroxisomal H_2O_2 metabolism can (i) modulate normal metabolic processes (e.g., energy metabolism) (Diano et al. 2011; Ruiz-Ojeda et al. 2016), (ii) activate protective mechanisms (e.g., upon renal ischemia-reperfusion injury) (Vasko 2016), or (iii) sensitize cells to cell death (e.g., upon exposure of pancreatic β-cells to long-chain saturated non-esterified fatty acids) (Elsner et al. 2011), it is not surprising that derangements in peroxisomal oxidant/antioxidant balance have also been linked to various oxidative stress-related disorders, such as inflammation, cancer, diabetes, neurodegeneration, noise-induced hearing loss, kwashiorkor, and aging. However, as this review is not intended to be exhaustive of the literature available on this topic, we refer the reader for more details to other recent reviews (Cipolla and Lodhi 2017; Pascual-Ahuir et al. 2017; Fransen and Lismont 2018) and references therein.

5 Conclusions, Challenges, and Perspectives

In a manner analogous to phosphorylation and ubiquitination, reversible oxidative protein modifications allow cells to respond rapidly to a multitude of physiological and environmental stimuli. As detailed above, accumulating evidence strongly indicates that also peroxisomes have the intrinsic ability to mediate and modulate redox-driven signaling events. However, the molecular mechanisms and physiological relevance of these events are only poorly understood and many open questions remain. For example, how do alterations in peroxisomal redox metabolism influence inter-organelle communication? How do alterations in cellular redox state affect peroxisome biology? What is the role of CAT in H_2O_2-mediated signaling? What are the molecular targets of peroxisome-derived H_2O_2? Do peroxisome-derived H_2O_2 and H_2O_2 produced in other cellular compartments have common and/or specific targets? And to what extent do alterations in peroxisomal H_2O_2 metabolism contribute to cellular and organismal physiology? All of these issues are further detailed in the following paragraphs.

Currently, there is ample evidence that peroxisomes can communicate with other cell organelles through redox signaling (see Sect. 4.1). This is perhaps best illustrated by the observations that peroxisomes and mitochondria share a redox-sensitive relationship, and that disturbances in peroxisomal redox metabolism impair mitochondrial fitness (Fransen and Lismont 2018; and references therein). Given that changes in peroxisomal H_2O_2 metabolism can modulate the activities and/or expression levels of various proteins displaying a cytoplasmic/nuclear distribution pattern (see Sect. 4.1), peroxisomes are also likely to communicate their changing metabolic and functional state to the nucleus. However, the molecular mechanisms controlling the peroxisome-to-mitochondria and peroxisome-to-nucleus communication pathways remain ill-defined. Note that

alterations in peroxisomal H_2O_2 metabolism are also likely to modulate the cytosolic NAD^+/NADH ratio (Farr et al. 2016), a factor influencing the (histone) deacetylation activity of sirtuins, a class of proteins involved in epigenetic signaling. In addition, it is well known that sirtuins can also be directly inactivated through oxidative modification by H_2O_2 (Caito et al. 2010). Among the transcription factors best-known for their redox-dependent activity regulation are NFE2L2 (Itoh et al. 2010), FOXO (Klotz et al. 2015), HIF-1α (Zepeda et al. 2013), and NFKB (Morgan and Liu 2011). In agreement with previous studies reporting increased NFKB activity upon peroxisomal H_2O_2 production (Li et al. 2000; Han et al. 2014), we recently found that also the NFKB1 and RELA subunits of NFKB are targets of peroxisome-derived H_2O_2 (Lismont et al. 2018). However, how peroxisome-derived H_2O_2 affects the activity of these proteins, remains to be determined. That is, redox modulation of transcription factor activity can be accomplished through modification of the transcription factor itself or any other member of the pathway, including regulatory proteins, kinases and phosphatases. In addition, depending on which cysteine is affected and the type of modification, a single oxidation event may result in inactivation or activation of the protein. This is beautifully illustrated by the observation that glutathionylation of Cys179 in the β subunit of the inhibitor of nuclear factor kappa B kinase (IKBK) inactivates this complex, while disulfide formation between the γ subunits of IKBK increases its activity (Morgan and Liu 2011).

As PEX5 plays a central role in peroxisome biogenesis and degradation (see Sect. 4.2), it is also necessary to consider the consequences of its oxidative modification by (peroxisome-derived) H_2O_2. Importantly, a compromised matrix protein import process mainly affects proteins containing a weak PTS1, such as CAT (Terlecky et al. 2006; Walton et al. 2017). However, the (patho)physiological consequences of a (partial) cytosolic mislocalization of CAT are still unclear. On one hand, it has been described that such mislocalization may result in a "self-sustaining peroxisome deterioration spiral", a phenomenon in which the mislocalization of CAT leads to a decrease in peroxisomal antioxidant capacity and hence increased cellular H_2O_2 levels (Terlecky et al. 2006). On the other hand, retaining CAT in the cytosol may also constitute a cellular defense mechanism to cope with cytosolic redox imbalance (Hosoi et al. 2017; Walton et al. 2017). Finally, given that (export-deficient) monoubiquitinated PEX5 may also function as a trigger for peroxisome removal (Zhang et al. 2015; Nordgren et al. 2015), alterations in cellular H_2O_2 levels may also induce changes in peroxisome number (see Sect. 4.2).

Currently, it is a subject of debate how peroxisome-derived H_2O_2 participates in cellular redox signaling. At the heart of the problem lies the fact that the H_2O_2 reactivity and abundance of signaling proteins is typically much lower than that of peroxidases (Stöcker et al. 2017). One hypothesis is the so-called "floodgate hypothesis", which proposes that peroxidases such as PRDXs mainly function as anti-oxidants and therefore have first to be inactivated before H_2O_2 can directly

modify redox-sensitive cysteines in signaling proteins (Wood et al. 2003). Another hypothesis is that H_2O_2 is first captured by peroxidases and that the resulting oxidative modifications are subsequently transferred to target proteins, making the peroxidase itself a mediator of the signaling event (Stöcker et al. 2017). In case the second mechanism is favored, the peroxisomal pools of CAT and PRDX5 may play non-overlapping roles in H_2O_2 scavenging. Indeed, given that (i) PRDXs can transfer oxidative equivalents from H_2O_2 to other proteins that are intrinsically less susceptible to oxidation (Stöcker et al. 2017), and (ii) CAT and PRDX5 display distinct kinetic parameters for H_2O_2 (see Sect. 2.2), PRDX5 may scavenge low levels of H_2O_2, thereby functioning as a H_2O_2 sensor and signal transducer, and CAT may prevent the accumulation of H_2O_2 to toxic levels.

As the precise molecular mechanisms underlying peroxisome-derived H_2O_2 signaling are still poorly understood, a key focus for future research should be the unbiased identification of its physiological target proteins by redox proteomics [e.g., by employing cell models in which H_2O_2 production can be tightly controlled in a spatiotemporal manner (Lismont et al. 2018)]. A major challenge will be to optimize the experimental conditions in such a manner that they mimic the physiological conditions as close as possible. In this context, it is reasonable to assume that H_2O_2 production in a well-defined area and within the physiological range does not result in a general oxidation of all cellular redox-sensitive cysteines, but instead causes specific thiol modifications located along the H_2O_2 gradient. For example, it can be expected that peroxisomal proteins are more readily oxidized by peroxisome-derived than mitochondria-derived H_2O_2. Therefore, an important criterion to determine the appropriate treatment conditions will be the number of targets shared in common and the extent of their oxidation upon H_2O_2 production in different locations. Finally, given that (i) reversible oxidative modifications are labile, transient, and dynamic in nature, and (ii) many proteins of biological interest are present in relatively low abundance, it is important to point out that such redox proteome-based profiling strategies pose complex technical challenges that can only be overcome through creative and bespoke solutions.

In summary, it is clear that peroxisomes harbor sophisticated redox control mechanisms that integrate environmental signals and affect various physiological and metabolic functions. However, many questions remain, and additional functional and proteomics studies are needed to expand our knowledge on how these organelles are precisely embedded in cellular redox signaling networks that drive different diseases such as cancer, diabetes, and neurodegeneration.

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Cell Death or Survival Against Oxidative Stress

Non Miyata, Kanji Okumoto and Yukio Fujiki

Abstract Peroxisomes contain anabolic and catabolic enzymes including oxidases that produce hydrogen peroxide as a by-product. Peroxisomes also contain catalase to metabolize hydrogen peroxide. It has been recognized that catalase is localized to cytosol in addition to peroxisomes. A recent study has revealed that loss of VDAC2 shifts localization of BAK, a pro-apoptotic member of Bcl-2 family, from mitochondria to peroxisomes and cytosol, thereby leading to release of peroxisomal matrix proteins including catalase to the cytosol. A subset of BAK is localized to peroxisomes even in wild-type cells, regulating peroxisomal membrane permeability and catalase localization. The cytosolic catalase potentially acts as an antioxidant to eliminate extra-peroxisomal hydrogen peroxide.

Keywords BAK · Catalase · CHO mutant · Membrane permeabilization Oligomerization · Oxidative stress · Peroxisome · Reactive oxygen species VDAC2, apoptosis

N. Miyata · K. Okumoto
Department of Biology, Faculty of Sciences, Kyushu University, 819-0395 Nishi-ku, Fukuoka, Japan

K. Okumoto
Graduate School of Systems Life Sciences, Kyushu University, 819-0395 Nishi-ku, Fukuoka, Japan

Y. Fujiki (✉)
Medical Institute of Bioregulation, Kyushu University, 812-8582 Higashi-ku, Fukuoka, Japan
e-mail: yfujiki@kyudai.jp

Present Address:
N. Miyata
Department of Chemistry, Faculty of Sciences, Kyushu University, 819-0395 Nishi-ku, Fukuoka, Japan

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1 Introduction

Peroxisomes are single membrane-bounded organelles that participate in many metabolic pathways, including β-oxidation of fatty acids (Wanders and Waterham 2006). Many metabolic pathways of peroxisomes lead to the production of hydrogen peroxide, which is subsequently decomposed by catalase (Titorenko and Terlecky 2011). Mitochondria are double membrane-bounded organelles functioning in many metabolic pathways as well as energy production. Under irreparable cellular stress such as oxidative stress, the cells are committed to apoptosis through compromising the integrity of the mitochondrial outer membrane by inducing mitochondrial outer membrane permeabilization (MOMP) (Newmeyer and Ferguson-Miller 2003; Tait and Green 2010; Wang 2001). MOMP leads to the release of intermembrane-space proteins such as cytochrome *c*, followed by activation of caspases responsible for the progression of apoptosis (Newmeyer and Ferguson-Miller 2003; Tait and Green 2010; Wang 2001). The BCL-2 family proteins regulate apoptosis by controlling MOMP (Czabotar et al. 2014). Pro-apoptotic BCL-2 effector proteins, BAK and BAX, undergo homo-oligomerization to induce MOMP (Wei et al. 2001). Anti-apoptotic BCL-2 proteins including BCL-2, BCL-X_L, and MCL-1 promote survival by sequestering the pro-apoptotic BH3-only molecules, BID, BIM, and PUMA, thus preventing the initiation of BAX and BAK activation (Cheng et al. 2001). The anti-apoptotic BCL-2 members can also sequester "BH3-exposed" BAX and BAK monomers to prevent the homo-oligomerization of BAX and BAK (Chen et al. 2015). Alternatively, it has been reported that BH-3-only proteins activate BAK and BAX by neutralizing anti-apoptotic BCL-X_L and MCL-1 (O'Neill et al. 2016). BH3-only proteins are activated by various cellular stress signals and promote MOMP (Czabotar et al. 2014; Kim et al. 2006). Mitochondrial outer membrane channel, voltage-dependent anion channel 2 (VDAC2), interacts with BAK and keeps BAK as an inactive monomer at mitochondria (Cheng et al. 2003). VDAC2 also stabilizes the mitochondrial localization of BAK (Setoguchi et al. 2006).

We very recently found that VDAC2 deficiency leads to destabilization mitochondrial localization of BAK, and relocalization of a part of BAK to peroxisomes, giving rise to permeabilization of peroxisomal membrane (Hosoi et al. 2017). A subset of BAK is also localized to peroxisomes in wild-type cells. Knockdown of BAK in wild-type cells increases latency of catalase. Taken together, BAK regulates peroxisomal permeability and catalase localization.

2 Interaction Between VDAC2 and BAK on the Mitochondria Outer Membrane

VDAC2 is a β-barrel channel protein in the mitochondrial outer membrane. BAK is a C-tail anchored (TA) protein that is inserted into membranes with a transmembrane domain located at its C-terminal region and exposes most part of the protein

to cytosol. The interaction between VDAC2 and BAK was initially reported by Cheng et al. (2003). VDAC2 directly binds to BAK and keeps BAK inactive monomer. BAK is more readily activated upon apoptotic stimuli in VDAC2-deficient cells. Thus, VDAC2 protect cells from apoptotic cell death in cooperation with anti-apoptotic Bcl-2 family proteins, BCL-X_L and MCL-1.

In addition, BAK localization to mitochondria is stabilized by VDAC2 but not VDAC1 (Setoguchi et al. 2006), whilst knockdown of VDAC2 strongly reduces BAK localization to mitochondria. In contrast, the C-terminal region of BAK that is sufficient for targeting to mitochondria is targeted to mitochondria independently of VDAC2. This suggests the mitochondrial-targeting sequence of BAK is inactivated or masked within the molecule, and is activated or unmasked by interaction of its cytoplasmic domain with VDAC2. Thereby, VDAC2 regulates the localization as well as activity of BAK via its interaction with BAK.

3 BAK Is Localized to Peroxisomes and Regulates Peroxisomal Membrane Permeability

Tateishi et al. (1997) earlier isolated a peroxisome-deficient Chinese hamster ovary (CHO) cell mutant, ZP114. ZP114 cells show the impaired import of matrix proteins, not membrane proteins. By functional complementation screening using a human kidney cDNA library, VDAC2 was identified as a complementing gene of ZP114 cells (Hosoi et al. 2017). ZP114 cells express neither mRNA nor protein of VDAC2, indicating that ZP114 is a VDAC2-null mutant. Another CHO mutant ZP126 (Ghaedi et al. 1999) is recently classified to the same complementation group as *vdac2* ZP114 (Fujiki et al., unpublished observation).

Notably, BAK inactivation by overexpression of BAK-inhibitors, BCL-X_L and MCL-1 and BAK knockdown restores the impaired localization of matrix proteins to peroxisomes in ZP114 cells, hence suggesting that BAK is directly involved in peroxisomal abnormality in VDAC2-deficient ZP114 cells (Hosoi et al. 2017). Mitochondrial localization of BAK is decreased in ZP114 cells, where BAK is localized to the cytosol and peroxisomes. Introduction of peroxisome-targeted BAK harboring C-terminal transmembrane region of Pex26 into wild-type cells results in the release of peroxisomal matrix proteins such as catalase to the cytosol. A mutation to the BH3 domain of BAK that is responsible for homo-oligomerization and pro-apoptotic activity of BAK abolishes the release of peroxisome matrix proteins to the cytosol. Taken together, BAK forms the pore on peroxisomal membrane and releases peroxisomal matrix proteins in VDAC2-deficient cells, similar to MOMP (Fig. 1).

Furthermore, fractionation studies revealed that BAK is indeed detected in highly purified peroxisome fraction from the wild-type HeLa cells. BAK knockdown in the wild-type CHO-K1 and HeLa cells significantly reduces cytosolic catalase (Hosoi et al. 2017). Overexpression of pro-apoptotic BH3-only proteins,

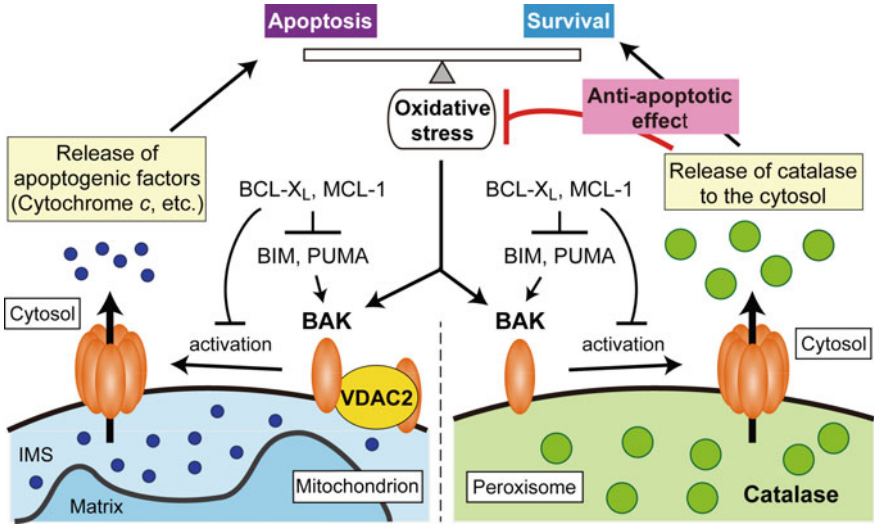

Fig. 1 BAK-dependent regulation of catalase release from peroxisomes. BAK is partly localized in peroxisomes in normal cells and regulates the permeability of peroxisomal membrane, leading to release of catalase into the cytosol. Pro-apoptotic BH3-only proteins including PUMA and BIM and anti-apoptotic BCL-2 family proteins such as BCL-XL and MCL-1 positively and negatively control the BAK-mediated catalase release, respectively. This finding strongly suggests a shared mechanism of BAK-dependent membrane permeabilization between mitochondria and peroxisomes. The catalase released from peroxisomes via the BAK pore eliminates H_2O_2, a toxic and major causative of the oxidative stress, in the cytosol. The Figure image is reproduced with permission from Fujiki et al. (2017)

BAK activators PUMA and BIM, permeabilizes peroxisomes and releases catalase to the cytosol in a BAK-dependent manner (Hosoi et al. 2017). Taken together, these observations strongly suggest that BAK is localized to peroxisomes in addition to mitochondria, regulates peroxisomal membrane permeability, and releases a part of catalase to the cytosol in normal cells (Fujiki et al. 2017, Fig. 1).

4 Dual Localization of C-Tail Anchored (TA) Proteins to Mitochondria and Peroxisomes

TA proteins are found in virtually all cellular and intracellular membranes and play essential roles in various biological processes such as protein translocation, membrane fusion, and viral response (Kutay et al. 1993). To ensure correct targeting and localization of TA proteins, eukaryotes have developed the mechanisms that recognize and target TA proteins to their destinations (Borgese et al. 2007). TA proteins destined for the ER are recognized by GET (Guided Entry of Tail-anchored proteins) pathway (Schuldiner et al. 2008; Hegde and Keenan 2011). TA proteins

such as Pex26 (Yagita et al. 2013) and acyl-CoA binding domain-containing 5 (ACBD5) (Yagita et al. 2017) destined to peroxisomes are recognized by Pex3-Pex19 pathway. In contrast, it was reported that TA proteins destined for the mitochondrial outer membrane was inserted to the mitochondrial outer membrane without any cytosolic factors in vitro (Setoguchi et al. 2006), suggesting that cytosolic chaperones are not essential for mitochondrial targeting of TA proteins, although it was also reported that Ubiquilin family proteins bind to newly synthesized mitochondrial TA proteins to protect them against aggregation in the cytosol (Itakura et al. 2016). This feature probably makes targeting of the mitochondrial TA proteins "fuzzy or enigmatic". The mitochondrial TA proteins are often localized to peroxisomes in addition to mitochondria (Kobayashi et al. 2007; Itoyama et al. 2013; Dixit et al. 2010; Delille and Schrader 2008; Costello et al. 2017). Fis1, a TA protein localized to and involved in fission of mitochondria and peroxisomes (Kobayashi et al. 2007), is targeted to peroxisomes in a Pex19-dependent manner (Delille and Schrader 2008). The other TA proteins including Mff (Itoyama et al. 2013) showing dual localization to mitochondria and peroxisomes are likewise transported to peroxisomes by the Pex3-Pex19 pathway. Within this context, BAK is also translocated to peroxisomes by the Pex3-Pex19 pathway (Fujiki et al., unpublished observation). It may be stochastic whether the dual-localized TA proteins are localized to peroxisomes by the Pex3-Pex19 pathway or mitochondria by the unassisted pathway.

5 Redox-Dependent Regulation of Peroxisome Targeting Signal 1 (PTS1) Receptor, Pex5

PTS1 receptor Pex5 shuttles between the cytosol and peroxisomes to import peroxisomal matrix proteins harboring PTS1 (Platta et al. 2005; Miyata and Fujiki 2005; Dammai and Subramani 2001). A highly conserved cysteine residue in the N-terminal region of Pex5, cysteine 11 at the alignment position in mammals, is a site of mono-ubiquitination, which is essential for Pex5 export to the cytosol (Platta et al. 2007; Okumoto et al. 2011; Carvalho et al. 2007). Protein ubiquitination usually takes place at lysine residues via isopeptide bond. In contrast, ubiquitin is conjugated to the cysteine 11 of Pex5 by thioester bond, which is highly redox-sensitive (Grou et al. 2012). It is noteworthy that, in cells which are defective in peroxisome assembly, the redox state of the cytosol is more reductive than that of the wild-type cells (Yano et al. 2010). In such cell mutants, the cysteine 11 of Pex5 is less sensitive to the redox state and becomes more stable. It is also reported that the cysteine 11 of Pex5 is highly susceptible to oxidants such as oxidized glutathione and that the ubiquitination at the cysteine 11 of Pex5 is readily inhibited by oxidative stress (Apanasets et al. 2014). Interestingly, the cysteine 11 of Pex5 was very recently suggested to act as a redox sensor and reduce the import of PTS1 proteins, especially catalase to retain catalase in the cytosol under oxidative stress

(Walton et al. 2017). Thus, it is more likely that catalase release from peroxisomes by BAK and import inhibition due to defective ubiquitination of cysteine 11 of Pex5 under oxidative stress (Apanasets et al. 2014) cooperatively contribute to retaining of catalase in the cytosol.

6 Function of Cytosolic Catalase in Elimination of Extra-Peroxisomal Hydrogen Peroxide

Catalase activity is detected in cytosolic fraction in wild-type cells such as liver cells (Eriksson et al. 1992, 1991). Catalase harbors noncanonical PTS1, KANL at its C-terminus. The PTS of catalase weakly binds to Pex5, resulting in lower import efficiency than matrix proteins with the canonical PTS1 such as SKL (Otera and Fujiki 2012). The C-terminal KANL of catalase is highly conserved between mammalian species, implying that the weak targeting signal plays a physiologically consequent role. Yeast, *Saccharomyces cerevisiae*, and a worm, *Caenorhabditis elegans*, possess cytosolic catalase, which is thought to play an important role in elimination of reactive oxygen species (ROS) in cells, together with glutathione peroxidase (Hartman et al. 2003). Mammalian cells lack cytosolic catalase and contain only peroxisomal catalase. Given the findings described above, we propose that mammalian catalase indeed has a physiological function in the cytosol (Fujiki et al. 2017). The inefficient import signal of catalase in concert with peroxisomal BAK and oxidation of cysteine 11 of Pex5 elevates cytosolic catalase that eliminates cytosolic hydrogen peroxide. Consistent with such a functional model, *pex* mutant cells including CHO *pex* mutants and those from patients with Zellweger spectrum disorders that are defective in peroxisomal matrix protein import are more resistant to exogenous hydrogen peroxide than normal cells (Hosoi et al. 2017). Such differential sensitivity to hydrogen peroxide between normal cells and *pex* mutants is completely abolished with a catalase inhibitor, 3-aminotriazole (Hosoi et al. 2017). Similar result is reported by Walton et al. (2017). These findings indicate that cytosolic catalase more efficiently and readily eliminates oxidative stress than peroxisomal catalase (Fujiki et al. 2017).

7 Concluding Remark

Studies using VDAC2-deficient CHO ZP114 cells revealed that VDAC2-BAK axis regulates peroxisomal membrane permeability and catalase localization. BAK promotes MOMP and cell death in mitochondria. To the contrary, BAK plays a pro-survival function in peroxisomes via generating cytosolic catalase, giving rise to efficient elimination of external hydrogen peroxide. Collectively, BAK functions as a checkpoint coordinating cell survival and cell death. Under normal condition or

weak oxidative stress, BAK acts towards pro-survival by releasing a part of catalase to the cytosol. In contrast, under irreparable oxidative stress, BAK induces MOMP to promote apoptotic cell death.

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A Role for RNS in the Communication of Plant Peroxisomes with Other Cell Organelles?

Francisco J. Corpas, Luis A. del Río and José M. Palma

Abstract Plant peroxisomes are organelles with a very active participation in the cellular regulation of the metabolism of reactive oxygen species (ROS). However, during the last two decades peroxisomes have been shown to be also a relevant source of nitric oxide (NO) and other related molecules designated as reactive nitrogen species (RNS). ROS and RNS have been mainly associated to nitro-oxidative processes; however, some members of these two families of molecules such as H_2O_2, NO or *S*-nitrosoglutathione (GSNO) are also involved in the mechanism of signaling processes mainly through post-translational modifications. Peroxisomes interact metabolically with other cell compartments such as chloroplasts, mitochondria or oil bodies in different pathways including photorespiration, glyoxylate cycle or β-oxidation, but peroxisomes are also involved in the biosynthesis of phytohormones including auxins and jasmonic acid (JA). This review will provide a comprehensive overview of peroxisomal RNS metabolism with special emphasis in the identified protein targets of RNS inside and outside these organelles. Moreover, the potential interconnectivity between peroxisomes and other plant organelles, such as mitochondria or chloroplasts, which could have a regulatory function will be explored, with special emphasis on photorespiration.

Keywords Nitric oxide · Nitration · Photorespiration · *S*-nitrosoglutathione *S*-nitrosylation · Reactive nitrogen species · Signaling

Abbreviations

CaM	Calmodulin
GSNO	*S*-nitrosoglutathione
GSH	Reduced glutathione
mARC	mitochondrial Amidoxime Reducing Component

F. J. Corpas (✉) · L. A. del Río · J. M. Palma
Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain
e-mail: javier.corpas@eez.csic.es

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NR	Nitrate reductase
NO	Nitric oxide
NO_2-FA	Nitro-fatty acid
NOFNiR	Nitric Oxide Forming Nitrite Reductase
$ONOO^-$	Peroxynitrite
PEX	Peroxin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNOs	*S*-nitrosothiols

1 Introduction

Plant peroxisomes are organelles present in the cells of all plant tissues. However, its enzymatic composition can fluctuate depending of the cell-type and tissue, development stage or in response to environmental conditions (Corpas and Trelease 1998; Hayashi et al. 2000; Mano and Nishimura 2005; Graham 2008; Hu et al. 2012; Kubo 2013; Sørhagen et al. 2013; Reumann and Bartel 2016). Consequently, one of the main characteristics of plant peroxisomes is its metabolic plasticity. More importantly, peroxisomes have an active metabolic interaction with other plant cell organelles to efficiently exert their physiological functions (Poirier et al. 2006; Goto-Yamada et al. 2015). Although metabolic compartmentalisation of the different cellular pathways is a mechanism of regulation, there are additional signals that participate in the crosstalk among subcellular compartments (Lodhi and Semenkovich 2014; Mast et al. 2015; Lismont et al. 2017). Thus, the integration of different signals requires identifying how the signals are emitted and received in a specific cellular place. In plant cells major advances have been made in some signals including phytohormones, calcium or H_2O_2 (Wrzaczek et al. 2013; Kmiecik et al. 2016; Noctor and Foyer 2016; Mignolet-Spruyt et al. 2016; Saxena et al. 2016). Accordingly, in animal cells, an efficient crosstalk between peroxisomes and mitochondria mediated by peroxisome-derived oxidative stress has been suggested (Wang et al. 2013; Demarquoy and Le Borgne 2015; Fransen et al. 2012, 2017). In this sense, accumulating data reveal that nitric oxide (NO) is also a new molecule with signal properties that could contribute to the interconnectivity among organelles where peroxisomes could have a special role considering that this organelle has a very active nitro-oxidative metabolism (Corpas et al. 2001, 2017a; del Río 2011, 2015; del Río et al. 2006; del Río and López-Huertas 2016). This chapter will explore the potential function of plant peroxisomes and other organelles which could be integrated in a functional crosstalk with organelle communication particularly relevant under adverse conditions (Sehrawat et al. 2013; Misra et al. 2014; Palma et al. 2015; Gupta and Igamberdiev 2016). In this case, plant peroxisomes could be considered as key regulatory elements in NO-based signaling networks in plant cells.

2 Plant Peroxisomal NO Metabolism

Nitric oxide (NO) is a free radical molecule that forms part of the family of molecules designated as reactive nitrogen species (RNS). Thus, NO or other NO-related molecules can exert actions affecting numerous physiological processes, including seed and pollen germination, root development, plant growth, stomata movement, organ senescence or fruit ripening (Shapiro 2005; Šírová et al. 2011; Domingos et al. 2015; Simontacchi et al. 2015; Airaki et al. 2015; Jiménez-Quesada et al. 2017; Rodríguez-Ruiz et al. 2017a, b), as well as the mechanisms of defense against biotic and abiotic stresses (Signorelli et al. 2013; Arasimowicz-Jelonek and Floryszak-Wieczorek 2014; He et al. 2014; Asgher et al. 2017; Mata-Pérez et al. 2016b, 2017).

Besides the relevance of the oxidative metabolism of peroxisomes, during the last two decades, research has also focused on the potential presence of NO and other RNS in plant peroxisomes, trying to find out how these molecules are generated, and how they could affect the peroxisomal metabolism and, consequently, its function.

NO has been shown to be present in plant peroxisomes by different technical approaches including spin trapping electron paramagnetic resonance (EPR) spectroscopy and fluorescence specific probes (Corpas et al. 2004, 2009a). Moreover, some NO-derived molecules including *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^-$) have also been demonstrated to be present in peroxisomes either by specific fluorescent probes or antibodies (Barroso et al 2013; Corpas and Barroso 2014a). In this sense, different reports have verified the direct participation of the peroxisomal NO in different physiological processes, including pollen germination and tube growth (Prado et al. 2004, 2008), leaf senescence (Corpas et al. 2004), fruit ripening (Chaki et al. 2015), auxin-induced root organogenesis (Schlicht et al. 2013), salinity (Corpas et al. 2009a), and mechanisms of response against toxicity by heavy metals like cadmium or lead (Corpas and Barroso 2014a, 2017a).

On the other hand, it should be mentioned that although the enzymatic source(s) of NO in plant systems are still controversial, the most likely candidates are nitrate reductase (NR) (Yamasaki et al. 1999; Desikan et al. 2002) and an L-arginine-dependent NO synthase (NOS) like activity (Barroso et al. 1999; Corpas et al. 2009b; Corpas and Barroso 2017b). In the case of the NOS-like activity, in higher plants no ortholog genes similar to any classic mammalian NOSs have been found. However, biochemical data as well as phylogenetic and structural analyses start to indicate that some species of algae could contain a NOS-like protein. In the green alga *Ostreococcus tauri*, a protein sequence with a relatively high homology to the human NOS has been identified including the binding sites for the necessary cofactors FAD, FMN, BH_4, and calmodulin (CaM), as well as the substrate L-arginine and NADPH binding sites (Foresi et al. 2010, 2015). Complementary studies have extended this NOS-like sequence to other species of algae (Jeandroz et al. 2016) supporting the existence of a NOS-like protein in the plant kingdom. In the specific case of the enzymatic source of NO in plant peroxisomes, there are

biochemical data supporting the presence of an L-arginine-dependent NOS-like activity that requires the same cofactors of animal NOSs (Barroso et al. 1999; Corpas et al. 2004, 2009d). This is reinforced by additional analyses which demonstrate the presence in peroxisomes of some of the cofactors needed for the NOS activity such as the capacity to generate NADPH (for a review see Corpas and Barroso 2018b), the presence of CaM (Chigri et al. 2012) and Ca^{2+} (Costa et al. 2010; Corpas and Barroso 2018a). Additionally, through the use of calcium channel blockers and CaM antagonists, it has been demonstrated that the peroxisomal import of the protein responsible of the NOS activity is strictly dependent on both Ca^{2+} and CaM. Moreover, the import of this protein is through a peroxisomal targeting signal type 2 (PTS2), which requires peroxin 12 (PEX12), as well as both PTS receptors, PEX5 and PEX7 (Corpas and Barroso 2014b). These data are in good agreement with the presence of a NOS protein in animal peroxisomes (Stolz et al. 2002; Loughran et al. 2005) which also has a PTS2 (Loughran et al. 2013). Figure 1 outlines a working model of NO metabolism in plant peroxisomes.

On the other hand, additional analyses are also providing alternative mechanisms and candidates of enzymatic NO generation. For example, using the unicellular alga *Chlamydomonas reinhardtii* as model photosynthetic organism, it has been demonstrated that the interaction between the mitochondrial Amidoxime Reducing Component (mARC) and nitrate reductase (NR) can generate NO from nitrite (NO_2^-) since the ARC catalyzed the NO production from nitrite using electrons from NR (Chamizo-Ampudia et al. 2016). This new characterized mARC protein which contains a molybdenum cofactor has been named NO Forming Nitrite Reductase (NOFNiR) (Llamas et al. 2017); however, these new components have not been detected yet in peroxisomes.

3 *S*-Nitrosylation and Nitration Are Two Major Protein Post-translational Modifications (PTMs) that Affect Peroxisomal Metabolism

NO and other RNS can directly or indirectly mediate post-translational modifications (PTMs) of different macromolecules, including proteins, fatty acids and nucleic acids. The most studied PTMs include *S*-nitrosylation and nitration.

3.1 S-*Nitrosylation Is an Additional Redox-Based Protein Cysteine Modification*

S-nitrosylation (or nitrosation) is a redox based post-translational modification that covalently binds an NO group to the thiol (–SH) side chain of cysteine

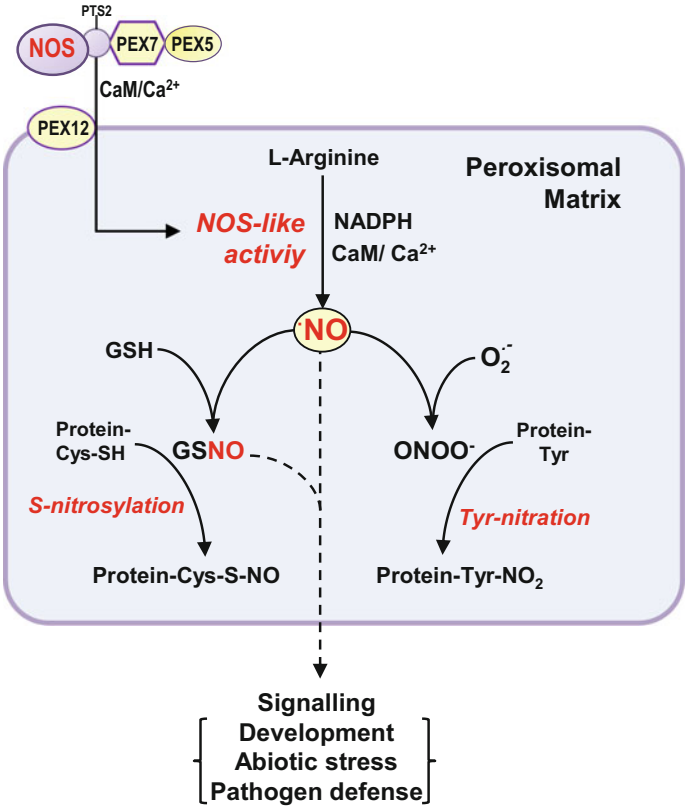

Fig. 1 Outline of the plant peroxisomal metabolism of reactive nitrogen species (RNS)The nitric oxide synthase (NOS)-like protein contains a peroxisomal targeting signal type 2 (PTS2). Import of the peroxisomal NOS protein requires calcium, calmodulin (CaM) and the involvement of several peroxins (PEXs), including PEX12, located in the membrane, and both PTS receptors (PEX5 and PEX7), located in the cytosol. Nitric oxide (NO) is generated by an L-arginine-dependent NO synthase (NOS)-like activity which requires NADPH, Ca^{2+} and CaM. NO can react with reduced glutathione (GSH), member of the ascorbate-glutathione cycle, to form *S*-nitrosoglutathione (GSNO) which can mediate *S*-nitrosylation processes. NO can also react with $O^{\cdot 2-}$ to generate the powerful nitrating molecule, peroxynitrite ($ONOO^-$), which can mediate the tyrosine nitration process. NO can function as a signal molecule outside the peroxisomes. Broken arrows indicate signalling processes. Tyr, tyrosine; Tyr-NO_2, nitro-tyrosine; Cys, cysteine

(Cys) present in peptides or proteins to produce *S*-nitrosothiols (SNOs). This process is selective, reversible and can modify protein conformation, properties and consequently its function (Stamler et al. 2001; Miersch and Mutus 2005; Wang et al. 2006; Astier et al. 2012; Astier and Lindermayr 2012; Lamotte et al. 2015). *S*-nitrosylation is quite labile under physiological conditions since its stability is influenced by the presence of trace metal ions (such as copper and iron) or reducing agents (such as thiols and ascorbate) which enhance its degradation (Askew et al.

1995; Vanin et al. 1997). Furthermore, SNOs can be transferred between small peptides and protein thiol groups through *S*-transnitrosylation mechanisms. Thus, the pool cellular of SNOs can act as a major intracellular NO reservoir and, in some cases, as a long distance NO signal. Among the different SNOs, *S*-nitrosoglutathione (GSNO) should be mentioned which is formed by the reaction between reduced glutathione (GSH) and NO (Broniowska et al. 2013; Corpas et al. 2013a) and has the capacity to modulate protein function as well as gene expression (Begara-Morales et al. 2014a, b).

The number of plant proteins reported to be susceptible of *S*-nitrosylation is increasingly growing (Lindermayr et al. 2006; Camejo et al. 2013; Sehrawat and Deswal 2014; Feng et al. 2013). Considering that a specific protein can have several Cys residues, the percentage of Cys residues that are needed to be *S*-nitrosylated to provoke a functional change or initiate a signaling event can be estimated. This question depends on the relevance of a specific Cys to the protein function. Table 1 shows a list of some identified *S*-nitrosylated proteins where the effect on its function and subcellular localization has been described. In the case of peroxisomes, it can be observed that the identified proteins are mainly related to antioxidant enzymes and some enzymes involved in photorespiration, which support a clear interaction between RNS and ROS metabolism. However, further studies are needed to identify additional potential peroxisomal proteins and their physiological relevance.

3.2 Nitration

Nitration consists in the addition of a nitro group ($-NO_2$) to different molecules such as proteins, lipids, and nucleic acids (Rubbo and Radi 2008; Souza et al. 2008). In plants, protein nitration has been the most studied (Corpas et al. 2009c; Kolbert et al. 2017) and very recently the nitration of fatty acids has begun to be analyzed due to its capacity to mediate a signaling response under physiological and stress conditions (Sánchez-Calvo et al. 2013; Mata-Pérez et al. 2016b).

3.2.1 Protein Nitration

In proteins, some amino acids such as tyrosine (Tyr), cysteine (Cys), methionine (Met), and tryptophan (Trp) are more favorably nitrated (Radi 2013). However, in plants most studies have been focused on tyrosine nitration (Tyr-NO_2), which involves adding a nitro group to one of the two equivalent ortho-carbons of the aromatic ring of Tyr residues. Nitration could have several potential effects on protein function such as function loss (which is the most common), gain, or no functional change. Under internal or external stress conditions, an increase in protein nitration or free nitrotyrosine could be regarded as a reliable marker of nitrosative stress, although in plant cells it has also been demonstrated that there is a

Table 1 Subcellular localization of some known protein targets of the NO-mediated post-translational modifications (NO-PTMs) nitration and *S*-nitrosylation

Subcellular location/Protein	NO-PTMs	Effect	Plant species	References
Peroxisome				
Catalase	Nitration *S*-nitrosylation	Inhibition	*Capsicum annuum* *Helianthus annuus* *A. thaliana* *Pisum sativum*	Chaki et al. (2015), Begara-Morales et al. (2013a, b), Corpas and Barroso (2017a), Ortega-Galisteo et al. (2012)
Ascorbate peroxidase (APX)[a]	Nitration *S*-nitrosylation	Inhibition Activation	*Pisum sativum* *A. thaliana*	Begara-Morales et al. (2014a, b), de Pinto et al. (2013)
Hydroxypyruvate reductase (HPR1)	Nitration	Decreased activity	*Pisum sativum* *A. thaliana*	Corpas et al. (2013a, b, c)
Monodehydroascorbate reductase (MDAR)[a]	Nitration *S*-nitrosylation	Inhibition Inhibition	*Pisum sativum* *Pisum sativum*	Begara-Morales et al. (2014a, b)
Serine-glyoxylate aminotransferase (SGAT)	*S*-nitrosylation	Unknown	*Pisum sativum*	Ortega-Galisteo et al. (2012)
Glycolate oxidase	*S*-nitrosylation	Inhibition	*Kalanchoe pinnata* *Pisum sativum*	Abat et al. (2008), Ortega-Galisteo et al. (2012)
Chloroplast				
Peroxiredoxin II E	*S*-nitrosylation	Inhibition	*A. thaliana*	Romero-Puertas et al. (2007)
Methionine synthase	Nitration	Decreased activity	*A. thaliana*	Lozano-Juste et al. (2011)
PSBA(D1) of Photosystem II complex	Nitration	Disassembly of PSII dimers	*A. thaliana*	Galetskiy et al. (2011a, b)
Rubisco large subunit	*S*-nitrosylation	Inhibition carboxylase activity	*A. thaliana* *Kalanchoe pinnata* *Brassica juncea*	Abat et al. (2008), Abat and Deswal (2009), Fares et al. (2014)
Photosystem I apoprotein A2	*S*-nitrosylation	Unknown	*A. thaliana*	Fares et al. (2014)
Ferredoxin-NADP reductase	*S*-nitrosylation	Unknown	*A. thaliana* *Pisum sativum*	Holzmeister et al. (2011), Begara-Morales et al. (2013a, b)
Adenylate translocater	*S*-nitrosylation	Unknown	*A. thaliana*	Fares et al. (2014)
Phosphoribulokinase	*S*-nitrosylation	Unknown	*Pisum sativum*	Begara-Morales et al. (2013b)

(continued)

Table 1 (continued)

Subcellular location/Protein	NO-PTMs	Effect	Plant species	References
Calvin-Benson cycle fructose-1,6-bisphosphatase	*S*-nitrosylation	Inhibition	*Pisum sativum*	Begara-Morales et al. (2013b), Serrato et al. (2017)
Plastidial glyceraldehyde-3-phosphate dehydrogenase	*S*-nitrosylation	Inhibition	*A. thaliana*	Wang et al. (2017)
Mitochondrion				
Phosphate transporter (PHT3;1)	*S*-nitrosylation	Unknown	*A. thaliana*	Fares et al. (2014)
Glycine decarboxylase subunits P1, P2, H1 and T	*S*-nitrosylation	Inhibition	*A. thaliana*	Palmieri et al. (2010)
Glycine decarboxylase subunit T	Nitration	Inhibition	*A. thaliana*	Lozano-Juste et al. (2011)
Ser hydroxymethyl transferase	*S*-nitrosylation	Unknown	*A. thaliana*	Palmieri et al. (2010)
Lipoamide dehydrogenases 1 and 2	*S*-nitrosylation	Unknown	*A. thaliana*	Palmieri et al. (2010)
NAD-malate dehydrogenase	*S*-nitrosylation	Unknown	*A. thaliana*	Romero-Puertas et al. (2008)
Subunits of complex I	*S*-nitrosylation	Unknown	*A. thaliana*	Burwell et al. (2006)
Formate dehydrogenase	Nitration	Unknown	*A. thaliana*	Lozano-Juste et al. (2011)
Serine hydroxymethyltransferase	Nitration	Unknown	*A. thaliana*	Lozano-Juste et al. (2011)
Cysteine synthase	Nitration	Unknown	*A. thaliana*	Lozano-Juste et al. (2011)
Mitochondrial elongation factor Tu	Nitration	Unknown	*A. thaliana*	Lozano-Juste et al. (2011)
Glutamine synthetase 2	Nitration	Unknown	*A. thaliana*	Lozano-Juste et al. (2011)
Cytosolic				
Glutathione reductase (GR)	Nitration *S*-nitrosylation	No effect	*Pisum sativum*	Begara-Morales et al. (2014a, b)
Phytochelatin (PC2, PC3 and PC4)	*S*-nitrosylation	No effect	*A. thaliana*	Elviri et al. (2010)
Carbonic anhydrase (β-CA)	Nitration	Decreased activity	*Helianthus annuus*	Chaki et al. (2013)
S-adenosyl homocysteine hydrolase (SAHH)	Nitration	Decreased activity	*Helianthus annuus*	Chaki et al. (2009)
NADP-isocitrate dehydrogenase	Nitration *S*-nitrosylation	Decreased activity Unknown	*Pisum sativum* *A. thaliana*	Begara-Morales et al. (2013a, b), Fares et al. (2014)

(continued)

Table 1 (continued)

Subcellular location/Protein	NO-PTMs	Effect	Plant species	References
NAD-dependent glyceraldehyde 3-P dehydrogenase (GAPDH)	*S*-nitrosylation	Inhibition	*A. thaliana,*	Lindermayr et al. (2005), Holtgrefe et al. (2008)
GSNO reductase	*S*-nitrosylation	Inhibition	*A. thaliana*	Guerra et al. (2016)
Phosphoenolpyruvate carboxylase	*S*-nitrosylation	Activation	*Sorghum bicolor*	Baena et al. (2017)
Metacaspase AtMC9[b]	*S*-nitrosylation	Inhibition	*A. thaliana*	Belenghi et al. (2007)
Nucleus				
Transcription factor MYB2	*S*-nitrosylation	Inhibition	*A. thaliana*	Serpa et al. (2007)
Transcription factor NPR1	*S*-nitrosylation	Inhibition	*A. thaliana*	Tada et al. (2008)
Transcription factor TGA1	*S*-nitrosylation	Activation	*A. thaliana*	Lindermayr et al. (2010)
Auxin receptor (TIR1)	*S*-nitrosylation	Increased activity	*A. thaliana*	Terrile et al. (2012)
Vacuole				
Vacuolar ATPase subunit	*S*-nitrosylation	Unknown	*A. thaliana*	Fares et al. (2014)
Cysteine protease RD21	*S*-nitrosylation	Unknown	*A. thaliana*	Fares et al. (2014)
Pathogenesis-related (PR)-1 (PR-1A and PR-1B), PR-3 (chitinases PR-P and PR-Q) and PR-5 (thaumatin-like proteins E22 and E2) proteins.	Nitration	Unknown	*Nicotiana tabacum*	Takahashi et al. (2016)
Plasma membrane				
NADPH oxidase H[c]	*S*-nitrosylation	Inhibition	*A. thaliana*	Yun et al. (2011)

[a]Present also in cytosol
[b]Present also in nucleus
[c]Also called respiratory burst oxidase homologue (RBOH)

basal physiological nitration (Corpas et al. 2007; Berton et al. 2012). Table 1 summarizes some of the identified nitrated proteins in plant cells where the effect of this PTM has been described as well as its function and subcellular localization.

3.2.2 Nitro-Fatty Acids (NO_2-FAs)

Polyunsaturated fatty acids (FAs) are an important source of energy as well as essential cellular membrane components which could be involved in the response mechanism to certain environmental stresses such as cold or salinity (Meï et al. 2015). For example, in *Arabidopsis* the most abundant polyunsaturated FAs are 18:2 (linoleic acid) and 18:3 (linolenic acid), representing about 80% of 18C and

nearly 55% of total FAs, whereas 18:1 (oleic acid) represents about 10% of total FAs (Bonaventure et al. 2003; Meï et al. 2015, Mata-Pérez et al. 2016b). These molecules can interact with some NO-related molecules, such as nitrogen dioxide ($^{\cdot}NO_2$) and peroxynitrite ($ONOO^-$), through non-enzymatic reactions and generate nitro-fatty acids (Trostchansky et al. 2013). Recently, in *Arabidopsis* it has been demonstrated that nitro-linolenic acid can release NO but also activate the heat shock proteins (HSPs) pathway acting as a molecular chaperone network under different stress conditions (Mata-Pérez et al. 2016a, b). Some preliminary data have also shown the presence of nitro-fatty acids in pea leaf peroxisomes (Mata-Pérez et al. 2017), which opens new avenues of research considering that peroxisomes have a very active NO metabolism, and include a fatty acid β-oxidation pathway that is a source of powerful signaling molecules, such as jasmonic acid (León 2013).

4 Potential Role of RNS in Peroxisome Communication with Other Subcellular Compartments

Plant peroxisomes have an active ROS and RNS metabolism but these organelles also have a close interaction with other cell organelles, participating in different pathways such as β-oxidation, glyoxylate cycle or photorespiration, and being directly involved in different stages of plant development from germination to senescence (Mano and Nishimura 2005). Additionally, peroxisomes have a role in the generation of signaling molecules, biosynthesis of salicylic acid, and metabolism of urate, polyamines, sulfite, and branched-chain amino acids (Hu et al. 2012; Leterrier et al. 2016; Corpas et al. 2017a, b). The interaction among organelles such as peroxisomes, oil bodies, mitochondria and chloroplasts is well established (Oikawa et al. 2015; Cui et al. 2016; Barton et al. 2018) and, besides the active metabolic interchange, some of the enzymes involved have been found to be targets of some of the previously described NO-dependent PTMs (see Table 1).

However, the modulation of a specific enzyme in a specific organelle will be dependent on the amount of the RNS and the physiological or stress situation of cells. In this context, among the different RNS studied in plant cells that could be involved in organelle crosstalk, NO, *S*-nitrosoglutathione (GSNO), nitrosothiols (SNOs) and peroxynitrite ($ONOO^-$) could be the best candidates. Nevertheless, behind these molecules, there is a complex biochemistry which is more intricate that it could be expected. Consequently, the action ratio of these molecules will depend on the diffusion distance in a specific cellular micro-environment which, at the same time, depends on their production rate and reactivity with the surrounding molecules. However, in plant cells there are not many experimental data showing the action ratio of these molecules produced in specific organelles but some information could be extrapolated from animal cells where the information is most profuse. Nitric oxide is a small molecule that could freely diffuse across membranes

from one compartment to another. However, as it has been mentioned, NO can react with other molecules such as oxygen (O_2), superoxide radicals ($O_2^{\cdot-}$), reduced thiols (–SH) or protein metal centers which can generate different nitrogen species (NO_2, N_2O_3, NO_2^-, NO_3^-), peroxynitrite, and *S*-nitrosothiols among others (Stamler et al. 1992; Pfeiffer et al. 1999; Pacher et al. 2007; Broniowska and Hogg 2012; Broniowska et al. 2013; Ferrer-Sueta and Radi 2009). For example, $ONOO^-$ is a powerful oxidant/nitrating molecule which is formed by the reaction between NO and $O_2^{\cdot-}$, which has a rate constant within the range $4–6 \times 10^9\ M^{-1}\ s^{-1}$. Peroxynitrite is very reactive and will probably react nearly in the same place where it is generated. In this sense, cellular approaches using specific fluorescence probes for NO, $O_2^{\cdot-}$ and $ONOO^-$ allowed to observe these molecules in *Arabidopsis* guard cells, where NO was present in peroxisomes but also in the rest of the guard cells, whereas superoxide and $ONOO^-$ were restricted to peroxisomes (Corpas and Barroso 2014a). However, $ONOO^-$ is also capable to partially cross cell membranes through anion channels (Ferrer-Sueta and Radi 2009), and this could be relevant under stress situations where these molecules are overproduced, and consequently could affect the function of target proteins located in closer subcellular compartments.

4.1 Photorespiration as an Example of Communication Between Chloroplasts, Mitochondria and Peroxisomes

Peroxisomes, chloroplasts, mitochondria and other subcellular compartments are multifunctional organelles that are interconnected metabolically to perform specific functions which depend on the cell type in a given organ (root, stem, leaf, cotyledon, flower or fruit), stage of development and environmental conditions (Troncoso-Ponce et al. 2013; Palma et al. 2015; Cui et al. 2016; Hagemann and Bauwe 2016). Nitric oxide (NO) and some derived molecules are generated in different subcellular compartments of plant cells, and they could have a signaling function within the same organelle but also in organelles closely associated (Jasid et al. 2006; Blokhina and Fagerstedt 2010; Igamberdiev et al. 2014; Kmiecik et al. 2016). Plant peroxisomes have the capacity to generate RNS under physiological and stress conditions (for a review see Corpas et al. 2017a; del Río 2011) and consequently, these RNS could modulate not only the own peroxisomal metabolism but also that of the surrounding organelles.

Photorespiration is a light-dependent metabolic process which consumes O_2 with the concomitant release of CO_2 (Florian et al. 2013). However, this process is also connected with other pathways such as photosynthesis, nitrate assimilation, amino acid metabolism, and the tricarboxylic acid cycle (Hodges et al. 2016). Photorespiration involves the participation of chloroplasts, peroxisomes and mitochondria, and clearly differs from the mitochondrial respiration which is light-independent and takes place exclusively within this organelle. Figure 2 outlines

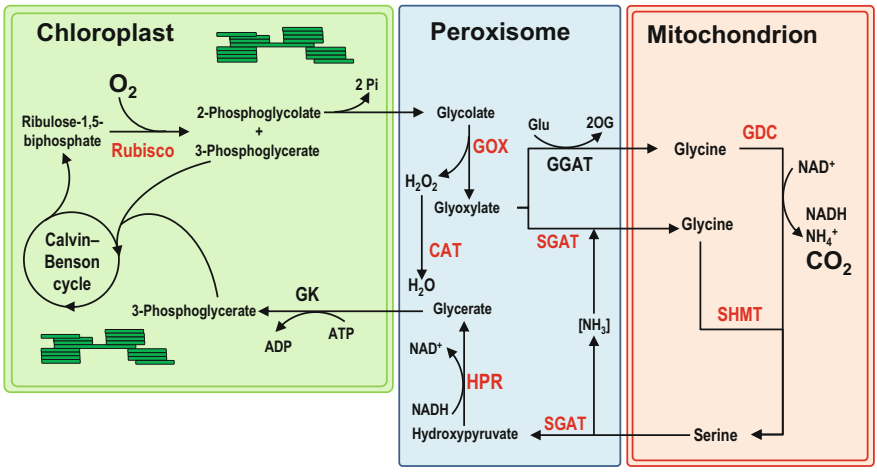

Fig. 2 Simple model indicating the identified enzymes of the photorespiratory pathway of higher plants that undergo NO-mediated post-translational modifications (PTMs) and its interconnection with the photosynthetic Calvin–Benson cycleAbbreviations: 2OG, 2-oxoglutarate; CAT, catalase; GDC, glycine decarboxylase complex; GGAT, glutamate:glyoxylate aminotransferase; GOX, glycolate oxidase; GK, glycerate kinase; HPR, hydroxypyruvate reductase; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SGAT, serine-glyoxylate aminotransferase; SHMT, serine hydroxymethyltransferase. The enzymes that undergo an NO-derived PTM, either nitration, *S*-nitrosylation or both, are written in red colour

a simple model of the photorespiratory pathway. The identified enzymes that undergo *S*-nitrosylation and/or nitration, have been highlighted (red colour) in the different organelles (see Table 1).

In *Arabidopsis* it has been found that the peroxisomal hydroxypyruvate reductase (HPR) undergoes a nitration which provokes a loss of function (Corpas et al. 2013b). A deeper analysis revealed that among the eleven Tyr residues present in the HPR only three are nitrated (Tyr-97, Tyr-108 and Tyr-198), and site-directed mutagenesis confirmed Tyr-198 as the primary site of nitration (Corpas et al. 2013b). It should be mentioned that under some stress conditions, such as cadmium or lead stress, where an increase of peroxisomal peroxynitrite production has been described, the HPR activity did not show significant changes (Corpas and Barroso 2014a, 2017a) suggesting that maybe the $ONOO^-$ content was not sufficient to affect this activity or that there is an alternative mechanism of protection. In this sense, very recently the presence of a peroxisomal protein immuno-related to peroxiredoxin, which could exert this protective function has been described (Corpas et al. 2017b). In the case of glycolate oxidase (GOX), which is a major source of H_2O_2 during photorespiration, it has been observed that under in vitro conditions this protein is susceptible to be *S*-nitrosylated which inhibited its activity (Abat et al. 2008; Ortega-Galisteo et al. 2012). However, as it has been mentioned above with HPR, in *Arabidopsis* exposed to heavy metals and with a significant increase of RNS metabolism, it was observed that GOX activity was not

significantly affected (Corpas and Barroso 2017b). This could be due to the redundancy of GOX since there are up to five GOX genes in *Arabidopsis* that could mitigate the potential inhibition under stress conditions. However, it should be mentioned that in these cases of heavy metal-induced oxidative stress, the activity of catalase, an enzyme which can be nitrated and *S*-nitrosylated, was down-regulated.

On the other hand, an unexplored area in the photorespiration pathway, especially in mitochondria, is the reaction of $ONOO^-$ with CO_2 to yield the adduct nitroso peroxycarbonate ($ONOOCO_2^-$), which then decomposes in two radical molecules, $^{\cdot}NO_2$ and $CO_3^{\cdot-}$, thus diversifying the action of peroxynitrite (Ferrer-Sueta and Radi 2009). This could be very relevant under nitro-oxidative stress conditions, but to our knowledge there is no information about this reaction in plant cells.

5 Conclusions

At present, new set of data have clearly demonstrated that besides some classical signal molecules including phytohormones, calcium and H_2O_2, the family of RNS molecules could also exert this function. Plant peroxisomes have the capacity to generate different RNS such as NO, GSNO or $ONOO^-$ (peroxynitrite) which could mediate PTMs in peroxisomal proteins involved either in photorespiration, or in its own antioxidant system, particularly catalase which regulates the peroxisomal H_2O_2 content, as well as in surrounding organelles. These relationships between the metabolism of RNS and ROS in peroxisomes and other cell organelles could be more relevant under adverse stress conditions and could be accompanied by a nitro-oxidative stress. However, this could also have positive consequences. For example, in plants under pathogen infection there is an accumulation of peroxisomes in the epidermal tissue, at the fungus penetration sites, possibly to participate in degrading generated ROS (mainly H_2O_2) and limit the extent of damage (Koh et al. 2005). Under these conditions, it seems reasonable to think that the peroxisomal generation of RNS, like $ONOO^-$, could be useful as a mechanism of defense, as it occurs in the mammalian immune system. Taken together, all these data open new lines of research on a possible role of RNS generated in plant peroxisomes in the communication with other cell organelles as well as in the mechanisms of defense of plants that deserve to be explored.

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