MOLECULAR MECHANISMS AND PHYSIOLOGICAL SIGNIFICANCE OF ORGANELLE INTERACTIONS AND COOPERATION

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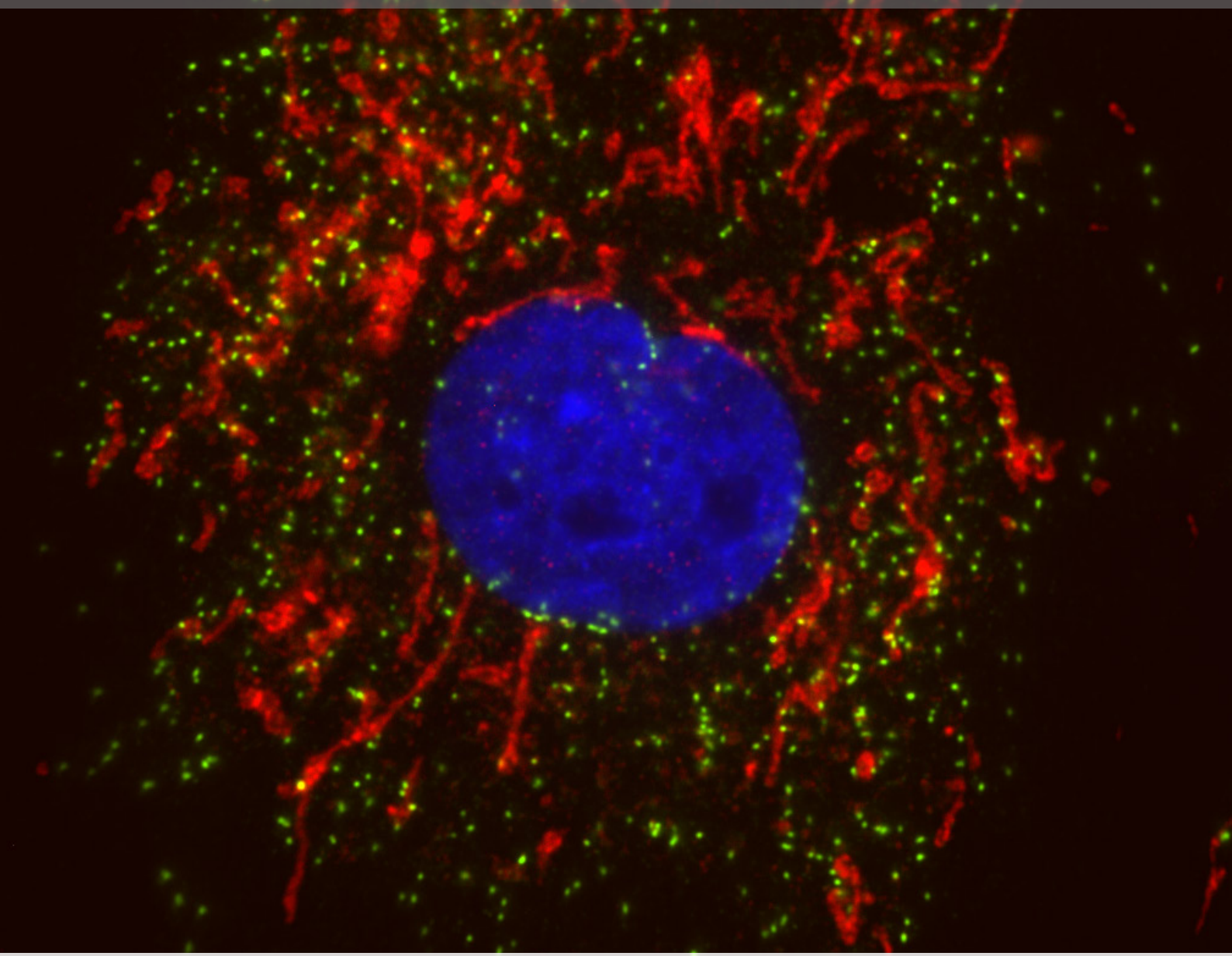

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MOLECULAR MECHANISMS AND PHYSIOLOGICAL SIGNIFICANCE OF ORGANELLE INTERACTIONS AND COOPERATION

Topic Editors:
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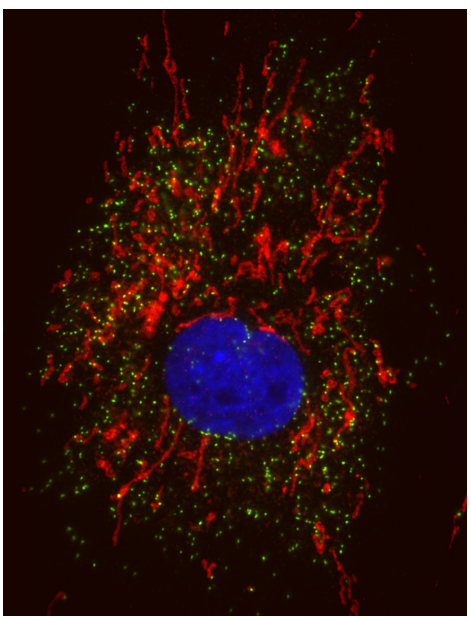

Organisation of mitochondria (red) and peroxisomes (green) in cultured human skin fibroblasts. Indirect immunofluorescence of fixed cells using antibodies directed to the mitochondrial outer membrane protein TOM20 and the peroxisomal membrane protein Pex14. Secondary antibodies were coupled to the fluorophores Alexa 488 and TRITC. The nucleus (blue) was stained with DAPI. Image owned by M. Schrader

Eukaryotic cells contain distinct membrane-bound organelles, which compartmentalise cellular proteins to fulfil a variety of vital functions. Many organelles have long been regarded as isolated and static entities (e.g., peroxisomes, mitochondria, lipid droplets), but it is now evident that they display dynamic changes, interact with each other, share certain proteins and show metabolic cooperation and cross-talk. Despite great advances in the identification and characterisation of essential components and molecular mechanisms associated with the biogenesis

and function of organelles, information on how organelles interact and are incorporated into metabolic pathways and signaling networks is just beginning to emerge. Organelle cooperation requires sophisticated targeting systems which regulate the proper distribution of shared proteins to more than one organelle. Organelle motility and membrane remodeling support organelle interaction and contact. This contact can be mediated by membrane proteins residing on different organelles which can serve as molecular tethers to physically link different organelles together. They can also contribute to the exchange of metabolites and ions, or act in the assembly of signaling platforms. In this regard organelle communication events have been associated with important cellular functions such as apoptosis, antiviral defense, organelle division/biogenesis, ROS metabolism and signaling, and various metabolic pathways such as breakdown of fatty acids or cholesterol biosynthesis.

In this research topic we will focus on recent novel findings on the underlying molecular mechanisms and physiological significance of organelle interaction and cooperation with a particular focus on mitochondria, peroxisomes, endoplasmic reticulum, lysosomes and lipid droplets and their impact on the regulation of cellular homeostasis. Our understanding of how organelles physically interact and use cellular signaling systems to coordinate functional networks between each other is still in its infancy. Nevertheless recent discoveries of defined membrane structures such as the mitochondria-ER associated membranes (MAM) are revealing how membrane domains enriched in specific proteins transmit signals across organelle boundaries, allowing one organelle to influence the function of another. In addition to its role as a mediator between mitochondria and the ER, contacts between the MAM and peroxisomes contribute to antiviral signaling, and specialised regions of the ER are supposed to initiate peroxisome biogenesis, whereas intimate contacts between peroxisomes, lipid droplets and the ER mediate lipid metabolism. In line with these observations it is tempting to speculate that further physical contact sites between other organelles exist. Alternatively, novel regulated vesicle trafficking pathways between organelles (e.g., mitochondria to peroxisomes or lysosomes) have been discovered implying another mode of organelle communication. Identifying the key molecular players of such specialised membrane structures will be a prerequisite to understand how organelle communication is physically accomplished and will lead to the identification of new regulatory networks. In addition to the direct transmission of interorganellar information, cytosolic messenger systems (e.g., kinase/phosphatase systems or redox signaling) may contribute to the coordination of organelle functions. This research topic will integrate new findings from both modes of communication and will provide new perspectives for the functional significance of cross-talk among organelles.

We would like to thank all the researchers who contributed their valuable work to this research topic. Furthermore, we are grateful to the reviewers and Associate Editors who contributed valuable comments and positive criticism to improve the contributions.

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EDITORIAL
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Editorial: Molecular Mechanisms and Physiological Significance of Organelle Interactions and Cooperation

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Keywords: membrane contact sites, organelle dynamics, peroxisomes, mitochondria, endoplasmic reticulum, intracellular signaling, organelle communication, lipid droplet

Editorial on the Research Topic

Molecular Mechanisms and Physiological Significance of Organelle Interactions and Cooperation

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Compartmentalization is a characteristic feature of eukaryotic life. Subcellular compartments such as mitochondria, peroxisomes, or lipid droplets have long been regarded as isolated and static entities which are mainly defined by their protein composition and specific metabolic function. However, it is now evident that they undergo dynamic changes, share certain proteins and interact with each other, showing metabolic cooperation and cross-talk. Effective communication between organelles is essential for cell function, viability and response to external stimuli. Despite great advances in the identification and characterization of key components and molecular mechanisms associated with the biogenesis and function of organelles, information on how organelles interact and are incorporated into metabolic and signaling networks is just beginning to emerge. Organelle cooperation requires sophisticated targeting systems which regulate the proper distribution of shared proteins to more than one organelle. Organelle motility and membrane remodeling support organelle interaction and contact. This contact can be mediated by membrane proteins residing on different organelles which can serve as molecular tethers to physically link different organelles. They can also contribute to the exchange of metabolites and ions, or act in the assembly of signaling platforms. In this regard organelle communication events have been associated with important cellular functions such as apoptosis, antiviral defense, organelle division and biogenesis, ROS metabolism and signaling, and various metabolic pathways such as breakdown of fatty acids or ether lipid (plasmalogen) biosynthesis.

The goal of this Research Topic is to review, present, compare, and debate recent novel findings on the underlying molecular mechanisms and physiological significance of organelle interaction and cooperation with a particular focus on mitochondria, peroxisomes, endoplasmic reticulum, and lipid droplets and their impact on the regulation of cellular homeostasis. The special topic thus combines a set of reviews, perspectives and research articles.

Our understanding of how organelles physically interact and use cellular signaling systems to coordinate functional networks between each other is still in its infancy. Recent work on membrane contact sites, for example the mitochondria-ER associated membranes (MAM), deciphered molecular players enriched in membrane domains and is thereby beginning to reveal mechanistic insights into organellar interaction systems, allowing one organelle to influence the function of

another. Identifying the key molecular players of such specialized membrane structures will be a prerequisite to understand how organelle communication is physically accomplished and will lead to the identification of new regulatory networks. The review by Schrader et al. provides a timely overview of organelle contact sites, the molecular components involved and discusses the potential functions of organelle interactions. I. Sparkes addresses the techniques applied to investigate membrane contacts with special emphasis on the use of optical tweezers for their biophysical characterization (Sparkes). Kunze and Berger review protein import mechanisms into different organelles, including mitochondria and peroxisomes, and their targeting signals. They focus on the similarity between N-terminal targeting signals, address dual targeting and bi-localization, and highlight its evolutionary relevance.

Wanders et al. focus on an important function of organelle interaction, namely the metabolic interplay between peroxisomes and mitochondria as well as the ER. They highlight the disease-relevant interplay between peroxisomes and mitochondria in the breakdown of fatty acids by β-oxidation, as well as the cooperative role of peroxisomes and the ER in the biosynthesis of ether lipids that are required for the myelin sheath in humans. An important question is how substrates and products of metabolic networks are exchanged between participating organelles. Gao and Goodman review our current knowledge on how and why cytoplasmic lipid droplets interact with other subcellular organelles. Important functions are seen in the transfer of lipids between compartments, the supply of lipids for membrane expansion, energy production, and signaling. The underlying mechanism and extent of activation of lipases by contact sites, and the mode of fatty acid transfer between organelles, still remain to be elucidated.

Mueller and Reski focus on mitochondrial dynamics and interactions in plants. Using the model moss *Physcomitrella patens,* they provide microscopic evidence for the existence of mitochondria-ER interactions in plants, their correlation with mitochondrial dynamics and a potential role for MELL1 in modulating mitochondrial association to the ER. In their research article, Woods et al. present experimental evidence for a role of the microtubule cytoskeleton and the protein CluA in mitochondrial dynamics in the soil-dwelling amoeba *Dictyostelium discoideum*, a lower eukaryotic model organism.

Jaipargas et al. provide new data on the interaction of plant peroxisomes with mitochondria. Using live cell imaging, they show that peroxisomes form thin membrane protrusions (peroxules) which interact with mitochondria as a result of high light irradiation and subsequent ROS production. These interactions with ROS-distressed mitochondria may provide factors to peroxisomes which facilitate their proliferation for enhancing the ROS-combating capability of a plant cell. In line with this, Lismont et al. review our current view on redox-interplay between peroxisomes and mitochondria in mammalian cells. The authors outline the pro- and anti-oxidant systems of both organelles, their role as redox signaling nodes and discuss emerging evidence that peroxisomes and mitochondria share an intricate redox-sensitive relationship and cooperate in cell fate decisions. Schönenberger and Kovacs critically explore how hypoxia-inducible factor (HIF-α) signaling regulates the abundance and function of major oxygen-consuming organelles such as mitochondria and peroxisomes.

The reviews and research articles presented in this special topic demonstrate the impressive breadth of research currently being undertaken to understand the molecular mechanisms and physiological significance of organelle interactions and cooperation. Advances in the field, both methodological and conceptual, will have profound implications for understanding the architecture, organization and regulation of cellular metabolic and signaling networks and their impact on health and disease. Future challenges in this research area are to identify and characterize the specific components of the individual organellar interaction sites and to unravel signaling pathways which are able to dynamically regulate these interorganellar contact systems.

AUTHOR CONTRIBUTIONS

MS and MI discussed and planned the content of the Editorial and wrote the manuscript.

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REVIEW
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The different facets of organelle interplay—an overview of organelle interactions

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Membrane-bound organelles such as mitochondria, peroxisomes, or the endoplasmic reticulum (ER) create distinct environments to promote specific cellular tasks such as ATP production, lipid breakdown, or protein export. During recent years, it has become evident that organelles are integrated into cellular networks regulating metabolism, intracellular signaling, cellular maintenance, cell fate decision, and pathogen defence. In order to facilitate such signaling events, specialized membrane regions between apposing organelles bear distinct sets of proteins to enable tethering and exchange of metabolites and signaling molecules. Such membrane associations between the mitochondria and a specialized site of the ER, the mitochondria associated-membrane (MAM), as well as between the ER and the plasma membrane (PAM) have been partially characterized at the molecular level. However, historical and recent observations imply that other organelles like peroxisomes, lysosomes, and lipid droplets might also be involved in the formation of such apposing membrane contact sites. Alternatively, reports on so-called mitochondria derived-vesicles (MDV) suggest alternative mechanisms of organelle interaction. Moreover, maintenance of cellular homeostasis requires the precise removal of aged organelles by autophagy—a process which involves the detection of ubiquitinated organelle proteins by the autophagosome membrane, representing another site of membrane associated-signaling. This review will summarize the available data on the existence and composition of organelle contact sites and the molecular specializations each site uses in order to provide a timely overview on the potential functions of organelle interaction.

Keywords: membrane contact sites, organelle dynamics, peroxisomes, mitochondria, endoplasmic reticulum, intracellular signaling, MAM, PAM

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Introduction

In eukaryotic cells sophisticated membrane-bound organelles have evolved which enable the cell to compartmentalize specialized biochemical reactions in specific locations within the cell (**Figure 1**). Historically, subcellular compartments were regarded as isolated, membrane bound biochemical entities, and individual organelles such as mitochondria, lysosomes, peroxisomes, or the endoplasmic reticulum (ER) have been associated with distinct cellular tasks including ATP production, protein degradation, lipid breakdown, and protein export. In recent years, a

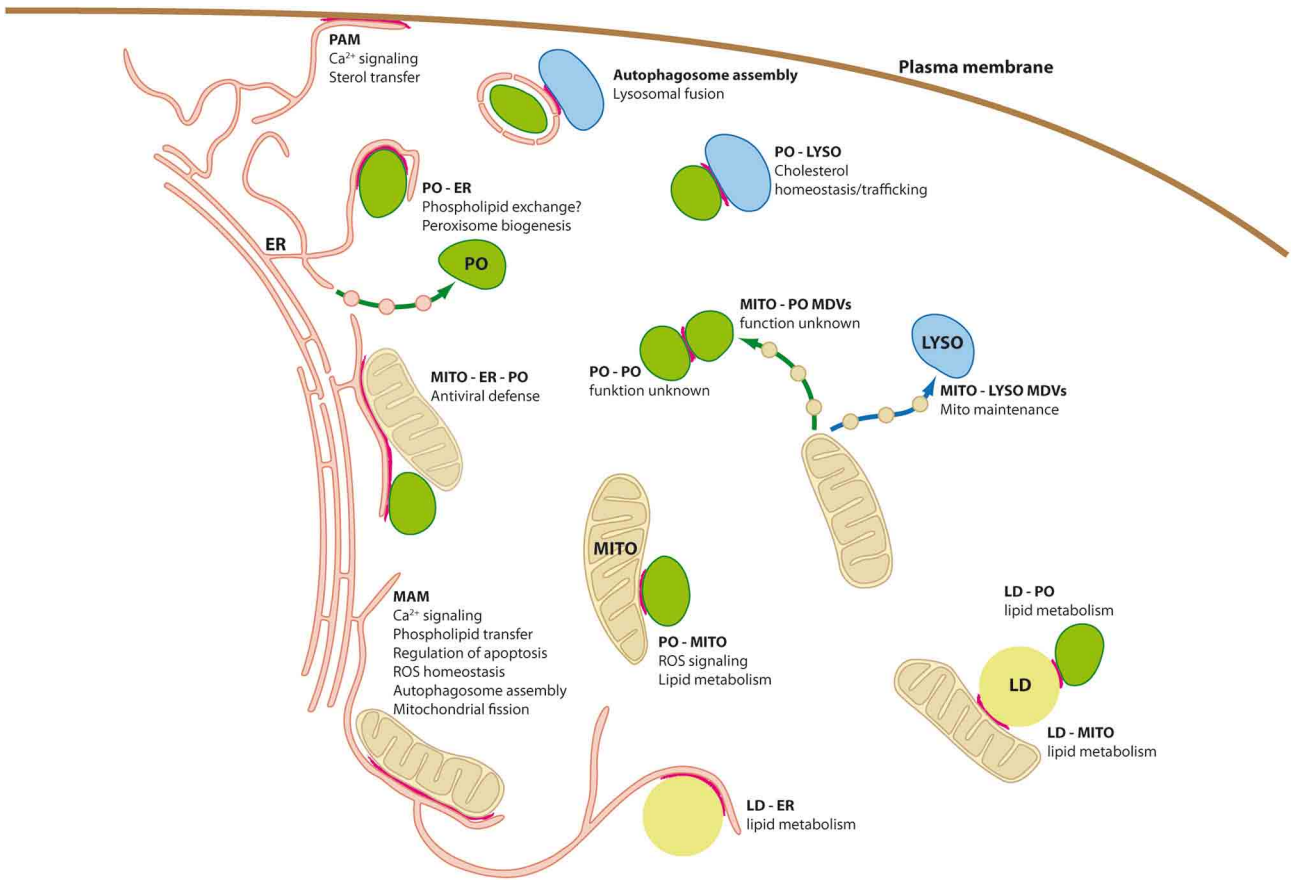

FIGURE 1 | Organelle interplay and interorganellar contacts. Schematic diagram of a mammalian cell depicting organelle interplay and interorganellar membrane contacts (highlighted by red lines). ER, endoplasmic reticulum; LD, lipid droplets; LYSO, lysosome; MAM, mitochondria associated-membrane; MDVs, mitochondria derived vesicles; MITO, mitochondrium; PAM, plasma membrane-associated membrane; PO, peroxisome.

combination of ultrastructural studies, fluorescence-based live cell imaging techniques, molecular cell biology, biochemistry, and modern proteomics approaches has substantially changed this view towards a highly dynamic, cooperative and complex network of interacting and communicating subcellular compartments (**Figure 1**). It is evident that intracellular compartments have to exchange material and transmit signals between each other to maintain and balance cellular activities. Cooperative functions of organelle networks include (1) metabolic interaction, (2) intracellular signaling, (3) cellular maintenance, (4) regulation of programmed cell death/cell survival, and (5) pathogen defence. Mechanistically, functional interplay can be established by vesicular transport (as initially revealed for organelles within the secretory pathway), by exchange of metabolites or signaling molecules through diffusion, or direct physical contacts which are mediated by specialized membrane contact sites (**Figure 2**). It is becoming evident that the cytoskeleton and molecular motors are not the sole organizers of cellular architecture, and that membrane contacts can influence the positioning and motility of organelles. Organelle interaction also depends on the total number of organelles which is regulated by organelle biogenesis/formation, membrane dynamics and autophagic processes. Remarkably, these processes also involve membrane contact sites, for example ER-mitochondria contacts which are supposed to contribute to mitochondrial division [see Sections The Mitochondria-associated Membrane of the ER (MAM) and Interplay between Peroxisomes and Mitochondria] or interactions with lysosomes during autophagy (see Section Lysosomal Interactions and Autophagy). Membrane contact sites involve tethering of two

Abbreviations: AIS, axon initial segment; ATP, Adenosine triphosphate; CMA, chaperone-mediated autophagy; EMC, ER membrane protein complex; EPCON, ER-peroxisome contact site; ER, endoplasmic reticulum; ERMES, ER mitochondria encounter structure; GPI, Glycophosphatidylinositol; IP3R, inositol 1,4,5-trisphosphate receptor; LD, lipid droplet; LPMC, lysosomal-peroxisome membrane contacts; MAM, mitochondria-associated membrane; MCU, mitochondrial low affinity calcium uniporter; MDVs, mitochondria-derived vesicles; MITO, mitochondria; NCLX, Na+/Ca2+ exchanger; ORPs, oxysterol-binding protein-related proteins; PAM, plasma membrane-associated membrane; PE, phosphatidylethanolamine; PS, phosphatidylserine; PIP, phosphatidylinositol phosphate; PI4P, phosphatidylinositol 4-phosphate; PKA, protein kinase; PM, plasma membrane; PML, promyelocytic leukemia; PMPs, peroxisomal membrane proteins; PO, peroxisome; PTS, peroxisomal targeting signal; ROS, reactive oxygen species; SERCA, ER sarcoplasmic/endoplasmic reticulum calcium ATPase; SOCE, store-operated calcium entry pathway; STIM, stromal-interacting molecule; UPR, unfolded protein response; VAPS, vamp-associated proteins.

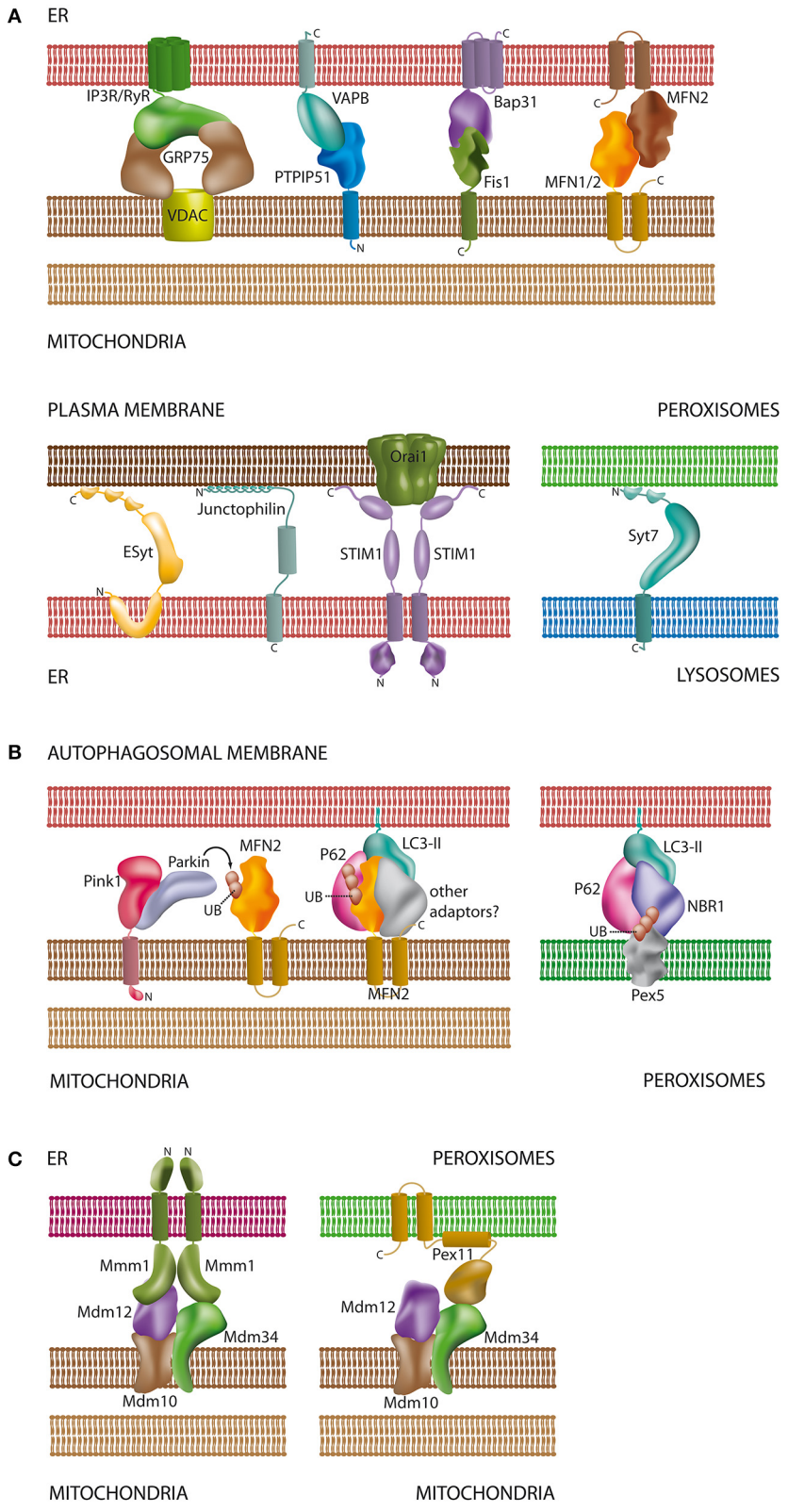

FIGURE 2 | Schematic overview of proteins and lipids involved in the interaction of organelles. (A) Tethering complexes in mammals: unlike in yeast species only a few protein complexes have been characterized at the molecular level and involve protein-protein and protein-lipid contacts [see Sections Connections

(Continued)

FIGURE 2 | Continued
between the ER and the Plasma Membrane, The Mitochondria-associated Membrane of the ER (MAM), Interplay between Peroxisomes and Mitochondria, and Lysosomal Interactions and Autophagy]. Part of the tethering complexes shown may only comprise core complexes, which will interact with additional proteins for regulatory purposes; **(B)** contacts between mitochondria/peroxisomes and the autophagosomal membrane: both organelles require ubiquitination of membrane proteins for recognition by the autophagosome. In addition to MFN2 (Mitochondria) and Pex5 (Peroxisomes) other ubiquitinated organelle proteins have been described to participate in autophagosomal contacts [see Sections The Mitochondria-associated Membrane of the ER (MAM) and Lysosomal Interactions and Autophagy]; **(C)** ERMES as a multifunctional tethering complex in yeast: unlike mammals, yeast species possess the ERMES oligomeric complex at the mitochondrial membrane. ERMES forms complexes with the ER and peroxisomes [see Sections The Mitochondria-associated Membrane of the ER (MAM) and Interplay between Peroxisomes and Mitochondria]. In addition, a considerable number of other tethering complexes (not shown) have been described in yeast (Prinz, 2014). For molecular details and references of the depicted complexes please refer to the corresponding sections of this review. Membrane spanning α-helices in the proteins are depicted as cylindrical segments; C- and N-termini are marked with the corresponding letters.

membranes in close apposition (typically within 30 nm) and the enrichment of specific proteins and/or lipids at these sites (see **Table 1**). In general, the tethered membranes do not fuse, but contact formation has an impact on the function or composition of one or both organelles. Although membrane contacts between organelles have been reported in early ultrastructural studies, their important functions in intracellular signaling, metabolite transport/metabolism, organelle dynamics and transport is just beginning to emerge. Furthermore, a growing number of proteins with potential tethering functions are being identified in yeast and mammals.

As an introduction to the Frontiers research topic on "Molecular mechanisms and physiological significance of organelle interactions and cooperation" this review aims at providing a general and timely overview of the new and fascinating mechanisms which convey the cellular plasticity required to react to metabolic and environmental changes in a spatial and temporal manner. We address processes of organelle interaction with a particular focus on membrane contact sites emerging at the cross roads of organelle research and intracellular signaling. In addition, we highlight novel findings on the functional aspects of organelle interaction with a special emphasis on mitochondria and peroxisomes. We particularly focus on organelle interplay in mammals but where appropriate also refer to recent discoveries in plants and fungi.

Connections between the ER and the Plasma Membrane

In striated muscle cells a close apposition between peripheral ER and the plasma membrane, now well known as the T-tubule system required for excitation-contraction coupling, was reported as early as the 1950's by the pioneers of cell biological research, Keith Porter and George Palade (Porter and Palade, 1957). Originally regarded as a specialization only found in muscle cells it has in the meanwhile become obvious, that specialized juxtaposed membrane stretches between the ER and the plasma membrane are ubiquitously distributed among eukaryotic cells (Stefan et al., 2013) (**Figure 1**). While the classical secretory pathway or endosomal trafficking between the ER and the plasma membrane involves the passage of further intermittent organelle structures, the so-called "plasma membrane-associated membrane of the ER" (PAM) represents a direct link between both subcellular compartments (**Figure 1**). Linked to the function of the peripheral sarcoplasmic reticulum, one of the specializations of the PAM comprises the control of Ca^{2+} dynamics between the extracellular space and the ER, which is the dominant Ca^{2+} storage compartment of the cell. In this respect the plasma membrane of T-tubules is enriched in voltage gated ion channels which activate juxtaposed ryanodine receptors in the PAM to elicit Ca^{2+} into the cytosol (Endo, 2009) (**Figure 2A**). Both membranes are interconnected by junctophilins, integral membrane proteins of the ER in excitable cells (**Figure 2A**). Junctophilins stabilize association between the plasma membrane and the ER at junctional complexes by binding to phosphatidylinositol phosphate (PIP) lipids at the cytoplasmic side of the plasma membrane (Takeshima et al., 2015). A more commonly distributed protein assembly found in less specialized cells is the "store-operated calcium entry pathway" (SOCE). This is composed of the ER Ca^{2+} sensor STIM (stromal-interacting molecule), which interacts with the plasma membrane Ca^{2+} channel Orai1 at ER/plasma membrane contact sites in order to replenish ER Ca^{2+} concentrations (Liou et al., 2005) (**Figure 2A**). Again this process involves the binding of PIP lipids at the plasma membrane by the ER resident STIM protein (Park et al., 2009). Opening of Orai1 channels leads to focally elevated Ca^{2+} concentration at the cytosolic face of the PAM facilitating its uptake by "ER sarcoplasmic/endoplasmic reticulum calcium ATPase" channels (SERCA).

Interestingly, SOCE assemblies have been recently described for the spine apparatus—a stack of smooth ER found in the necks of dendritic spines of principal cortical and hippocampal neurons (Korkotian et al., 2014). An essential component of the spine apparatus is the actin-associated protein synaptopodin (Deller et al., 2000a). Functional studies indicate that the spine apparatus acts as a dynamic intracellular calcium store (Vlachos et al., 2009), involved in regulation of homeostatic synaptic plasticity and memory (Deller et al., 2000a; Vlachos et al., 2013; Korkotian et al., 2014). In line with such a function, characteristic Ca^{2+} ryanodine and inositol tris-phosphate 3-receptors (IP3R) have been described in the ER of dendritic spines (Satoh et al., 1990) (**Figure 2A**). Thus, plasma membrane/ER associations may act to regulate the Ca^{2+} concentrations in the spine apparatus in order to dynamically control postsynaptic signal transmission. A putative axonal homolog of the SA is comprised of the so-called cisternal organelle which is specifically localized in the axon initial segment (AIS) (Deller et al., 2000b). Structurally, the cisternal organelle is comprised of stacks of smooth ER frequently found in apposition to the AIS plasma membrane. Similar to the SA, synaptopodin is also an essential component for the cisternal organelle (Bas Orth et al., 2007). Additional proteins characteristic for the PAM,

TABLE 1 | Summary of the protein components found at organelle contact sites in mammalian cells.

Organelles	Contact site	Contact site function	Confirmed components	Putative components	References
ER-PM	Junctional membrane complexes (JMC)	Various, including coupling electric excitation of the PM with myofilament contraction	Junctophilin1-4 (JP1-4): Bind PIP lipids at cytoplasmic side of PM		Takeshima et al., 2015
	Store-operated calcium entry pathway (SOCE)	Replenishment of ER Ca^{2+} levels	STIM1: ER resident, binds PIP lipids and Orai1 at plasma membrane, Orai1: PM resident, forms channel facilitating Ca^{2+} uptake.		Liou et al., 2005; Park et al., 2009
	ORPS	Generating focal lipid exchange sites	ORP1/2: Sterol transport from PM to ER		Ngo et al., 2010; Jansen et al., 2011
	Extended Synaptotagmins	Implicated in the mediation of lipid transfer	E-Syt1-3: ER proteins mediating Ca^{2+}-dependent tethering of the ER to PM		Giordano et al., 2013; Fernández-Busnadiego et al., 2015
ER-MITO	MAM	Various, including lipid metabolism, Ca^{2+} signaling, and regulation of mitochondrial maintenance	PEMT: converts PE to PC	DGAT2: triglyceride synthesis, TMX: thioredoxin, calnexin: protein chaperone, Acsl4: enzyme in steroidogenesis, PTDSS1/2: formation of PS from PC/PE, Mfn2: MITO fusion, DRP1: Mito fission, Atg14, autophagy receptor, Ero1α: oxidoreductase, IP3R, Ca^{2+} channel, MAVS: antiviral signaling	Cui et al., 1993; Stone et al., 2009; Lynes et al., 2012
	VAPB-PTPIP51 tether	Physical tether, may be involved in Ca^{2+} homeostasis	VAPB: ER/MAM protein plays role in UPR, PTPIP51: MITO protein, various functions		Stoica et al., 2014
	Fis1-Bap31 tether	Recruitment of procaspase-8 to MAM leading to induction of apoptosis	Fis1: MITO/PO TA protein involved in MITO/PO fission, Bap31: ER protein involved in quality control		Iwasawa et al., 2011
MITO-LD	Periphilin 5 tether	Physical and metabolic linkage	Periphilin 5: LD-associated scaffold protein		Wang et al., 2011

including IP3R channels and SERCA pumps, have been found in the cisternal organelle (Benedeczky et al., 1994; Sánchez-Ponce et al., 2011). Although, the precise function of the cisternal organelle is still unknown, it may act as a distinct axonal ER Ca^{2+} storage compartment which mediates calcium-dependent signal transmission in cooperation with apposed ion channels in the plasma membrane (King et al., 2014). In this respect both the spine apparatus and the cisternal organelle may represent neuron-specific specialized PAM regions which create the Ca^{2+} microenvironments required in specific subcellular compartments of highly polarized neurons.

Comparable to the specialization of the mitochondria associated membrane of the ER (MAM), the PAM is also supposed to be involved in the transfer of lipids to the opposing plasma membrane. While the MAM delivers phosphatidylserine to mitochondria [see Section The Mitochondria-associated Membrane of the ER (MAM)], the PAM is involved in the transport of sterol compounds between the ER and the plasma membrane (Toulmay and Prinz, 2011) (**Figure 1**). In yeast, oxysterol-binding protein (OSBP)-related proteins (ORPs) have been proposed as shuttles between apposed ER/plasma membrane sites (Schulz et al., 2009) and deletion of all ORPs in a yeast strain has been shown to decrease sterol exchange significantly (Beh et al., 2001). A subset of yeast ORPs possess Pleckstrin homology domains and a motif containing two phenylalanine residues in an acidic tract (FFAT), which bind PIPs of the plasma membrane and Vamp-associated proteins of the ER membrane (VAP), respectively (Roy and Levine, 2004; Loewen and Levine, 2005). Both structures ensure that the proteins target to ER/plasma membrane contacts thereby generating focal lipid exchange sites. Sterol lipid exchange between the opposing membranes appears to function in both directions and implies a complex lipid sensing system which is still not completely understood (see Toulmay and Prinz, 2011; Stefan et al., 2013 for detailed information). In this context, yeast ORPs (Osh proteins) appear to fulfill a role beyond mere sterol shuttles, also acting as lipid sensors, transmitting signals to upstream regulators. The Osh proteins localize to ER/PM contacts after phosphatidylinositol 4-phosphate (PI4P) binding and interaction with the VAP Scs2 (Stefan et al., 2011). PI4P binding to Osh prevents sterol loading at the plasma membrane, which exhibits high PI4P levels (Stefan et al., 2013). In this context, a further interaction of Osh with the PIP phosphatase Sac1 could act as a reciprocal regulation circuit facilitating the extraction of sterols from the plasma membrane by reduction of PI4P levels (Stefan et al., 2011).

ORPs (like VAPs) are conserved in higher eukaryotes (Ngo et al., 2010) and a role for mammalian ORPs in the trafficking of sterols from the plasma membrane to the ER and lipid droplets has been described recently (Jansen et al., 2011). Thus, comparable regulation mechanisms may exist in higher eukaryotes.

Three protein families have been recently identified to physically link the ER with the plasma membrane at contact sites in yeast: tricalbins, VAPs, and Ist2 (related to mammalian TMEM16 ion channels) (Manford et al., 2012). Knockout of all tethering proteins not only disrupted PIP signaling but also caused a constitutive activation of the ER unfolded protein response (UPR). Recently, the three mammalian tricalbin homologs, the extended synaptotagmins E-Syt1-3 have been functionally characterized (**Figure 2A**). All three were shown to tether the ER to the plasma membrane by binding to $PI(4,5)P_2$ emphasizing the importance of ER/PM contact sites across species (Giordano et al., 2013; Fernández-Busnadiego et al., 2015). Thus, ER/PM contact sites appear to be required for maintenance of ER physiology, which imply that they are integrated into signaling pathways which cope with the general regulation of cellular homeostasis.

Remarkably, in addition to the ER/plasma membrane contact sites described above, mitochondria are also frequently observed in several cell types in proximity to the cellular surface; e.g., in HeLA cells up to 10% of mitochondria are found beneath the plasma membrane (Frieden et al., 2005). In contrast to the ER, however, mitochondria do not seem to be frequently directly connected to the plasma membrane but appear to be linked via discrete ER-cisternae (Csordás et al., 2010) or filamentous adherence plaques associated to additional vesicular structures along neuronal synapses (Spirou et al., 1998; Rowland et al., 2000). Functionally, these structures may distribute calcium waves from the extracellular space to these calcium storing organelles. In this respect, neuronal mitochondria show specific vulnerability to the elevated excitatory influx of Ca^{2+}, which can eventually impair mitochondrial functions (Connolly and Prehn, 2015). Mitochondria from individuals with mutations in the Surfeit locus protein 1 (Surf1) gene show only partially assembled cytochrome C oxidase complexes (3rd complex of the electron transport chain) resulting in the lethal Leigh syndrome in humans (Zhu et al., 1998). Remarkably, neurons of Surf1 KO mice, which show no Leigh-like phenotype, are refractory against glutamate induced Ca^{2+} stress and exhibit an increased life span and enhanced cognitive abilities (Dell'agnello et al., 2007; Lin et al., 2013). Interestingly, the lack of Surf1 leads to decreased Ca^{2+} influx into mitochondria in response to glutamate-induced excitotoxicity. The authors speculated that the reduced buffering capacity of Surf1 KO mitochondria could determine the saturation of the Ca^{2+} microdomains in the contact sites between mitochondria and the plasma membrane or the ER, thereby promoting the feedback closure of their Ca^{2+} channels (Dell'agnello et al., 2007). Thus, distinct molecular changes in the regulatory organelle framework underneath the neuronal plasma membrane appear to have a direct impact on general neuronal physiology and survival demonstrating the functional significance of organelle contact sites.

The Mitochondria-associated Membrane of the ER (MAM)

The increasing application of the transmission electron microscope in the field of cell biology during the 1960s and 1970s already revealed that mitochondria and the ER are often found in close proximity to each other (Copeland and Dalton, 1959; Ruby et al., 1969; Franke and Kartenbeck, 1971; Morré et al., 1971). Co-sedimentation experiments using density gradients further

implied that both organelles are indeed physically associated (Pickett et al., 1980; Montisano et al., 1982). In 1990, however, J. Vance discovered that the microsomes co-sedimenting with mitochondria represent a specialized cellular subcompartment and proposed the name "mitochondria associated membrane of the endoplasmic reticulum" (MAM) (Vance, 1990) (**Figure 1**). In the decades after this ground-breaking discovery our understanding of the functional significance of the MAM has greatly advanced revealing that this special ER compartment communicates with mitochondria in order to fulfill a plethora of functions associated with, among others, lipid metabolism and Ca^{2+} signaling but also the regulation of mitochondrial maintenance and programmed cell death/cell survival reflecting different levels of complexity (Raturi and Simmen, 2013; Vance, 2014; van Vliet et al., 2014). As these diverse functions imply, it is still not clear if there is one single MAM compartment or if there are several MAMs equipped with a specialized sub-proteome in order to fulfill different functions. The protein assembly found at the MAM is a subset of *bona fide* ER proteins, which are, however, enriched if compared to classic smooth or rough ER fractions. Thus, the enzymatic activities found at the MAM can be also found at other ER sites, but seem to be focused at this specific location. To date only one protein has been described as specific to the MAM—the phosphatidylethanolamine-N-methyltransferase-2 (Cui et al., 1993), which appears to be only expressed in liver (Cui et al., 1995). Proteomic approaches to define the MAM in different tissues led to the identification of approximately 1000 proteins each (Poston et al., 2013; Horner et al., 2015). However, the overlap between proteins identified in different tissues using different approaches is far lower. A significant number of these identifications may arise from contaminating microsomes and mitochondria, which cannot be entirely separated from the MAM fraction. Thus, to define a specific MAM proteome, sophisticated isolation strategies combined with quantitative mass spectrometry approaches are required in the future. Nevertheless, to date a considerable number of proteins is generally accepted to be significantly enriched in the MAM and can be used as marker proteins for this subcompartment (Vance, 2014; van Vliet et al., 2014). Since the MAM is continuous with the remaining ER it is also pertinent to discuss the mechanisms which lead to enrichment of specific proteins in this membrane subcompartment. Commonly, conserved amino acid stretches target specific proteins to their designated compartment, a mechanism which was reported for the MAM-enriched transmembrane protein acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) (Stone et al., 2009). Corresponding targeting sequences have not yet been confirmed for other MAM proteins but cysteine palmitoylation was recently reported to be required for the sorting of the two MAM-enriched membrane proteins of the thioredoxin family, TMX and calnexin (Lynes et al., 2012). However, there is currently no consensus on a *bona fide* sorting signal for the different types of MAM protein constituents. Other targeting information as well as the lipid membrane environment may ensure that individual proteins are retained in the MAM or even enriched in specific raft-like subdomains of the compartment.

A closer look at the group of enriched proteins, which include, amongst others, long-chain acyl-CoA synthetase-4, phosphatidylserine synthase-1 and -2, mitofusin 2 (MFN2), dynamin-related protein 1 (DRP1), calnexin, autophagy-related protein 14 (ATG14), the oxidoreductase Ero1α and IP3R, reveals that the MAM is a multifunctional compartment which is involved in several metabolic but also regulatory pathways of the cell. In this respect, the MAM is currently supposed to be involved in the processes of (1) phospholipid synthesis and transfer, (2) calcium signaling, (3) mitochondrial fission, (4) mitophagy, (5) ER-stress response, (6) regulation of apoptosis, and (7) inflammatory/antiviral responses (**Figure 1**), which will be described in more detail in the following paragraph.

Historically, the first function associated with the MAM was its contribution to lipid metabolism (Vance, 1990). The production of phospholipids in order to supply the remaining endomembrane system of the cell is a well-known task of the ER. After synthesis, the phospholipids can be transferred to their destinations by vesicle-mediated transport. However, not all subcellular compartments—e.g., mitochondria and peroxisomes—are supposed to receive phospholipids via such a process, but may rely on a direct transfer between juxtaposed membranes (Voelker, 2009; Prinz, 2010; Schlattner et al., 2014). Mitochondrial membranes are characterized by a high content of phosphatidylethanolamine (PE). PE can be synthesized at the site of the inner mitochondrial membrane from phosphatidylserine (PS) via the PS decarboxylation pathway (Shiao et al., 1995; Birner et al., 2001). PS, however, is synthesized and supplied by the ER (Vance, 1991). Consequently, the MAM, as a site of close apposition between the ER and mitochondria, was found to be strongly enriched in the two PS synthases 1 and 2 (Stone and Vance, 2000). There is strong evidence that PS synthesized at the MAM is subsequently channeled to the mitochondrial inner membrane for further processing into PE, which can be subsequently exported back to the ER or to other subcellular compartments (Vance, 2014). Besides PE, other mitochondrial membrane lipids like phosphatidylcholine or cardiolipin are at least partially supplied in the form of precursor molecules to mitochondria by the ER (Vance, 2014). Since a significant number of lipid-metabolizing enzymes are enriched at the MAM, it is likely that further lipids are transferred between mitochondria and this specialized ER subcompartment (Raturi and Simmen, 2013).

Both, the ER and mitochondria are important intracellular calcium stores and cyclical calcium exchange between both organelles is crucial for cell life and death (Raturi and Simmen, 2013; Marchi et al., 2014). However, Ca^{2+} concentrations of approximately 1 mM inside the ER (de la Fuente et al., 2013) by far exceed those in mitochondria, which are highly dynamic and react to even small Ca^{2+} changes in the cytosol (Giacomello et al., 2007). Calcium ions in mitochondria are required to regulate mitochondrial energy homeostasis by activating the rate limiting enzymes of the Krebs cycle. Moreover, Ca^{2+} is involved in the regulation of mitochondrial motility and apoptosis (Giacomello et al., 2007; Rowland and Voeltz, 2012). Ca^{2+} uptake by mitochondria is electrochemically driven by the electron potential across the inner mitochondrial membrane and

facilitated by the mitochondrial low affinity calcium uniporter MCU (Baughman et al., 2011; De Stefani et al., 2011; Chaudhuri et al., 2013). To still allow rapid and highly dynamic Ca^{2+} changes in mitochondria, the close proximity between the MAM and mitochondria creates locally elevated cytosolic Ca^{2+} concentrations (Rizzuto et al., 1998). For this reason the MAM is highly enriched in inositol-1,4,5-tris-phosphate sensitive Ca^{2+} channels (IP3R) which release calcium into the local surrounding cytosol in response to IP3 signaling (Rizzuto et al., 1993; Hayashi et al., 2009) (**Figure 2A**). Indeed, changes in the distance between the MAM and mitochondrial membranes lead to alterations in the efficiency of Ca^{2+} transfer (Csordás et al., 2010). Moreover, IP3R activity is inhibited by low and very high cytosolic Ca^{2+} concentrations in an autoregulative system (Bezprozvanny et al., 1991). Creating a reciprocal cycle, Ca^{2+} ions can also be released from mitochondria via the Na^{+}/Ca^{2+} exchanger NCLX (Palty et al., 2010) and taken up by the calcium pumps of the SERCA family, which may also be concentrated at the MAM (Lynes et al., 2012). To interfere with mitochondrial energy homeostasis, MAM Ca^{2+} release is reciprocally coupled to cytosolic ATP concentration. Specifically, IP3R3 channel activity has been shown to closely depend on free ATP concentrations in the surrounding cytosol (Mak et al., 1999). In addition, IP3R activity is further modulated by numerous control systems. For example, phosphorylation by cAMP-dependent protein kinase (PKA) promotes IP3R1 activity, whereas protein phosphatases 1 and 2A have an inhibitory effect (DeSouza et al., 2002). Moreover, IP3R activity is modulated by further regulatory proteins like the sigma-1 receptor, promyelocytic leukemia (PML) tumor suppressor protein or GRP75/VDAC1 (see Raturi and Simmen, 2013; Marchi et al., 2014 for details) (**Figure 2A**).

A deregulation of cellular calcium signaling is supposed to be involved in the development of insulin resistance in type 2 diabetes (Guerrero-Hernandez and Verkhratsky, 2014). IP3R calcium release activities were reported to be influenced by an interaction with the GRP75/VDAC1 complex at the MAM (Szabadkai et al., 2006). Interestingly, disruption of MAM integrity and correspondent VDAC1/IP3R1 and Grp75/IP3R1 interactions are associated with altered insulin signaling in mouse and human primary hepatocytes (Tubbs et al., 2014). Likewise, the authors observed that ER—mitochondria contact sites are decreased in established diabetic mouse models. An induction of ER-mitochondria contact sites by pharmacologic treatment or overexpression of the mitochondrial MAM protein cyclophilin D, however, partially restored insulin sensitivity in the mice. Thus, the MAMs role in Ca^{2+}-mediated organelle communication does not appear to be restricted to a direct regulation of mitochondrial physiology but may represent a signaling hub, which interferes with higher level networks controlling cellular energy homeostasis.

Considering its role in the regulation of energy homeostasis, it is not surprising that the MAM is also involved in the complex signaling network controlling cell fate decision. Generally, the ER responds to cellular stress, paralleled by accumulation of unfolded protein, with a signal transduction mechanism called "unfolded protein response" (UPR). In this process the ER stops protein translation and activates chaperones assisting protein folding (Schröder and Kaufman, 2005). Chaperone-mediated protein folding is a massively energy demanding process. To maximize ATP production under ER-stress, cells exhibit an increasing number of ER/mitochondrial contact sites leading to elevated mitochondrial Ca^{2+} concentrations and thus higher oxidative ADP-phosphorylation rates (Bravo et al., 2012). However, if the UPR is not able to reduce cellular stress, lethal signaling pathways will be activated finally triggering apoptosis. In such a situation truncated isoforms of SERCA1 localizing to the MAM were reported to be upregulated. This process leads to an increase in ER/mitochondria contact sites, elevated Ca^{2+} leakage and inhibition of mitochondrial movement, thereby causing mitochondrial Ca^{2+} overload which triggers apoptosis (Chami et al., 2008).

Mitochondria are not a static cellular compartment but constantly change their morphology by fusion and fission. Fission of mitochondria is mediated by cytosolic Drp1 which is recruited to mitochondrial constriction sites by several membrane proteins like Fis1, MiD49/MiD51, or Mff (Lee and Yoon, 2014) (see Section Interplay between Peroxisomes and Mitochondria). ER tubules have been found to wrap around mitochondria marking sites of fission by inducing actin assembly at these ER-mitochondria contacts (Friedman et al., 2011; Korobova et al., 2013). These events seem to precede Drp1-induced fission by preformation of a constriction site to which Drp1 is subsequently recruited (Friedman et al., 2011). Indeed, Drp1 was found to colocalize in significant amounts with these ER/mitochondria contacts (Friedman et al., 2011). In yeast the "ER mitochondria encounter structure" (ERMES), a multiprotein complex, has been described as a tethering structure participating, amongst other functions, in lipid transfer and mitochondrial fission (Murley et al., 2013) (**Figure 2C**). With the conserved "ER membrane protein complex" (EMC), a second tethering complex involved in lipid transport has been described in yeast recently (Lahiri et al., 2014). In higher eukaryotes a direct ERMES homolog has not been identified, whereas candidates for EMC homologs exist but are yet not functionally characterized. Very recently, syntaxin17 was reported to reside at ER/mitochondria contacts and to promote mitochondrial fission by participating in Drp1 assembly at the mitochondrial constriction site in mammalian cells (Arasaki et al., 2015). Mitochondrial fusion is mediated by the dynamin-related proteins Mfn1/2 or Opa1 found at the outer and inner mitochondrial membrane, respectively (Lee and Yoon, 2014). Mfn2 is also a *bona fide* constituent of the MAM, generally supposed to physically tether ER/mitochondria contact sites by interacting with Mfn2 or Mfn1 of the outer mitochondrial membrane (**Figure 2A**). However, this view has recently been questioned (Cosson et al., 2012; Filadi et al., 2015). In contrast to the general view, the authors come to the conclusion that Mfn2 acts as a negative regulator of organelle apposition (Filadi et al., 2015). In addition to Mfn2, VAPB of the MAM has been recently described to interact with the outer mitochondrial membrane protein PTPIP51 representing an additional physical linker pair between both organelles (Stoica et al., 2014) (**Figure 2A**). However, the tethering function of Mfn2 at the contact sites between ER and mitochondria appears

to be independent from its role in mitochondrial fusion events. Thus, whilst there is already considerable knowledge on the architecture of the MAM, its role in regulation of mitochondrial fission and fusion remains fragmentary. Nevertheless, there is clear evidence that the mitochondrial dynamics are crucial for the regulation of metabolic homeostasis and cell survival (Ni et al., 2015). Mitochondrial elongation rescues mitochondria from autophagy whereas damaged mitochondria appear to lose their fusion capacity preventing their incorporation into the healthy mitochondrial network (Twig et al., 2008). Besides its role as a mediator of mitochondrial fission, the MAM also seems to be more directly involved in the process of mitophagy. There is increasing evidence that the ER supplies membrane material for the formation of autophagosomes (Tooze and Yoshimori, 2010). Interestingly, the pre-autophagosomal protein Atg14 relocalizes from a homogenous ER distribution to the MAM during autophagy-inducing starvation conditions (Hamasaki et al., 2013). Likewise after starvation Atg5 accumulated at ER/mitochondria contact sites. In contrast, disruption of ER/mitochondrial contacts by Mfn2 or PACS2 knockdown attenuated the formation of autophagosomes, implying a role for the MAM in autophagosome formation (**Figure 2B**). Thus, the MAM may act as a direct linker between phagosome formation and mitochondrial fragmentation, thereby regulating mitochondrial homeostasis. As described above the interaction of the MAM and mitochondria on different mechanistic levels is involved in determining cell survival or death, significantly contributing to the regulation of apoptosis. The communication systems involved in apoptosis described so far predominantly transmit signals from the MAM to mitochondria. A sophisticated regulation system, however, involves feedback loops between communicating cellular entities. In this respect, mitochondrial Fis1 was recently reported to interact with ER Bap31 in order to recruit procaspase-8 to the MAM facilitating its activation into caspase-8 (Iwasawa et al., 2011) (**Figure 2A**). Subsequently, Ca^{2+} emission from the MAM further elevates mitochondrial Ca^{2+} concentration stimulating the induction of apoptosis.

Further signaling networks, in which mitochondria and the ER were reported to cooperate at the MAM are involved in the activation of the antiviral innate immune response (Marchi et al., 2014; van Vliet et al., 2014). Cytosolic pathogen recognition receptors RIG-I are able to detect cytosolic foreign RNA and subsequently induce the production of type I interferons and proinflammatory cytokines (Sumpter et al., 2005). To this end RIG-I receptors assemble in a multiprotein complex by docking to mitochondria antiviral signaling protein (MAVS)—an adaptor protein located at the outer membrane of mitochondria and peroxisomes (Belgnaoui et al., 2011) (see Section Interplay between Peroxisomes and Mitochondria). In a recent publication the MAVS were shown to reside on the MAM, where they appear in close proximity to peroxisomal and mitochondrial MAVS during viral infection (Horner et al., 2011). This organelle connecting assembly was suggested to act as signaling hub for the regulation of mitochondrial and peroxisomal innate immune responses after viral infection (Horner et al., 2011). In a subsequent publication the authors further reported that the MAM proteome dynamically changes after virus infection in particular increasing the amounts of individual MAVS interacting proteins (Horner et al., 2015). Evaluating their findings, the authors speculated that the MAM may be used to coordinate mitochondrial and peroxisomal metabolism according to the requirements during virus infection.

The Nod-like receptor NLRP3-inflammosome is a large multiprotein complex serving as a platform mediating the activation of interleukins IL1β and IL18 and contributing to innate immunity (Schroder and Tschopp, 2010; Gurung et al., 2015). To this end the NLRP3 senses pathogen- and danger-associated molecular patterns which activate the assembly of the inflammosome. In this respect, signals for mitochondrial dysfunction like ROS or elevated Ca^{2+} efflux stimulate inflammosome assembly (Gurung et al., 2015). Inactive NLRP3 was reported to localize to the ER, but upon inflammosome activation redistributes to ER-mitochondrial clusters comprising MAM sites (Zhou et al., 2011). These events occur in response to elevated mitochondrial ROS production after inhibition of mitochondrial autophagy. Interestingly, knockdown of VDAC1, which promotes mitochondrial Ca^{2+} uptake at the MAM, thus elevating mitochondrial ATP production, significantly reduced inflammosome activation. In this respect, inflammosome formation at the MAM may be a reaction to elevated ROS production during mitochondrial ATP production.

The intriguing diversity of functions associated with the MAM described above vividly illustrates how the cell connects the metabolic control of cellular functions to control circuits of higher order and complexity which finally contribute to the decision of cellular survival and death. In this respect, the findings that the MAM cooperatively interconnects peroxisomal and mitochondrial MAVS signaling (Horner et al., 2011) (see Section Interplay between Peroxisomes and Mitochondria) further directs our view on organellar cooperation to cross-compartment signaling networks which may integrate cellular homeostasis and dysfunction in different locations of the cell.

The Peroxisome-ER Connection

The intricate relationship between the ER and peroxisomes (**Figure 1**) includes cooperation in various metabolic pathways, for example the biosynthesis of ether-phospholipids (e.g., myelin sheath lipids), which starts in peroxisomes and is completed in the ER, the formation of GPI-anchored proteins in the ER, and the production of polyunsaturated fatty acids (e.g., docosahexaenoic acid) (for a detailed review see Schrader et al., 2013). It is now clear that the ER also has a role to play in the generation of peroxisomes as well as regulation of their function. Study of this relationship began with ultrastructural studies in the 1960's which demonstrated a close proximity between the smooth ER and peroxisomes (Novikoff and Shin, 1964; Novikoff and Novikoff, 1972; Reddy and Svoboda, 1972, 1973). These early images show peroxisomes entwined and engulfed by the tubules of the ER, suggesting an intimate, physical interaction (which may not even leave sufficient space

for vesicle-based interaction). Indeed, in those TEM images both organelles appear to be interconnected by electron-dense intermembrane cross-bridges, spanning a distance between 10 and 15 nm (Kartenbeck and Franke, 1974; Zaar et al., 1987), which resemble the ultrastuctural appearance of known organellar contact sites, like the association between MAM and the outer mitochondrial membrane. Importantly, the electron-dense cross-bridges and attached ER tubules could even be visualized, and biochemically verified, in isolated peroxisome fractions (Zaar et al., 1987). Despite this clear and long-held evidence for a specialized ER-peroxisome contact site, its protein composition and physiological function remain obscure but may be broadly associated to two cellular processes: (1) the biogenesis of peroxisomes as derivatives from the ER or (2) the exchange of metabolites from shared biochemical pathways, for example the ether phospholipid biosynthesis.

Confidence in the level of this intimacy, with regards to the ER as the site of peroxisome production has fluctuated over the years. Over 40 years ago Christian De Duve suggested that it was "almost textbook knowledge" that peroxisomes were derived from the ER and that peroxisomal proteins were delivered intraluminally via ER channels (De Duve, 1973). This view was, however, subsequently replaced by the growth and division model of peroxisome biogenesis established by Fujiki and Lazarow (Fujiki and Lazarow, 1985). This model proposed that, although the phospholipids required to form the peroxisome membrane could be provided by the ER, peroxisomal proteins were synthesized on cytoplasmic ribosomes and delivered directly to peroxisomes. There is general agreement that this applies to peroxisomal matrix proteins, whereas delivery of peroxisomal membrane proteins (PMPs) became a matter of ongoing debate.

Over the years a wide variety of evidence has been presented in support of both models and there has been considerable debate as to which mechanism predominates in wild type cells (Hoepfner et al., 2005; Kim et al., 2006; Motley and Hettema, 2007; Nagotu et al., 2008; Delille et al., 2010; Rucktäschel et al., 2010; Van der Zand and Reggiori, 2012). Much of the debate has stemmed from the observation that cells lacking, or carrying mutations in, the peroxisome biogenesis factor Pex3 do not contain peroxisomes (Baerends et al., 1996; Muntau et al., 2000). Pex3 is a membrane protein which, along with its cytoplasmic partner Pex19, forms an import complex required for insertion of peroxisomal membrane proteins (Götte et al., 1998; Rottensteiner et al., 2004). When Pex3 is re-introduced into Pex3 deficient cells, the protein was observed to route first to the ER and then be released in pre-peroxisomal vesicle structures, which were then supplied with PMPs from the ER (Van der Zand et al., 2010; Van der Zand and Reggiori, 2012). This concept was questioned by a recent ultrastructural study which demonstrated that such pre-peroxisomal structures are already present in cells lacking Pex3 (Knoops et al., 2014). The authors suggested that the ER-localization of re-introduced Pex3, and other proteins, could be due to limitations in the resolution of fluorescence microscopy.

Further data in support of a model in which PMPs transit via the ER comes from studies investigating co-translation insertion at the ER membrane. An early study in yeast suggested that PMP50 was synthesized on ER-associated ribosomes (Bodnar and Rachubinski, 1991). This was supported by a more recent global study which investigated the extent of co-translational delivery of proteins to the ER and found a clear enrichment of genes coding for PMPs at ER-anchored ribosomes in yeast and, to a lesser extent, in mammals (Jan et al., 2014). Jan et al. interpreted this finding to show that PMPs are translated at the ER membrane and are presumably inserted into the ER before being delivered to peroxisomes. An exception to this are tail-anchored membrane proteins which are translated on cytoplasmic ribosomes before being delivered to the appropriate organelle (Borgese and Fasana, 2011) and appear to be targeted by species-specific systems. Accordingly yeast peroxisomal tail-anchored proteins go either direct, or via the ER using the "Guided Entry of Tail-anchored Proteins" (GET) system (Mariappan et al., 2010) but mammalian tail-anchored proteins are delivered directly to peroxisomes (Chen et al., 2014; Kim and Hettema, 2015).

Overall there are still some aspects of peroxisome biogenesis which require clarification but the most recent data supports a growth and division model with a role for the ER (dependent on conditions and species) in delivery of phospholipids and some specific PMPs, such as Pex3.

Having established that at least a portion of PMPs can be delivered by the ER another unresolved issue is the mechanism of transport of such proteins, as well as the essential phospholipids required for the peroxisomal membrane. Vesicular transport of PMPs has been demonstrated in an *in vitro* cell-free system (Agrawal et al., 2011) and may involve the Sec16B protein in mammalian cells (Yonekawa et al., 2011), whilst non-vesicular mechanisms have also been reported to exist (Lam et al., 2010). Removal of Sec16B in mammalian cells results in peroxisome elongation, disruption of ER exit sites and redistribution of Pex16 from peroxisomes to the ER (Yonekawa et al., 2011). Based on these observations Yonekawa and colleagues speculated that Sec16B is involved in forming Pex16-containing vesicles in a peroxisome-like domain of the ER. A recent report also highlighted the potential importance of Pex16 in ER-peroxisomal trafficking (Hua et al., 2015). However, the validity and scope of such a mechanism, and the precise role for Sec16B in this process remains unclear.

Although it is generally accepted that the phospholipids generating peroxisomal membranes come from the ER there are relatively few studies on this process. One such study in yeast, supporting a non-vesicular mechanism, used an engineered strain in which the PTS1 enzyme responsible for the decarboxylation of phosphatidylserine (PS) to phosphatidylethanolamine (PE) was artificially targeted to peroxisomes (Raychaudhuri and Prinz, 2008). In a strain where the endogenous PTS1 genes were removed this allowed monitoring of lipid transfer by measuring the conversion of radiolabelled PS (generated exclusively in the ER) to PE which could now only occur in peroxisomes in this system. The authors found that PS transfer to peroxisomes occurred under normal conditions and also under conditions where vesicular transport was compromised.

Despite a wealth of evidence suggesting a direct, physical interaction between peroxisomes and the ER, understanding of the molecular basis of such contacts is limited. So far there are only a small number of studies reporting a physical tether between the ER and peroxisomes analogous to the complexes which anchor the ER to other organelles such as mitochondria, the PM or lysosomes [see Sections Connections between the ER and the Plasma Membrane, The Mitochondria-associated Membrane of the ER (MAM), Interplay between Peroxisomes and Mitochondria, and Lysosomal Interactions and Autophagy for details and (Prinz, 2014) for a comprehensive review on membrane contact sites]. However, by comparison with other ER-anchoring systems it is likely that there are several tethers connecting the ER to peroxisomes (Stefan et al., 2013). So far in yeast two potential tethering complexes have recently been identified. A complex involving Pex30 has been implicated as a facilitator between peroxisomes and the ER along with a tether involving Pex3 and Inp1 (David et al., 2013; Knoblach et al., 2013). The Pex30 anchoring complex is involved in the regulation of peroxisome proliferation and requires the integrity of the ER tubular network. Through interaction between, among others, Pex30 and the ER proteins, Rtn1, Rtn2, and Yop1 an "ER-peroxisome contact site" (or EPCON) is generated to facilitate ER-peroxisome interactions (David et al., 2013). However, a detailed interaction map of this macromolecular complex bridging both organelles remains to be specified. The authors speculate that these EPCONs could represent a platform from which peroxisomes could be formed. The Pex3-Inp1 tethering system is based on Pex3 being resident in both the ER and peroxisomal membrane and Inp1 acting as a molecular hinge interacting directly with both Pex3 proteins. This tether reportedly functions to regulate the maintenance of peroxisome numbers during budding (Knoblach et al., 2013). Knoblach and colleagues postulate that this occurs by the anchoring, via Inp1 and Pex3, of peroxisomes to the cortical ER prior to division. When division is signaled the peroxisomal division machinery assembles (see Section Interplay between Peroxisomes and Mitochondria) leading to a pulling force which elongates the peroxisome, eventually leading to fission. The newly-formed peroxisomal structure can then move from the mother cell and into the bud. There is no homolog of Inp1 in metazoa so the relevance of a similar tether in other systems is unclear and may be specific to budding yeast.

As initially indicated, ER-peroxisome contacts are extensively observed in mammalian cells and likely represent functionally specialized contact sites comparable to the MAM or PAM described above. However, it remains to be determined if these numerous appositions between both organelles predominantly mirror the process of peroxisome biogenesis or if they mainly contribute to several other cellular processes including exchange of metabolites, such as precursors of ether phospholipids, polyunsaturated fatty acids and cholesterol or even regulation of viral defence (see Section Interplay between Peroxisomes and Mitochondria). Thus, their contribution to peroxisome biogenesis is just one aspect of their multiple functions and it will be challenging to unravel their actual function in different experimental models and set ups.

Interplay between Peroxisomes and Mitochondria

In recent years, convincing evidence for a close connection between peroxisomes and mitochondria has been obtained (Schrader and Yoon, 2007; Camões et al., 2009; Delille et al., 2009; Schrader et al., 2012, 2015) (**Figure 1**). Peroxisomes and mitochondria cooperate in cellular lipid metabolism, in particular the breakdown of fatty acids via their organelle-specific β-oxidation pathways and can both act as subcellular source, sink or target of ROS (Schrader and Fahimi, 2006; Wanders and Waterham, 2006; Antonenkov et al., 2010; Ivashchenko et al., 2011; Fransen et al., 2013). Although peroxisomes and mitochondria can be observed in close proximity, e.g., in ultrastructural studies in mammalian cells and can also be co-purified at distinct buoyant densities (Hicks and Fahimi, 1977; Islinger et al., 2006), studies on the molecular background of physical interactions and their physiological importance are scarce (Horner et al., 2011, 2015; Van Bergeijk et al., 2015). Recent studies in yeast localized peroxisomes to specific mitochondrial subdomains such as mitochondria-ER junctions and sites of acetyl-CoA synthesis (Cohen et al., 2014). In line with this, a genome-wide localization study of peroxisome-mitochondria interactions in yeast identified Pex11, a membrane-bound peroxin (peroxisome biogenesis factor) involved in peroxisome division and proliferation, and the mitochondrial ERMES complex (Mattiazzi Ušaj et al., 2015) (**Figure 2C**). The ERMES complex is supposed to provide a tether and to facilitate the exchange of molecules between the ER and mitochondria. In particular, Pex11 was found to physically interact with Mdm34 to establish the contact sites between peroxisomes and mitochondria (**Figure 2C**). Interestingly, this interaction was only observed in glucose media, but not after induction of peroxisome proliferation by fatty acids in the absence of glucose. The authors speculate that besides its role in elongation and fission of the peroxisomal membrane, Pex11 may also be a sensor of the metabolic state of peroxisomes. Thus, metabolic stimuli may modulate the peroxisome-mitochondrion tether in yeast. Tethering of both organelles may enhance metabolism by reducing the distance for efficient transport of metabolites from one organelle to another. Mammalian cells lack ERMES, and another tethering complex is supposed to perform similar functions in higher eukaryotes.

Tethering might also play a role in the coordinated movement of both organelles, in particular for organelle inheritance. Whereas in budding yeast distinct organelle-specific membrane proteins are involved in the actin-myosin dependent inheritance of peroxisomes and mitochondria (Knoblach and Rachubinski, 2015), in the fission yeast *Schizosaccharomyces pombe* peroxisome movement in association with mitochondria has been reported (Jourdain et al., 2008). Another example is the red algae *Cyanidioschyzon merolae*, which possesses

only one peroxisome and one mitochondrion. During coordinated organelle-division the peroxisome interacts with the mitochondrion to partition into the daughter cell (Miyagishima et al., 1999). Note that in budding yeast tethering of peroxisomes and mitochondria to the ER is crucial for organelle retention and inheritance (see Section The Peroxisome-ER connection).

Another interesting twist of the peroxisome-mitochondria connection is the discovery that peroxisomes and mitochondria share key proteins of their division machinery (Schrader et al., 2012), namely the dynamin-related GTPase Drp1/DLP1/(Koch et al., 2003; Li and Gould, 2003), its membrane adaptor proteins Fis1 and Mff (Koch et al., 2005; Gandre-Babbe and Van Der Bliek, 2008; Otera et al., 2010; Koch and Brocard, 2012; Itoyama et al., 2013) as well as GDAP1, a putative GST-transferase (Huber et al., 2013) in mammals. Fis1 and Mff are supposed to recruit the mechanochemical enzyme Drp1 to distinct spot-like division sites at the organelle membrane prior to fission. Sharing of key division components is conserved in mammals, fungi, yeast, and plants (Delille et al., 2009; Schrader et al., 2012), The first patients with defects in different division proteins (e.g., Drp1, Mff, Pex11β) and thus, an abnormal elongated organelle morphology, have been identified underlining the biomedical importance of membrane deformation and fission (Waterham et al., 2007; Ebberink et al., 2011; Ribeiro et al., 2012; Shamseldin et al., 2012; Schrader et al., 2014). Unraveling how a cell is able to timely coordinate the distribution of shared components of the mitochondrial and peroxisomal division machinery in order to meet the requirements of increased organelle-specific proliferation will be a challenging task for future research activities and may involve hitherto undetected networks of organelle cross-talk.

In addition to the key division proteins, the division of mitochondria involves ER-mitochondria contacts (Friedman et al., 2011), and actin filaments (Li et al., 2015). ER tubules were observed to wrap around mitochondria in yeast and mammalian cells, to mark fission sites and to drive mitochondrial constriction (Friedman et al., 2011; Korobova et al., 2013). It is unknown if peroxisomal membrane fission is also ER-assisted. Recent *in vitro* studies using liposomes and recombinant Pex11β imply that membrane constriction may occur unassisted by ER (Yoshida et al., 2015).

The constitutive formation of organelles also requires degradation of faulty or surplus organelles. This is achieved by autophagic processes (pexophagy, mitophagy). The size of the organelle is a critical factor for the efficient engulfment by the sequestering compartment, the phagophore. Organelle fission is critical for the efficient elimination of mitochondria (Gomes and Scorrano, 2013) and peroxisomes (Mao et al., 2014). In *S. cerevisiae*, it was reported that pexophagy-specific fission, mediated either by the dynamin-like GTPases Dnm1 or Vps1, occurred at mitochondria-peroxisome contact sites. The authors suggest that whereas division of mitochondria requires the participation of the ER, the fission of yeast peroxisomes may involve mitochondria (Mao et al., 2014). It should be noted that as both organelles are in intimate contact with the ER (see Section The Peroxisome-ER Connection), potential peroxisome-mitochondria contacts might be indirect and mediated by ER membranes.

Mitochondria, and increasingly also peroxisomes, are now recognized as important signaling nodes in the cell and cooperative functions in anti-viral and redox signaling are emerging (Dixit et al., 2010; Fransen et al., 2013; Odendall and Kagan, 2013; Nordgren and Fransen, 2014). With the discovery of the dual distribution of mitochondrial antiviral signaling protein (MAVS) to both peroxisomes and mitochondria, a novel role for peroxisomes in the innate immune response of the host cell to combat viral and bacterial infections, either alone or in cooperation with mitochondria, was revealed (Dixit et al., 2010; Odendall et al., 2014). MAVS functions as an adaptor protein for retinoic acid-inducible gene 1 protein (RIG-I) and transmits downstream signaling of antiviral immunity. Interestingly, MAVS localizes to mitochondria-associated ER membranes (MAM) and dynamic MAM tethering to mitochondria and peroxisomes is supposed to coordinate MAVS localization to form a signaling synapse between membranes. It could regulate the interaction between positive and negative regulators distributed on different organelles in order to fine-tune the RIG-1 induced innate immune response (Horner et al., 2011) (**Figure 1**). Proteomic analysis of MAM during RNA virus infection revealed an increased presence of peroxisomal proteins if compared to control cells, supporting physical interactions between peroxisomes and mitochondria (or MAM) during anti-viral response (Horner et al., 2015).

It is becoming increasingly evident that peroxisomes and mitochondria also share an intricate redox-sensitive relationship. Both organelles are crucial for cellular redox homeostasis (Nordgren and Fransen, 2014). Interestingly, disturbances in peroxisomal lipid and ROS metabolism have an impact on the mitochondrial redox balance (Koepke et al., 2008; Ivashchenko et al., 2011; Walton and Pizzitelli, 2012). It is hypothesized that such peroxisomal disturbances can trigger redox-related signaling events that ultimately result in increased mitochondrial stress and the activation of mitochondrial stress pathways (Titorenko and Terlecky, 2011; Beach et al., 2012; Fransen et al., 2013). It is, however, unknown, how those signals are transmitted between peroxisomes and mitochondria. Interorganellar communication may involve diffusion of signaling molecules from one organelle to another, communication via membrane contact sites or vesicular transport. With respect to direct membrane contact, it is tempting to speculate that the MAM may contribute to the transmission of ROS and stress responses from peroxisomes to mitochondria. It should be noted, that loss of peroxisomal biogenesis and metabolism, a hallmark of Zellweger syndrome, is associated with impaired mitochondrial integrity. Recent studies in Zellweger-mouse models revealed impaired mitochondrial respiration, DNA depletion, PGC-1α independent proliferation of mitochondria and perturbed carbohydrate metabolism in peroxisome-deficient hepatocytes (Peeters et al., 2011, 2015). These findings suggest an impact on organelle interplay in Zellweger spectrum patients. Concerning vesicular transport, mitochondria have been reported to generate so called mitochondria-derived vesicles (MDVs) that can transport specific mitochondrial proteins to either

peroxisomes or to lysosomes for degradation (Neuspiel et al., 2008; Soubannier et al., 2012). The physiological role for peroxisome-directed MDVs is currently unclear. Peroxisomes may also be able to generate and target vesicles to mitochondria, but experimental evidence for this phenomenon is missing. Finally, live cell imaging of peroxisomes in mammals and plants revealed that peroxisomes can form tubular membrane protrusions, which vividly extend and retract, and are thought to mediate interactions with other peroxisomes and organelles. Very recently, peroxisomal membrane extensions were reported to mediate contact with oil bodies (see Section Lipid Droplets) in the model plant *Arabidopsis thaliana* and to deliver a membrane-bound lipase, required for lipid mobilization during seedling establishment (Thazar-Poulot et al., 2015). Membrane protrusions may also be involved in the transfer of membrane lipids. Remarkably, transient contacts between peroxisomes and lysosomes are thought to mediate transfer of cholesterol from lysosomes to peroxisomes (Chu et al., 2015) (**Figure 1**). Contacts are mediated by synaptotagmin VII on lysosomes which binds to the lipid $PI(4,5)P_2$ at the peroxisomal membrane (**Figure 2A**). LDL-cholesterol enhances such contacts, whereas peroxisome dysfunction results in cholesterol accumulation in lysosomes (Chu et al., 2015). This cholesterol trafficking blockage may contribute to the pathology of peroxisome disorders. An intriguing idea is that peroxisomes may associate with other organelles and deliver cholesterol to them (Chu et al., 2015). This can be mediated by transient organelle contacts or by membrane protrusions. Interestingly, transient contacts between individual peroxisomes have been reported (Bonekamp et al., 2012). These contacts do not result in the exchange of peroxisomal matrix or membrane proteins, but have been suggested to contribute to the equilibration of the peroxisomal compartment in the cell and might instead mediate the transfer of lipids or cholesterol between peroxisomes for further modification. These exciting novel findings underline the role of peroxisomal membrane dynamics in inter-organelle communication and protein/lipid transport and highlight the clinical relevance of these processes.

Lipid Droplets

Lipid droplets (LDs) are specialized organelles involved in the storage of neutral lipids, mainly triacylglycerols, and sterol esters, for energy and membrane homeostasis. LDs have been found in all eukaryotic and some prokaryotic cells, since lipids are essential for life and the capacity to store lipids confer an evolutionary advantage to the organism. The concept that LDs are simple, inert lipid-storage containers has now been dismissed. Today it is widely accepted that LDs are dynamic organelles which are involved in multiple cellular processes including lipid metabolism, but also protein sequestration/degradation and pathogen replication (Palacpac et al., 2004; Welte, 2007; Sorgi et al., 2009; Vogt et al., 2013). LDs are thought to originate from the ER and grow by fusion through a SNARE-mediated process (Böstrom et al., 2007; Murphy, 2012; Walther and Farese, 2012). They are known to move bi-directionally on microtubules and there is significant evidence showing that LDs dynamically interact with other organelles (**Figure 1**). LDs have been found in close association with ER, peroxisomes, mitochondria, endosomes, and the plasma membrane (Goodman, 2008; Murphy et al., 2009; Dugail, 2014). Lipid-exchange is likely to be the functional linkage between LDs and ER, peroxisomes and mitochondria. The association between the ER and LDs seems to occur even after budding of the LDs, with permanent contacts between these organelles being reported in different cells types (Blanchette-Mackie et al., 1995; Szymanski et al., 2007). Peroxisomes and mitochondria are frequently found in close association with LDs (Novikoff et al., 1980; Schrader, 2001; Binns et al., 2006; Sturmey et al., 2006; Shaw et al., 2008). Those contacts may link fatty acid supply by lipolysis in LDs with peroxisomal and mitochondrial fatty acid β-oxidation. In addition, exchange of lipids between LDs and peroxisomes or mitochondria may also serve membrane replenishment or storage in LDs. Defects in peroxisomal β-oxidation or absence of peroxisomes have been associated with enlarged LDs (Dirkx et al., 2005; Zhang et al., 2010). Inhibition of lipid mobilization in plants resulted in enlarged LDs and clustering of peroxisomes around them (Brown et al., 2013). Recently, it was reported that fatty acids stored in LDs in well-fed cells travel from LDs into mitochondria when cells are kept under starvation conditions. This transfer was dependent on mitochondrial fusion dynamics and close proximity to LDs (Rambold et al., 2015). Endosomes have also been observed to enwrap LDs, potentially promoting the delivery of LDs to lysosomes allowing for the transfer of cholesterol (Martin and Parton, 2005; Ouimet et al., 2011). Even though the interaction of LDs and other organelles (e.g., endosomes, ER, and vacuole) appears to be regulated by several Rab GTPases (Liu et al., 2007; Murphy et al., 2009; Bouchez et al., 2015), and the fusion events between LDs themselves, or LDs and mitochondria likely involves SNARE-mediated homotypic fusion (Goodman, 2008; Jägerström et al., 2009; Olofsson et al., 2009), the underlying molecular mechanisms remain largely unknown. In this respect, the LD-associated protein perilipin 5 is regarded as a candidate for the physical and metabolic linkage of mitochondria to LDs (Wang et al., 2011), whereas the molecular basis for a peroxisome—LD interaction remains elusive. A remaining question is the contribution of protein-protein and/or protein-phospholipid interactions to LD-organelle contacts. Hemi-fusion-like mechanisms would, however, represent an efficient way with low energy cost to exchange lipids between LDs and other organelles (Murphy et al., 2009; Olofsson et al., 2009).

Lysosomal Interactions and Autophagy

The first sign of the existence of an organelle with lytic function, known today as lysosomes, arose from the lab of Christian de Duve in 1949. Later on, the first electron microscopy image of lysosomes was obtained in collaboration between de Duve and Novikoff (Novikoff et al., 1956). Through ultrastructural studies researchers observed that lysosomes show pronounced cellular heterogeneity and individual polymorphism. In these pioneering cell biological studies vacuoles containing various organelles in different stages of degradation were observed in the

proximity of the ER and the endosomal-lysosomal compartment (Novikoff, 1959; Novikoff and Essner, 1962). As these membrane-surrounded structures were soon discovered to contain the lysosomal marker enzyme acid phosphatase, de Duve proposed that they may be involved in the constitutive removal of cellular material and named them autophagic vacuoles/autophagosomes (De Duve, 1963). Not much later he already hypothesized that the process of autophagy could represent a tightly regulated process involving an autophagic membrane originating from the ER segregating impaired organelles from the remaining pool and a subsequent fusion with primary lysosomes in order to digest the enclosed material (De Duve and Wattiaux, 1966). Thus, even if the origin of the autophagic membrane is still not resolved, it is quite obvious, that the process of autophagy involves controlled interaction between (1) the segregation membrane and an impaired organelle and (2) the autophagosome and a primary lysosome/endosome. Consequently, autophagy can be regarded as a highly specialized process of organellar interactions organizing cellular maintenance (**Figure 1**).

Cellular homeostasis can be disturbed due to cellular damage caused by nutrient deprivation, genetic alterations, or aging. To prevent cellular damage, a large array of quality control processes is available to the cell. Autophagy is one such process, consisting of the removal/recycling of cytoplasmic materials (e.g., protein aggregates, lipids, ribosomes, and organelles) by delivering them to the lysosome (Mizushima et al., 2011; Choi et al., 2013). Autophagy can be divided into 3 types: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy is the most understood autophagic process of the three, largely due to the extensive yeast genetic studies which have led to the identification of more than 35 autophagy-related (ATG) genes, along with their corresponding mammalian homologs (Mizushima et al., 2011). In macroautophagy components of the cytoplasm are engulfed by the phagophore (the so-called isolation membrane) leading to the formation of the autophagosome (double-membrane structure) (Mizushima et al., 2011). Maturation of the autophagosome occurs by fusing to endosomes and eventual engulfment by lysosomes, where it is degraded along with the cytoplasmic materials present in it. The autophagosome-lysosome fusion was found to be mediated by the SNARE Syntaxin 17 protein (Itakura et al., 2012). Special types of macroautophagy have been reported depending on the type of organelle; mitochondria (mitophagy), peroxisomes (pexophagy), lipid droplets (lipophagy), ER (reticulophagy), and microbes (xenophagy) (Klionsky et al., 2007).

Peroxisome and mitochondria homeostasis is attained by ensuring equilibrium between organelle biogenesis and degradation. The selective degradation of superfluous or damaged organelles is achieved by either a non-selective or selective autophagic process. During starvation or nutrient deprivation, non-selective autophagy is the predominant process, in order to ensure cell survival by providing essential amino acids and nutrients to the cell. However, under nutrient-rich conditions selective autophagy usually occurs to ensure the removal of damaged or superfluous organelles (Nordgren et al., 2013; Sureshbabu and Bhandari, 2013). In the selective autophagy pathway the specific phagophore membrane required for each form of selective autophagy recognizes the specific cargo prior to delivering it to the vacuole/lysosomal for degradation. The origin of the phagophore membrane still remains controversial. Recent studies have pointed to several organelles as potential membrane source (PM, Golgi, ER, and mitochondria) (Hailey et al., 2010; Mari et al., 2011; Bernard and Klionsky, 2013; Hamasaki et al., 2013). In yeast the mechanism of recognition of specific cargo for both pexophagy and mitophagy is well understood. For methylotrophic yeasts (e.g., *P. pastoris*) the pexophagy receptor is Atg30, which interacts with peroxisomal membrane proteins Pex3, Pex14, and Atg37 (Till et al., 2012; Nazarko, 2014). However, for *S. cerevisiae* and related yeasts the pexophagy receptor is Atg36 and appears to interact solely with Pex3 (Motley et al., 2012). Both Atg30 and Atg36 need to be activated by phosphorylation in order to interact with the scaffold protein Atg11 and the autophagosome via Atg8 (Farré et al., 2013). Surprisingly, Atg30 and Atg36 display no similarities at the amino acid level even though they exhibit similar function (Van der Zand and Reggiori, 2012). In yeast mitophagy the mitochondria outer membrane protein Atg32 was identified as the mitophagy receptor (Kanki et al., 2009; Okamoto et al., 2009). When phosphorylated it interacts with Atg11 and Atg8 on the autophagosome (Farré et al., 2013). Recent reports have shed some light over the signaling events that govern pexophagy/mitophagy, which are still largely unknown. In *S. cerevisiae*, two MAPK kinases, Hog1 and Pbs2, are exclusively required for mitophagy (Mao et al., 2011), whereas the MAPK kinase Slt2 was shown to be required for pexophagy (Manjithaya et al., 2010). Recently the Hrr25 kinase was identified as the responsible kinase for the phosphorylation of Atg19 and Atg36. Hence, enhancing the interactions between these receptors and the mutual adaptor Atg11 (Tanaka et al., 2014). Despite the fact that for methylotrophic yeasts the kinase responsible for phosphorylation of Atg30 is still unknown, a distinct At30-binding domain was recently identified in Pex3 which was important for the phosphorylation of Atg30 and the recruitment of Atg11 by Atg30 (Burnett et al., 2015). Furthermore, it was recently reported that a MAP kinase phosphatase 1 (MKP1) harboring a novel PTS1 (SAL) is targeted to peroxisomes under stress conditions in *Arabidopsis thaliana*. Whether this phosphatase is involved in plant pexophagy is still unknown since the regulatory role of MKP1 was not identified (Kataya et al., 2015).

In contrast to yeast, mechanistic understanding of pexophagy in mammals is more limited. Three pathways have been proposed for degradation of peroxisomes (**Figure 2B**): (1) p62-mediated detection of an ubiquitinated, unknown peroxisomal membrane protein, followed by autophagosome recruitment via p62 and LC3-II interaction (Kim et al., 2008), (2) direct binding of LC3-II to Pex14, by competing with the binding of Pex5 to Pex14 depending on the nutrient conditions (Hara-Kuge and Fujiki, 2008), (3) binding of NBR1, another adaptor protein like p62, to an ubiquitinated peroxisomal membrane protein or through direct binding to the peroxisomal membrane (Deosaran et al., 2013). This last pathway also includes p62 as another interacting protein, but downstream of the obligate NBR1, supposedly acting

as an accessory interaction partner in the tethering complex (**Figure 2B**).

Also, for mammalian cells a two-step model for priming mitochondria for mitophagy has been proposed: RING-between-RING E3 ubiquitin ligase Parkin dependent or Parkin independent (Ding and Yin, 2012) (**Figure 2B**). In the Parkin dependent pathway, PINK1 is constitutively cleaved by the mitochondrial protease PARL (Jin et al., 2010). Inactivation of PARL, due to mitochondria membrane depolarization, blocks PINK1 cleavage and access to the inner mitochondrial membrane and subsequently the PINK1 precursor is stabilized at the outer mitochondrial membrane (Meissner et al., 2011). At the outer mitochondrial membrane, PINK1 recruits and activates cytosolic Parkin which then promotes ubiquitination of mitochondria outer membrane proteins (Lazarou et al., 2013; Kane et al., 2014). P62 recognizes ubiquitinated proteins and through its direct interaction with LC3-II recruits autophagosomal membranes to the mitochondria. Parkin and PINK1 have been reported to interact with several other cellular proteins that might be involved in their regulation. For the Parkin independent mechanism, mitophagy is mediated by FUNDC1, Nix, and BNIP3 which interact directly with LC3-II promoting the recruitment of autophagosomes to mitochondria (Ding and Yin, 2012; Jin and Youle, 2012). Interestingly, cardiolipin, a phospholipid of the inner mitochondrial membrane, is transferred to the outer mitochondrial membrane of compromised mitochondria (Chu et al., 2013, 2014). There it can be bound by LC3 via several clusters of basic amino acids on the protein's surface, thereby triggering autophagy. The authors further speculated that cardiolipin peroxidation, resulting from excessive mitochondrial ROS production, could serve to switch between the processes of mitophagy and programmed cell death (Chu et al., 2014). More recently, two other pathways to target mitochondria for mitophagy have been reported. The formation of mitochondria-derived vesicles (MDVs) (Soubannier et al., 2012) (**Figure 1**), and mitochondria spheroids (Ding et al., 2012) that may delivery mitochondrial components for degradation to the lysosomes and the direct recruitment of p62 via choline dehydrogenase (CHDH) in response to mitochondrial membrane depolarization (Park et al., 2014). In selective autophagic processes, required for degradation of faulty or surplus organelles, the size of the organelle is a critical factor for obtaining efficient engulfment by the autophagosome (Müller and Reichert, 2011; Mao et al., 2014). Peroxisomes and mitochondria fission/fragmentation is a requirement for both selective autophagy processes, and the dynamin-like GTPase DLP1 has been reported to be recruited and activated before either pexophagy or mitophagy occur (Twig and Shirihai, 2011; Mao et al., 2014). However, not all the fragmented organelles are triggered for elimination, indicating that there must be a mechanism that regulates which organelles need to be eliminated. For mitochondria it has been revealed that fission followed by selective fusion of mitochondria and tubular network formation under nutrient deprivation conditions protects mitochondria from mitophagy (Twig et al., 2008; Rambold et al., 2011). In addition, mitophagy is avoided if the membrane potential of the mitochondria is sustained after fission events (Twig et al., 2008). On the other hand, peroxisomes are not able to fuse with one another (Bonekamp et al., 2012) and also do not possess a membrane potential, so the regulatory mechanism must be distinct from the ones available to mitochondria. One hypothesis to discriminate healthy peroxisomes from the ones that need to be degraded might be via asymmetric fission/division of the organelle (Nordgren et al., 2013). A recent study showed that removal of protein aggregates present in the lumen of peroxisomes and its subsequent elimination by autophagy, was achieved by asymmetric peroxisome fission to separate the aggregate from the mother peroxisome (Manivannan et al., 2013). Besides the physical interactions between peroxisomes and autophagosomes, required for pexophagy and subsequent fusion to lysosomes, a very recent study has shown for the first time the existence of lysosomal-peroxisome membrane contacts (LPMC) essential for the cellular trafficking of cholesterol (Chu et al., 2015) (**Figure 1**). In a well-designed set of experiments the authors showed that the lysosomal Syt7 protein binds peroxisomal PI(4,5)P2 (phospholipid), bridging the organelles and allowing cholesterol to transfer from lysosomes to peroxisomes (**Figure 2A**). Furthermore, the authors propose a central role for peroxisomes in intracellular cholesterol trafficking and that intracellular cholesterol accumulation may underlie the pathological mechanism of peroxisome disorders (Chu et al., 2015). Thus, lysosomes not only interfere with other subcellular compartments in terms of removal of compromised organelles but appear to be involved also in functional networks which guarantee cellular maintenance.

Concluding Remarks

The current examples for organelle interaction in mammalian cells, as discussed in sections Connections between the ER and the Plasma Membrane, The Mitochondria-associated Membrane of the ER (MAM), The Peroxisome-ER Connection, Interplay between Peroxisomes and Mitochondria, Lipid Droplets, and Lysosomal Interactions and Autophagy, clearly illustrate that subcellular organelles are integrated in cooperating cellular networks. Although intimate physical contacts between organelles were described some time ago, we are just beginning to reveal the key components involved and their physiological importance. A major role of organelle interaction is clearly in metabolite exchange, but exciting new functions in organelle distribution and membrane dynamics have been discovered. Furthermore, increasing evidence points to an important function in signaling and the assembly of dynamic signaling platforms according to cellular requirements. In this respect, higher ordered complexes between more than two organelles may exist as exemplified by antiviral signaling via MAVS involving the ER, mitochondria and peroxisomes. A common principle may be the involvement of structurally similar or overlapping protein complexes for the physical tethering of different organelle membranes. Future studies will reveal if organelle interplay and cooperation is primarily mediated via those hubs, or if indirect mechanisms via the cytosol are more prevalent.

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OPINION
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Using Optical Tweezers to Characterize Physical Tethers at Membrane Contact Sites: Grab It, Pull It, Set It Free?

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Keywords: membrane contact sites, tether, optical tweezer, MCS, biophysics

Compartmentalisation is a defining feature of eukaryotic life. Effective communication between organelles is essential for cell maintenance, growth and response to external stimuli. Static snapshots provided through ultrastructural studies of preserved tissue highlight that certain organelles are in intimate contact at membrane contact sites (MCSs), also referred to as inter-organellar tethering sites. However, live cell imaging indicates that these interactions are not necessarily stable with organelles frequently "colliding," moving in unison and then separating. This dramatic intracellular "waltz" between organelles with ever changing partners (organelles) indicates that the molecular factors controlling MCSs are highly regulated. Key questions therefore relate to defining which organelles physically interact, deciphering the molecular components that control MCS formation, and ultimately deciphering the specific functional role that the interaction provides to the cell (**Figure 1**).

Reviews on the roles of MCSs are covered elsewhere (Elbaz and Schuldiner, 2011; Helle et al., 2013; Prinz, 2014; Islinger et al., 2015; Phillips and Voeltz, 2016). Readers are also directed to the Frontiers special topic (Schrader and Islinger, 2016) and the special issue of Current Opinion in Cell Biology dedicated to cell organelles including MCSs (Schuldiner and Guo, 2015). Here, I will provide an overview of the techniques used to interrogate MCSs and how optical tweezers could provide a future platform for characterizing the biophysical nature of MCSs.

Tethers have been isolated using multiple techniques, however a difficulty has been being able to discriminate between a role in physical tethering versus a role in transferring components at the MCS itself. For example, tethering sites are required for signaling, trafficking and biogenesis. Proteins located at MCSs could provide physical stability and MCSs formation, whereas others could collocate to and function in the actual transfer of molecules such as lipids and calcium. These generic roles are not necessarily mutually exclusive as evidenced by studies with OSBP (Mesmin et al., 2013). One way to discriminate between these two generic roles is if disruption of the potential tether affects the subsequent physical association between organelles. A clear example of this is observed during division of budding yeast where tethering can play a role in organelle inheritance into the bud cell. Dependent on the organism and cell type, organelles can be highly motile during interphase. Here, organelles are not clearly partitioned in a similar manner during cell division, and so seemingly random motion could result in organelles occupying similar physical regions without it being as a result of direct physical interaction *per se*. Organelle movement in higher plants is even further complicated by fast cytoplasmic streaming events.

Attempts to isolate and probe the nature of physical tethers include biochemical fractionation, genetic screens and microscopy. Applications of these techniques to investigate MCSs are covered in more depth in the review by Helle et al. (2013).

Biochemical fractionation and concentration of membrane enriched fractions have resulted in mitochondrial associated membranes (MAMs), plasma membrane associated membranes (PAMs)

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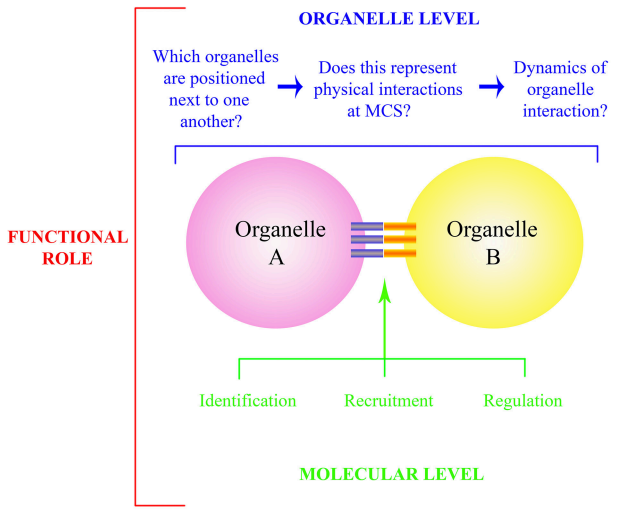

FIGURE 1 | Schematic representation of questions relating to organelle interactions and MCS. The functional role of organelle interaction (red) is determined at the whole organelle (blue) and molecular level (green). At the organelle level, *bona fide* physical interactions need to be identified, and the regulation of such is governed through identification, recruitment and regulation of the molecular components that comprise the MCS itself (blue and orange bars). These components include both physical tethers and those which allow exchange at the MCS.

and plastid associated membranes (PLAMs). Here, enriched fractions highlight differential lipid and protein content between opposing organelle membranes and an intermediary fraction which contains components from both organelle membranes. The latter is thought to represent MCS enriched regions. In principle, this is a straight forward process, however in practice, identification and characterization of the molecular factors that are enriched in these fractions, can be problematic (e.g., reconciling subcellular location with function at MCS).

Novel ways to isolate tethers have included a synthetic screen in yeast which pulled out the ER-mitochondria tethering complex, ERMES (Kornmann et al., 2009). Tethering components have also been isolated using more traditional genetic screens. For example, components of the Store-Operated Calcium Entry (SOCE) at the ER-PM were isolated through independent RNAi screens (Liou et al., 2005; Roos et al., 2005; Feske et al., 2006; Vig et al., 2006; Zhang et al., 2006).

Transmission electron microscopy (TEM) allows ultra-structural observations of MCSs between organelles to be quantified in terms of the number and length of the MCS, and the distance between the interacting organelles. Conventional analysis of single TEM images limits interpretation of organelle interactions to the plane that has been sectioned. Therefore, relatively large numbers of sections need to be quantified to provide statistically robust conclusions on the number of MCSs. TEM tomography however determines spatial relatedness through analysing serial sections of a fixed sample, or by tilting the block *in situ*, to provide a three dimensional overview of the intimate association and connections between organelles. Examples of the use include determination of the interaction between the ER and mitochondria, and endosomes in yeast (Friedman et al., 2011; Alpy et al., 2013).

Visualization of MCS in dynamic living tissue is difficult. They typically bridge a gap of up to 30 nm between organelles, and so are below the limit of resolution of conventional light microscopy. However, a clear advantage over EM and tomography is that the tissue is live allowing dynamic events to be observed, and also negates any potential artifacts that may have been introduced during the fixation procedure. Technological advances in imaging are now beginning to combine the advantageous properties of live cell imaging with the ultra-structural resolution offered through EM. By breaking the diffraction limit of light, and using algorithms to compute spatial positioning and relatedness between imaged structures, super resolution light microscopes can provide enhanced spatial resolution with sufficient scan speeds to capture organelle movement; for example STORM and RESOLFT systems have been used to image ER dynamics (Grotjohann et al., 2012; Shim et al., 2012). These imaging systems are not commonplace, and the dynamic range may not capture fast movement events. Traditionally, conventional light microscopy has been used to quantify organelle movement and correlate movement patterns of organelles which appear closely associated and / or move in tandem. The open question here is whether this reflects true physical association of the two organelles, coordinated movement through co-regulated motors or organelles that are traversing the same cytoskeletal track in a densely packed cytoplasmic environment?

The techniques highlighted above (biochemical fractionation, genetic screens and microscopy) cover certain aspects of MCS research. However, none of these techniques directly probe the biophysical nature of organelle interaction. Spatial relatedness could be caused by many reasons, not just through the role of the tethering process itself. For example, decreasing the cytoplasmic volume for organelles to occupy could result in increased "interactions" through mere random collisions of the organelles in a more highly constrained region, perhaps even changes in cytoplasmic viscosity may artificially elevate observed interactions through sheer issues of physically moving the organelles through a more viscous medium. Biophysical techniques which allow the user to physically "pull" apart organelle pairings *in vivo* are therefore advantageous.

Optical tweezers allows the user to physically trap an object which has a significantly different refractive index to the surrounding media, in this case the organelle in the cytoplasm. The trapped organelle can then be micromanipulated and moved laterally within the cytoplasm and interactions with neighboring organelles interrogated; Is more force required to move an organelle if it is next to a certain organelle indicating physically interaction? How does the interaction change in response to altering the properties of the tethers themselves?

Optical tweezers have been used to trap and move Golgi bodies in Arabidopsis leaf epidermal cells (Sparkes et al., 2009). This qualitative approach highlighted that movement of trapped Golgi, in turn remodeled the ER indicating a physical association between the two organelles. Furthermore, observations of the remodeled ER indicated that it could be

"hooked" or anchored in place at regions within the cell, indicative of anchoring to the plasma membrane (PM). Further studies have highlighted the molecular components involved in the ER-PM sites, with the sites themselves being implicated in mechanosensing (Wang et al., 2014; Perez-Sancho et al., 2015). Contacts between the chloroplast and ER also appeared to occur in laser ablated Arabidopsis protoplasts and pea leaves (Andersson et al., 2007).

More recently, Gao et al. (2016) developed a quantifiable platform for using optical tweezers to measure organelle interactions in intact cells, more specifically the interaction between peroxisomes and chloroplasts. Here, using an automated platform to trap a peroxisome, users moved it a set distance at a set speed, and then monitored and quantified the effects on the organelle during this process; was it trapped? Did it stay in the trap during the lateral automated motion at a set speed? How did these characteristics vary with changes in optical trap strength? Was more force required to move and separate a peroxisome from a neighboring chloroplast? Based on these observations at low optical trap strength peroxisomes either escaped the trap during the lateral movement or were not trapped at all. As optical trap strength increased the percentage of trapped organelles increased with a concomitant decrease in organelles that escaped the trap or could not be trapped. These characteristics for two populations of peroxisomes, which were either next to a chloroplast or far away from a chloroplast, were monitored and compared. Results indicated that it was physically "harder" to trap and move chloroplast associated peroxisomes compared to those that were not associated with chloroplasts, indicative of a tethering mechanism between the two compartments. By doing this type of quantitative analysis, and making comparisons between juxtaposed organelles and control measurements of organelles which are not near one another, provides a clear indication of physical interaction between the two compartments. Furthermore, by monitoring the movement of the peroxisomes after turning the trap off, the authors were able to model the motion which relates to the tethering process itself.

It is also worth noting an alternative biophysical approach to quantifying organelle interactions. By using a femtosecond laser to generate a pressure wave within the cell, users can estimate the force required to effectively move or "push" an organelle. This is quite different to optical tweezers which uses submicron precision to specifically "pull" rather than "push" an organelle. Both approaches have been used to establish physical connections between peroxisomes and chloroplasts (Oikawa et al., 2015; Gao et al., 2016).

Optical tweezers can therefore be used to monitor and probe physical interactions between organelles. By using a quantifiable platform (such as that descried by Gao et al.) it also has the potential to interrogate the role of molecular components that drive the interaction itself. Here, one might expect that tethering efficiency at the MCS may be affected upon altered tether expression; overexpression may increase tethering, whereas mutations in the tether could determine the functional domains / critical residues required to maintain the physical interaction between organelles. Quantification of interactions in this way is laborious, and so it is not advisable to attempt a genetic screen to identify novel tethers using an optical tweezer strategy. Similar to its use in measuring force values exerted by molecular motors *in vivo* (for example Hendricks et al., 2012, PNAS; Rai et al., 2013, Cell), optical tweezers could also quantify the forces involved in organelle interactions.

The future of MCS research will be shaped through a combination of several complementary techniques. By understanding the limitations and advantages that each technical approach provides, users will break through the barriers in understanding MCS structure and regulation. It will be interesting to see if technological advances will allow multiple techniques to be combined into the one modular system to allow attributes of individual MCSs to be probed simultaneously. For example, being able to measure the dynamics of interactions between components of the tether complex, whilst ascertaining the force imparted by the interactions to maintain spatial positioning of the organelles. Our basic picture of eukaryotic life consisting of discrete membrane bound compartments is certainly being challenged by MCS studies. One looks forward to seeing the results from future endeavors in deciphering this layer of subcellular complexity.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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The similarity between N-terminal targeting signals for protein import into different organelles and its evolutionary relevance

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The proper distribution of proteins between the cytosol and various membrane-bound compartments is crucial for the functionality of eukaryotic cells. This requires the cooperation between protein transport machineries that translocate diverse proteins from the cytosol into these compartments and targeting signal(s) encoded within the primary sequence of these proteins that define their cellular destination. The mechanisms exerting protein translocation differ remarkably between the compartments, but the predominant targeting signals for mitochondria, chloroplasts and the ER share the N-terminal position, an α-helical structural element and the removal from the core protein by intraorganellar cleavage. Interestingly, similar properties have been described for the peroxisomal targeting signal type 2 mediating the import of a fraction of soluble peroxisomal proteins, whereas other peroxisomal matrix proteins encode the type 1 targeting signal residing at the extreme C-terminus. The structural similarity of N-terminal targeting signals poses a challenge to the specificity of protein transport, but allows the generation of ambiguous targeting signals that mediate dual targeting of proteins into different compartments. Dual targeting might represent an advantage for adaptation processes that involve a redistribution of proteins, because it circumvents the hierarchy of targeting signals. Thus, the co-existence of two equally functional import pathways into peroxisomes might reflect a balance between evolutionary constant and flexible transport routes.

Keywords: Peroxisomes, PTS2, targeting signals, preprotein, transit peptide, signal peptide, specificity, ambiguous targeting signals

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Mechanisms of Protein Translocation across Cellular Membranes

In eukaryotic cells, an elaborate endomembrane system separates the cytosolic space[1] from sealed compartments such as mitochondria, peroxisomes, chloroplasts (in plants), and the secretory (endoplasmic reticulum (ER), golgi, trans golgi network (TGN), and lysosome) and endosomal (early and late endosomes) system, which we generally summarize as organelles within this review. The individual compartments of the secretory and endosomal system are interconnected, whereas chloroplasts, mitochondria, and peroxisomes are considered more separate in spite of an exchange of metabolites and membrane constituents between these organelles. This separation can serve various functions such as the local enrichment of specific metabolic intermediates,

[1] As cytosol and nucleus continuously exchange solutes and proteinaceous material, we do not explicate the differences here.

the sequestration of toxic compounds or the separation of oppositely directed reactions (e.g., biosynthesis and degradation of fatty acids). Some of the enzymatic activities that cohabitate the same organelle cooperate in coupled reactions within certain metabolic pathways to perform complex reactions such as respiration (in mitochondria), photosynthesis (in chloroplasts), or the degradation of fatty acids (in peroxisomes and mitochondria). This implies that the co-localization of diverse enzymes within the same organelle is a prerequisite for an efficient metabolic flux of compounds that are degraded or synthesized. Thus, the proper distribution of proteins among different subcellular compartments is essential for the functionality of a cell. While nearly all proteinaceous components of peroxisomes, mitochondria, or chloroplasts are synthesized by cytosolic ribosomes and transported into the organelles by specific import machineries, the ER is the entrance site for proteins destined for any place along the secretory or endosomal pathway. Only a few cellular proteins are encoded by mitochondrial or chloroplast DNA and are synthesized locally without the need to be imported. Thus, the distribution of proteins is critically dependent on a reliable protein transport system, which requires the cooperation between information specifying the cellular destination of an individual protein and cellular transport machineries, which recognize and process all proteins that harbor such information and need to be transported. The destination of individual proteins is encoded within their primary sequence in the form of short peptides called *targeting signals*, which can be considered postal codes necessary and sufficient to determine the intracellular location. These targeting signals are recognized by *receptor proteins*, which are the frontline of the organellar import machinery and initiate transport of their cargo proteins (Blobel and Dobberstein, 1975). The import mechanisms by which soluble proteins are translocated across the membrane(s) of peroxisomes, mitochondria, chloroplasts, or of the ER are remarkably different. However, the targeting signals for mitochondria, chloroplasts, or the ER appear structurally similar, because they all involve an α-helical domain in proximity to the N-terminus. In contrast, the majority of peroxisomal proteins is equipped with a targeting signal that resides at the extreme C-terminus of the protein. However, a peroxisomal destination can also be encoded by an independent second targeting signal that resides proximal to the N-terminus, but occurs less frequently and has attracted less interest (Schatz and Dobberstein, 1996; Fujiki et al., 2014).

In this review, we compare the different import systems translocating soluble proteins from the cytosol into the lumen of peroxisomes, mitochondria, chloroplasts, or the ER. The receptor proteins of these transport systems all recognize targeting signals encoded within N-terminal sequences that involve an α-helical domain. In particular, we highlight the recent finding that the second peroxisomal targeting signal (PTS) is also encoded by a sequence element that forms an α-helical domain. The similarity to other N-terminal targeting signals distinguishes this PTS (PTS2) from the predominant PTS (PTS1) residing at the C-terminus, which could serve as explanation for the existence of two completely independent PTS that exceeds simple redundancy. In this context, we discuss the specificity of targeting signals, the hierarchy of transport routes and the possibility to change the subcellular location of a protein in evolutionary adaptation processes.

Abbreviations: PEX, peroxin; RNC, ribosome nascent chain complex; TOM, translocon of the outer mitochondrial membrane; TIM, translocon of the inner mitochondrial membrane; TOC, translocon of the outer chloroplast envelope; TIC, translocon of the inner chloroplast envelope.

Mechanisms of Protein Import from the Cytosol into Endomembrane Systems

Complex protein machineries guide newly generated soluble proteins equipped with suitable targeting signals across the single membrane of peroxisomes and the ER and across the double membrane of chloroplasts and mitochondria. Although these transport machineries act on membrane proteins as well, we restrict ourselves to transport routes of soluble proteins, because this allows a comparison of different organelles within the given space. Moreover, we do not consider further intraorganellar transport processes that act on proteins in the mitochondrial matrix or the chloroplast stroma.

In spite of major differences between the import mechanisms of the above-mentioned organelles, the key steps are similar. Receptor proteins select suitable cargo proteins by specific interaction with targeting signals, but this selection can occur either during translation or after translation and can act either on unfolded or folded proteins (**Table 1**). In all cases, the receptor initiates the interaction of the cargo protein with a complex translocation machinery that can involve the receptor protein(s) itself. Moreover, all cargo proteins are translocated through pore-like structures, but this occurs either in an unfolded linear state or as fully folded protein. After transport the N-terminal sequences encoding targeting signals are processed by specific peptidases within the organelles. Each receptor protein mediates the import of many proteins, which necessitates a recycling of these receptor proteins. The targeting signals for mitochondria, chloroplasts, and the ER are encoded within N-terminal sequences with different denominations (*presequence*, *transit sequence*, and *signal peptide*), whereas *peroxisomal targeting signals* determine proteins for peroxisomes (**Table 1**). A comparative overview of the import mechanisms for soluble proteins into different organelles is depicted (**Figure 1**) and highlights the major steps of protein import. For further details of the import mechanism the readers are referred to excellent reviews that have been published elsewhere [peroxisomes (Hettema et al., 2014; Platta et al., 2014) mitochondria (Chacinska et al., 2009; Schulz et al., 2015); chloroplasts (Li and Chiu, 2010), and ER (Akopian et al., 2013; Johnson et al., 2013b)].

Protein Import into Peroxisomes

All soluble peroxisomal proteins are encoded by nuclear DNA, produced by free ribosomes and folded in the cytosol before they are translocated across the membrane (**Figure 1A**) (Léon et al., 2006). This folding might include co-factor binding and oligomerization. Even cross-linked proteins and labeled gold particles up to a size of 9 nm can be imported (Walton et al., 1995;

TABLE 1 | Transport processes mediated by the N-terminal targeting signal.

Organelle	Peroxisome	Mitochondria	Chloroplast	ER Co-translational	ER Post-translational
Targeting signal	**PTS2**	**Presequence**	**Transit peptide**	**Signal peptide**	**Signal peptide**
Structure	Amphiphilic α-helix	Amphiphilic α-helix	Amphiphilic α-helix	Hydrophobic α-helix	Less hydrophobic α-helix
Consensus sequence	Yes	No	No	No	No
Linker domain	Yes	No	No	No	No
Processing of the N-terminus	Yes	yes	yes	yes	Yes
Number of cargo proteins[a]	< 30	> 1000	> 1000	> 1000	Unclear
Receptor	Pex7 Soluble	Tom20 Membrane bound	Toc34/159 Membrane bound	SRP-complex Soluble	
Translocon[b]	Pex14/Pex5	Tom40	Toc75	Sec61	Sec61
Ribosomes	Free	Free	Free	Membrane bound	Free
Transfer to organellar membrane	Co-receptor mediated	Chaperone mediated	Chaperone mediated	SRP-mediated	Chaperone mediated
Import	Post-translational	Post-translational	Post-translational	Co-translational	Post-translational
Protein state (During translocation)	Folded	Unfolded	Unfolded	Unfolded	Unfolded
Import mode	Globular	Linear (N→C)	Linear (N →C)	Linear (N →C)	Linear (N →C)
Energy[c]					
Protein translocation	ATP	ATP	ATP	GTP	ATP
Transfer of the targeting signal		$\Delta\psi$	GTP		
Energy consuming process	Receptor recycling	Protein translocation	Protein translocation	Protein translocation	Protein translocation
Processing peptidase[d]	PPP/GPP	MPP	SPP	SP	SP

Protein transport mediated by N-terminal targeting signals.

[a] *Protein numbers are a rough estimation for complex animal or plant organisms based on the assumption that more than at least half of the organellar proteins are soluble: mitochondria (Homo sapiens, Pagliarini et al., 2008), chloroplasts (Arabidopsis thaliana, Richly and Leister, 2004), ER and secretory apparatus not considering the secreted proteins (Rattus norvegicus, Gilchrist et al., 2006) and peroxisomes (Arabidopsis thaliana, Reumann et al., 2007), but in the latter only a third of the proteins encodes a PTS2.*

[b] *Translocon: proteins forming the pore forming unit for the translocation of the preproteins.*

[c] *Energy is consumedin form of ATP hydrolysis (ATP), GTP hydrolysis (GTP), or derived from the electrochemical gradient ($\Delta\psi$).*

[d] *Processing peptidases: PPP/GPP, peroxisomal, or glyoxysomal processing peptidase; MPP, mitochondrial processing peptidase; SPP, stromal processing peptidase; SP, signal peptidase.*

Subramani, 2002; Léon et al., 2006). Peroxisomal proteins harbor a peroxisomal targeting signal (PTS), which is encoded by a peptide sequence either at the extreme C-terminus (type 1, PTS1) (Gould et al., 1988) or proximal to the N-terminus (type 2, PTS2) (Swinkels et al., 1991; Osumi et al., 1991), although, sporadically proteins have been described in peroxisomes that do not encode any of these sequences. The import of a protein requires the interaction of the PTS1 with the soluble receptor protein Pex5[2] (peroxin 5, Distel et al., 1996; Van der Leij et al., 1993; Dodt et al., 1995; Wiemer et al., 1995; Kragler et al., 1998) or of the PTS2 with the soluble receptor protein Pex7 (Marzioch et al., 1994; Braverman et al., 1997; Woodward and Bartel, 2005). Cargo-loaded receptor proteins translocate to the peroxisomal surface and interact with the docking complex (DC), which is part of the peroxisomal import machinery (PIM) (**Figure 1A**). The primary docking of Pex5 is driven by a lipid-protein interaction (Kerssen et al., 2006), but the functional interaction is dependent on specific sequences within Pex5 that mediate the interaction with proteins of the DC (Saidowsky et al., 2001; Otera et al., 2002). In contrast, Pex7 cannot move to the peroxisomal surface by itself, but requires the interaction with a co-receptor protein, which encodes the sequence elements required for the interaction with the proteins of the docking complex (Schliebs and Kunau, 2006; Grunau et al., 2009; Kunze et al., 2015). This co-receptor function for Pex7 is exerted in many organisms (metazoa and plants) by the PTS1 receptor Pex5 (Braverman et al., 1998; Otera et al., 1998; Khan and Zolman, 2010), whereas in fungi independent proteins exist for this function (Purdue et al., 1998; Titorenko et al., 1998). Cargo binding was found to be a prerequisite for the interaction of human Pex7 with its co-receptor protein Pex5 (Mukai and Fujiki, 2006; Kunze et al., 2015) and, thus, only cargo-loaded Pex7 can be transported to peroxisomes (Kunze et al., 2015). This resembles the cargo-induced translocation of Pex5 in PTS1-mediated import (Gouveia et al., 2003b).

At the docking complex, both import pathways converge, and thus, will be discussed together highlighting only specific differences. Cargo bound Pex5 integrates into the peroxisomal membrane in an ATP-independent step that is probably driven by protein-protein interactions (Oliveira et al., 2003). During this process Pex5 interacts with the N-terminus of Pex14 proteins via several copies of a conserved sequence motif involving two aromatic amino acids (Schliebs et al., 1999), which fits to the

[2] To facilitate reading, we use one nomenclature for proteins from all species using standard abbreviations with the first letter capitalized.

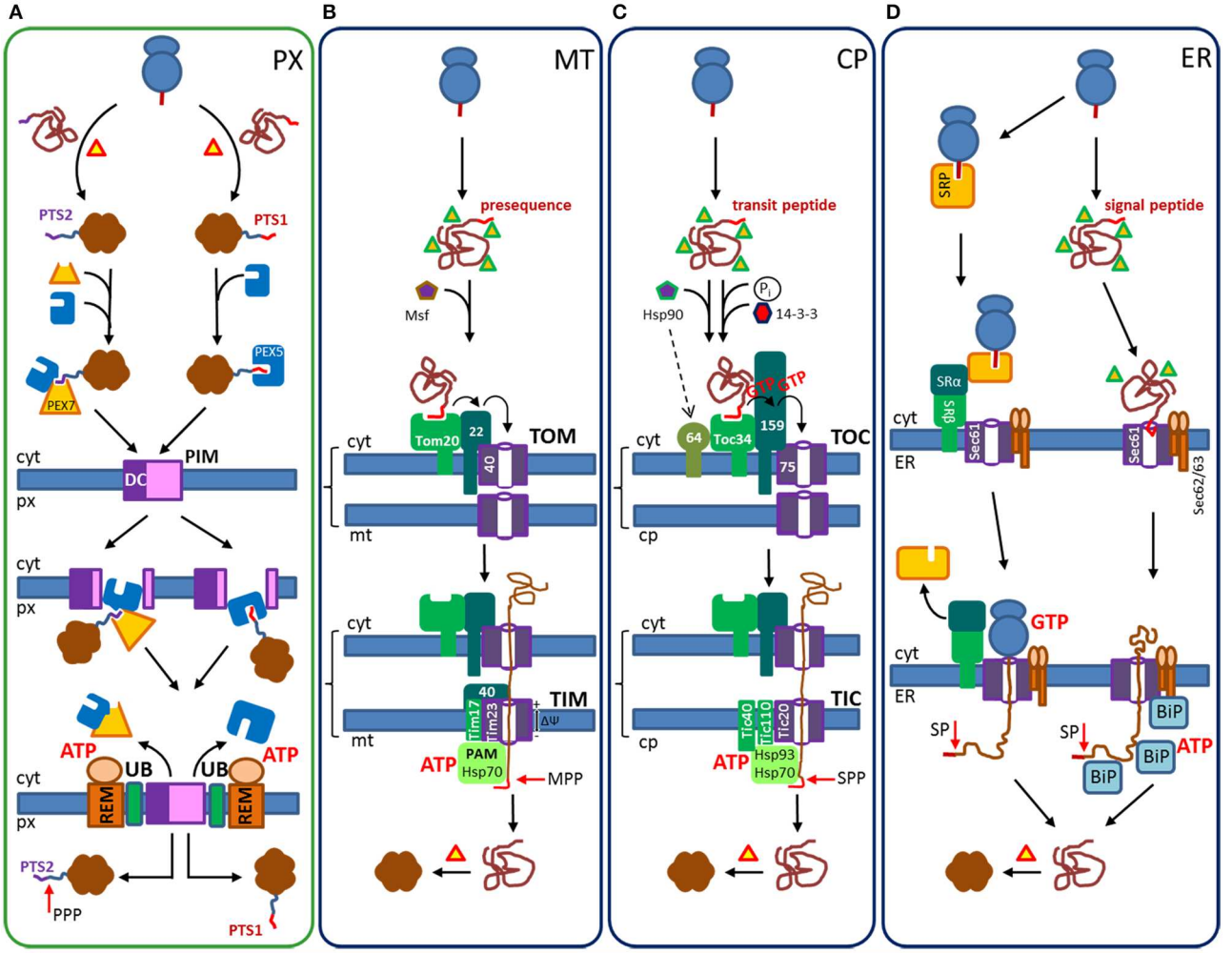

FIGURE 1 | Continued

FIGURE 1 | Protein transport routes from the cytosol into peroxisomes, mitochondria, chloroplasts, and the ER: The transport routes are depicted schematically to highlight certain players[3]. (A) *Peroxisomes*. Proteins encoding either a PTS1 or a PTS2 are folded within the cytosol and interact with the appropriated receptor proteins, Pex5 or the Pex7/co-receptor complex. This induces the translocation of cargo loaded receptors to the docking complex (DC), where they integrate into the peroxisomal membrane and release the cargo into the lumen. Finally, Pex5 and the Pex7/co-receptor complex are ubiquitylated by a specific ubiquitination machinery (UB) and recycled into the cytosol by an ATP driven extraction exerted by the receptor extraction machinery (REM). Soluble proteins reach the peroxisomal matrix in a folded state, but PTS2-carrying proteins are processed by the peroxisomal processing peptidase (PPP). **(B)** *Mitochondria*. Proteins encoding a presequence are translated within the cytosol, but remain in an unfolded state due to their association with proteins of the Hsp70 family. These complexes are transferred by the help of an additional cytosolic factor to a protein complex at the outer mitochondrial membrane (TOM), where the preprotein sequentially interacts with the receptors for soluble proteins (Tom20 and Tom22), before it is handed over to the pore forming translocon (Tom40). The preprotein crosses the outer mitochondrial membrane in an unfolded state and interacts with a protein complex in the inner mitochondrial membrane (TIM). The binding to Tim40 mediates the interaction with the pore forming unit of the inner membrane (Tim23) and the electrochemical gradient ($\Delta\Psi$) drags the presequence across the membrane. At the matrix side, the "presequence translocase-associated motor" (PAM)-complex, ropes the preprotein into the matrix by an ATP-driven mechanism that is based on the sequential interaction of mitochondrial chaperones. Next, the N-terminal sequence of the preprotein is cleaved off by the mitochondrial processing peptidase (MPP) and the protein folds within the matrix with the help of mitochondrial folding chaperones. **(C)** *Chloroplasts*. Proteins encoding a transit peptide are translated by cytosolic ribosomes and kept in an unfolded state by proteins of the Hsp70 family. Proteins of the 14-3-3 family, which bind selectively to phosphorylated transit peptides and Hsp90 proteins support the transfer to the chloroplast surface. The outer chloroplast membrane contains multi-protein complexes (TOC) that involve members of two receptor families (Toc34 and Toc159 family), a specific binding factor for Hsp90 proteins (Toc64) and the channel forming translocon Toc75. Transit peptides are translocated via sequential receptor binding from Toc34 to Toc159 and Toc75, which requires the cooperation between the GTPase domains of Toc34 and Toc159. Unfolded preproteins pass the translocon and bind to the multiprotein complex at the inner chloroplast membrane (TIC), involving the pore forming protein Tic20, Tic110, and Tic40, which allow the transfer of the transit peptide across the inner membrane. In the stroma a complex machinery of CpHsp70, Hsp90, and Hsp93, which is attached to the inner side of the chloroplast membrane by the interaction with TIC-proteins, supports the import of the preprotein by an ATPase driven mechanism. Within the chloroplast the transit peptide is cleaved off and the imported proteins are folded. **(D)** *ER*: *Co-translational (left part)*. A functional signal peptide sequence initiates the binding of the signal recognition particle (SRP complex) upon its appearance at the ribosomal exit site. SRP binding stalls translation until the trimeric complex consisting of a nascent chain harboring a signal peptide, a ribosome and a SRP binds to the heterodimeric SRP-receptor (SR) on the surface of the ER. Subsequently, the signal peptide and the ribosome become transferred to the outer side of the Sec61 complex, which is the channel forming translocon. The release of the SRP is coupled to the resumption of translation and the newly synthesized protein is directly inserted into the lumen of the ER. This complex mechanism involves the cooperation of GTPase domains within the SRP and the SR, whereby the hydrolysis of GTP is coupled to diverse conformational changes. However, the major energy consuming step that drives the translocation of preproteins across the ER membrane is the energy of translation (GTP hydrolysis). At the inner side of the ER the signal peptide is cleaved off by the signal peptidase and the protein is folded by the help of luminal chaperones. *Post-translational (right part)*. Proteins with N-termini that are not recognized by the SRP in spite of a functional signal peptide are translated to completion in the cytosol, but their folding is prevented by the interaction with cytosolic Hsp70 proteins. The preprotein interacts with the Sec61 complex in the ER membrane and becomes translocated across the membrane by ATP driven pulling mechanism exerted by luminal chaperones. Inside the ER the preproteins are processed by a signal peptidase (SP) and the proteins fold with the help of chaperones. Protein complexes are indicated in capital letters, proteins are indicated according to the nomenclature used in this manuscript.

overrepresentation of Pex14 in the membrane complex involving Pex5 (Gouveia et al., 2000). Interestingly, not only the C-terminal part of Pex5 that encodes the cargo-binding domain reaches into the peroxisomal matrix (Gouveia et al., 2003a), but also the N-terminal sequence (Dammai and Subramani, 2001). Similarly, Pex7 is imported into the peroxisomal matrix in an ATP-independent step that causes a complete enclosure of the receptor within the organelle (Rodrigues et al., 2014). Pex5 accumulates at the peroxisomal surface, integrates into the membrane and becomes part of a multiprotein complex before it is recycled (Dodt and Gould, 1996). The mechanism of cargo protein translocation across the peroxisomal membrane has not been resolved, but probably involves a dynamic pore-like core-structure consisting of Pex5 and Pex14 proteins. In reconstitution experiments utilizing protein complexes isolated from peroxisomal membranes and embedded into artificial lipid membranes, the addition of cargo bound receptor protein Pex5 is accompanied by a transient increase in ionic membrane permeability supporting the existence of a pore-like structure (Meinecke et al., 2010). The mechanisms by which receptor proteins release their cargo into the peroxisomal lumen are still unclear, but while in yeast the release of PTS1-carrying cargo from Pex5p involves of the yeast specific peroxin Pex8 (Ma et al., 2013), in mammals the release of cargo proteins is stimulated by a fragment of Pex14 (Freitas et al., 2011). After Pex5 has released its cargo, the receptor protein is recycled back to the cytosol to be available for another round of matrix protein import and possibly to create space for further integration of cargo loaded Pex5. This involves ubiquitination of a conserved cysteine close to the N-terminus of Pex5, the ATP-dependent extraction of Pex5 from the protein complex within the peroxisomal membrane and, finally, the removal of the ubiquitin moiety to regenerate a soluble and cargo-free receptor Pex5 (Platta et al., 2005; Francisco et al., 2014). Ubiquitination is exerted by specific machinery involving an ubiquitin conjugating enzyme (UBC, type E2) and a ubiquitin ligase (type E3). In yeast the UBC activity is exerted by Pex4 (Wiebel and Kunau, 1992; Platta et al., 2007) attached to the outer side of the peroxisomal membrane and the ubiquitin ligase activity is performed by the peroxisomal membrane proteins Pex10 and Pex12 (Platta et al., 2009, 2014). In mammals three homologous cytosolic proteins (UbcH5a-c) exert the UBC activity (Grou et al., 2008), whereas the ubiquitin ligase activity requires the peroxisomal proteins Pex2, Pex10, and Pex12, which might cooperatively exert the ligase activity (Francisco et al., 2014). The extraction of the mono-ubiquitinated Pex5 is exerted by the receptor extraction module (REM) consisting of a peroxisomal transmembrane protein (Pex26/Pex15) and two members of the AAA-ATPase family (Pex1 and Pex6), which utilize the energy of ATP hydrolysis to extract Pex5 from the membrane (Costa-Rodrigues et al., 2004; Platta et al., 2005) (**Figure 1A**) (for review see, Francisco et al., 2014; Platta et al., 2014). Deubiquitination of Pex5 is exerted by deubiquitinating

[3]For the sake of clarity some simplifications had to be made concerning the completeness of members of the membrane protein complexes and disregarding the import machineries for membrane proteins.

enzymes (Usp9x/Ubp15) (Debelyy et al., 2011; Grou et al., 2012). Importantly, membrane binding and integration of Pex5 neither requires ATP hydrolysis nor the cysteine in Pex5 nor a functional extraction module. This suggests that the whole energy demand of the peroxisomal import cycle is consumed during receptor extraction. Pex7 necessitates its interaction with the co-receptor not only for its import into peroxisomes, but also for its recycling that depends on the extraction of the co-receptor (Hensel et al., 2011; Liu and Subramani, 2013; Rodrigues et al., 2014). In most organisms, PTS2 carrying proteins are processed inside peroxisomes by the peroxisomal processing peptidase (PPP) releasing a prepeptide harboring the PTS2 (Helm et al., 2007; Kurochkin et al., 2007; Schuhmann et al., 2008). This peptidase is not only required for a functional processing, but also for a continuous peroxisomal protein import (Mizuno et al., 2013). Cytosolic chaperones are involved in the folding of cargo proteins before their transport, but should not be required inside peroxisomes.

Protein Import into Mitochondria

More than 99% of mitochondrial proteins are encoded by nuclear genes and translated by free ribosomes. Most soluble mitochondrial matrix proteins contain a targeting signal within their N-terminal amino acid sequence, termed the *presequence*, which mediates the interaction with membrane-bound receptor proteins that are part of a multi-protein complex, the *translocon of the outer mitochondrial membrane* complex (TOM complex; Tom20, 22, 40, 70, 5, 6, 7) (**Figure 1B**) (**Table 1**) (Neupert and Herrmann, 2007). Although proteins destined for the mitochondrial matrix are transported after translation is completed (post-translational), their folding is prevented by cytosolic chaperones of the Hsp70 family, because mitochondrial proteins are imported in an unfolded state. These chaperones and other proteinaceous factors of the 14-3-3 family (MSF, mitochondrial import stimulatory factor) support the translocation of preproteins to the mitochondrial membrane (Deshaies et al., 1988; Murakami et al., 1988; Hachiya et al., 1993; Komiya et al., 1997). There, the preprotein interacts sequentially with the mitochondrial receptor proteins Tom20 and Tom22 via different elements of its presequence (Brix et al., 1997; Saitoh et al., 2007). These receptors mediate the transfer of the presequence to the pore forming protein Tom40 (translocon) that channels the preprotein across the outer mitochondrial membrane in a linear mode from the N- to the C-terminus (N→C) (Model et al., 2008). This transfer is probably driven by the increasing affinity of the presequence to different components of the TOM-complex (*acid chain hypothesis*), which also involves a domain of Tom22 in the intermembrane space (Komiya et al., 1998; Kanamori et al., 1999). In the intermembrane space, the presequence interacts with the *translocon of the inner mitochondrial membrane* complex (TIM complex; Tim23, 50, 17, 21). First, the presequence binds to the primary receptor protein Tim50 (Yamamoto et al., 2002; Mokranjac et al., 2009) and is then transferred to the channel forming protein Tim23 (Truscott et al., 2001) that also interacts with the preprotein (Alder et al., 2008). During protein translocation TOM and TIM complexes are transiently linked to facilitate the transfer of a polypeptide across the double membrane (Chacinska et al., 2005; Tamura et al., 2009). The translocation of the presequence across the inner membrane is driven by the electrochemical force across this membrane ($\Delta\Psi$) acting on the positive charges of the presequence (Schleyer et al., 1982; Martin et al., 1991). The subsequent translocation of the complete polypeptide is facilitated by a dragging mechanism from the luminal side of the inner mitochondrial membrane enforcing the directionality of the import process. When the preprotein appears at the inner side of the Tim23 pore it is grasped by intramitochondrial Hsp70 proteins (mtHsp70). This requires the interaction of Tim23 with a multi protein complex, the ATP-coupled import motor (PAM, presequence translocase associated motor: Tim44, 14, 16, mtHsp70, Mge1), on the inner side of the membrane (Neupert and Herrmann, 2007). Within this protein complex the mtHsp70 proteins interacts with the preprotein in an ATP dependent manner, which prohibits sliding back of the preprotein (Neupert and Brunner, 2002). Further import of the preprotein exposes additional sequences that are again covered by mtHsp70 causing a net-onward movement either by ATP hydrolysis or simply due to the avoidance of backsliding (Neupert and Brunner, 2002). Inside the mitochondrial matrix, the preprotein is processed by the mitochondrial processing peptidase (MPP) releasing the N-terminal sequence (Gakh et al., 2002; Teixeira and Glaser, 2013). Finally, the processed protein is folded inside mitochondria by specific chaperones of the Hsp60 family (Cheng et al., 1989; Ostermann et al., 1989).

Protein Import into Chloroplasts

Chloroplast proteins that are encoded in the nucleus are equipped with a targeting signal within the N-terminal amino acid sequence termed *transit peptide* (Bruce, 2000). The proteins are synthesized in the cytosol and remain in an unfolded state until they interact with membrane bound receptor proteins at the surface of chloroplasts (**Figure 1C**) (Schleiff and Becker, 2011). This is supported by cytosolic chaperones of the Hsp70 protein family (Flores-Pérez and Jarvis, 2013) that cooperate either with proteins of the Hsp90 family that bind non-phosphorylated transit peptides and dock at a specific protein of the outer chloroplasts membrane (Toc64) (Qbadou et al., 2006; Fellerer et al., 2011) or with proteins of the 14-3-3 family that specifically bind to phosphorylated sequences within the transit peptides (Waegemann and Soll, 1996; May and Soll, 2000) (for review see, Lee et al., 2013). At the chloroplast surface, the transit peptide interacts sequentially with the receptor proteins Toc34 and Toc159 (Ma et al., 1996; Sveshnikova et al., 2000; Smith et al., 2004), which are only representatives of larger receptor protein families (Toc33 and Toc90, 120, or 132) (Jelic et al., 2003; Smith et al., 2004). All members can contribute to protein import, but have been characterized with different profiles of transit peptide recognition (Kubis et al., 2004; Demarsy et al., 2014). These receptor proteins are part of a large protein complex (TOC: translocon of the outer envelope of chloroplasts, TOC34, 159, 75, 64, 12) involving the specific binding protein for Hsp90 proteins (Qbadou et al., 2006) and the pore-forming Toc75 protein that performs the translocation of the transit peptide across the chloroplast outer membrane (Hinnah et al., 2002).

Importantly, both chloroplast receptor types are GTPases that can form homo- and heterodimers via their GTPase domain and they are able to couple nucleotide hydrolysis with the binding of transit peptides and a change in the dimerization status (Smith et al., 2002; Sun et al., 2002; Rahim et al., 2009). Accordingly, non-hydrolyzable GTP interferes with protein import (Schnell et al., 1994; Young et al., 1999), although the GTPase activity of individual receptor proteins is dispensable (Agne et al., 2009; Aronsson et al., 2010). Preproteins are handed over to the translocon Toc75 by a well-defined cycle of events, in which both receptor proteins change their dimerization status, their interaction partner and the phosphorylation state of the bound guanine nucleotide. The transit peptide opens the Toc34 dimer, stimulates its GTPase activity, and initiates its heterodimerization with Toc159, which is prerequisite for the transfer of the transit peptide to Toc159 (Paila et al., 2015). The transit peptide has to be dephosphorylated to bind to Toc159 and the sequence recognized by Toc159 overlaps with the Toc34 binding site, although it is not identical (Schleiff et al., 2002; Becker et al., 2004; Smith et al., 2004; Lee et al., 2009). Finally, the transit peptide is transferred by Toc159 in its GTP-bound state to the translocon Toc75 (Wang et al., 2008), which opens for the translocation of the transit peptide upon GTP hydrolysis in Toc159 (Schleiff et al., 2003). The transit peptide directly interacts with Toc75 (Perry and Keegstra, 1994; Hinnah et al., 2002), but then reaches through the Toc75 channel to interact with a chaperone in the intermembrane space (IAP70, Schnell et al., 1994; Ma et al., 1996), which supports the transfer of the preprotein across the outer envelope membrane. Next, the transit peptide interacts with Tic22 (Kouranov et al., 1998) and finally with a protein complex in the inner membrane (TIC: translocon of the inner envelope membrane; Tic100, Tic214, Tic56, Tic20/Tic21, and Tic40) that mediates the translocation of the preprotein across the chloroplast inner envelope membrane (Kikuchi et al., 2013; Nakai, 2015; Paila et al., 2015). This protein complex can appear with slightly different components, but shares Tic20 (Kouranov et al., 1998; Kovács-Bogdán et al., 2011) or its functional homolog Tic21 (Teng et al., 2006), and Tic110 (Heins et al., 2002). These proteins have been suggested as the key components of the TIC channel and have been directly linked to the channel function (Heins et al., 2002; Balsera et al., 2009). The stromal part of Tic110 interacts with transit peptides as they emerge from the pore (Inaba et al., 2003). Moreover, it forms a platform together with the membrane-bound co-chaperone Tic40, which links the pore with a complex protein machinery that supports preprotein import. This machinery consists of Hsp90 (Inoue et al., 2013), the motor chaperone Hsp93 (Chou et al., 2003, 2006), and the stromal Hsp70 (CpHsp70) (Latijnhouwers et al., 2010). The latter two proteins interact directly with transit peptides *in vitro* (Ivey et al., 2000) and a lack of these proteins interferes with preprotein import (Su and Li, 2008, 2010). Protein import into chloroplasts requires GTP hydrolysis during the early steps of transit peptide insertion (Young et al., 1999), but the translocation of the whole preprotein is driven by ATP hydrolysis by stromal chaperones and partially of a chaperone in the intermembrane space (Flügge and Hinz, 1986). When soluble proteins reach the stroma they are processed by the stromal processing peptidase (SPP) (Richter and Lamppa, 1998; Trösch and Jarvis, 2011) and protein folding is supported by members of the Hsp60 family (Cnp60, chaperonin 60) (Lubben et al., 1989; Kessler and Blobel, 1996).

Protein Import into the ER

Soluble proteins that are determined for an insertion into the ER harbor an N-terminal signal peptide, which is often cleaved off upon import (Blobel and Dobberstein, 1975; Schatz and Dobberstein, 1996). However, the recognition of the signal peptide can occur either during translation inducing a translational arrest until the ribosome has docked to the ER (cotranslational protein import) or after translation is completed (post-translational protein import) requiring the contribution of cytosolic chaperones that retain the proteins in an import competent unfolded state (**Figure 1D**) (Walter and Lingappa, 1986; Zimmermann et al., 2011; Johnson et al., 2013b). The choice of the transport route is influenced in the yeast by the hydrophobicity of the targeting signal (Ng et al., 1996) and in metazoa by the size of the protein (Johnson et al., 2013a).

The *co-translational protein import* is initiated by the interaction between the signal peptide and the soluble signal recognition particle (SRP) representing the cognate receptor protein. This SRP is a GTP-hydrolyzing ribonucleoprotein complex comprised of one (prokaryotes) or more (e.g., six in metazoa) proteinaceous components and an RNA (Akopian et al., 2013). This system is functionally equivalent to the bacterial protein export machinery and many contributions have been initiated by findings in this field. One domain (M-domain) of the key subunit (Srp54) exerts the binding to the signal peptide (Clemons et al., 1999), whereas the other domain (NG-domain) mediates the interaction with the membrane-bound docking site (SRP receptor, SR) (Schwartz and Blobel, 2003; Halic et al., 2004). The SRP and the SR contain GTPase domains and interact via these domains (Akopian et al., 2013). The recognition of a signal peptide occurs within a large protein complex consisting of the ribosome, the nascent chain of the cargo protein appearing at the ribosomal exit tunnel [together forming the ribosome nascent chain complex (RNC)] and the SRP scanning the N-terminus of the newly synthesized protein. A suitable signal peptide initiates a conformational change in the SRP that stalls translation and allows the interaction of the SRP with the membrane bound SR at the ER surface (docking site). In eukaryotes this SR is a heterodimer consisting of a soluble α- and a membrane bound β-subunit (Tajima et al., 1986; Schwartz and Blobel, 2003), which is directly linked to the Sec61 complex involving the pore forming Sec61α protein (translocon) (Wiedmann et al., 1987). Both the SRP and the SR contain GTPase modules that mediate their interaction, but also regulate the interaction between these protein complexes by switching between the GTP- and GDP-bound state (Focia et al., 2004). GTP-bound SR binds to cargo-loaded SRP and hydrolysis of SR bound GTP is coupled to the release of the SRP into the cytosol for recycling. Moreover, the rate of GTP hydrolysis in the SRP affects the interaction time with the RNC, the attachment of the SRP-RNC complex at the ER membrane and the release of the signal peptide from the SRP. In addition, structural rearrangements within this large protein

complex occur independently of local conformational changes upon GTP hydrolysis, which generate a complex cycle of events. During this process the RNC is transferred from the SRP to the Sec61 complex, which initiates the insertion of the nascent chain into the Sec61α channel and the sealing of Sec61α by the ribosome. Finally, GTP hydrolysis by the SRP is associated with a conformational change that initiates the release of the SRP from the ribosome, which allows the resumption of translation for an efficient coupling of protein synthesis and the transport of the newly synthesized protein across the ER membrane.

Post-translational protein import acts on proteins that pass the scan of the N-terminal sequence by the SRP, e.g., because hydrophobicity is below a certain threshold. These proteins remain unfolded and translocate independently to the surface of the ER, which requires cytosolic proteins of the Hsp70 and the Hsp40 family (Chirico et al., 1988; Dierks et al., 1993; Ngosuwan et al., 2003). There, the signal peptide interacts with the Sec61 translocon (Johnson et al., 2012) and releases cytosolic chaperones (Plath and Rapoport, 2000). In yeast, the transfer across the membrane is exerted by the Sec61 complex (αβγ) in cooperation with additional proteinaceous factors that have been described as Sec62/Sec63 complex (Panzner et al., 1995), which is comprised of Sec62p, Sec63p, Sec71p, and Sec72p (Lyman and Schekman, 1997). However, the latter two proteins are not essential and absent in mammals. In contrast to co-translational protein import, the energy for translocation is provided by luminal chaperones of the Hsp70 family (Kar2p/Grp78/BiP) that bind to the Sec62/63 complex and pull preproteins through the Sec61 channel, which renders the process ATP-dependent (Hansen et al., 1986).

Thus, the energy required for preprotein translocation following the initial transfer of the signal peptide is provided either by the GTPase activity of the ribosome during translation, which pushes the linear protein through the Sec61-translocon (co-translational) or by the ATPase activity of the luminal chaperone (Kar2p/Grp78/BiP) that drags the proteins into the ER. Two models have been suggested to account for the directionality of the translocation, which is accomplished by luminal chaperones. Either the chaperone utilizes the energy of ATP hydrolysis to exert a series of individual dragging steps or it progressively covers those parts of the preprotein, which appear at the luminal side and thereby prohibits the back-slipping of the preprotein (Elston, 2002). Independently of the import mode, the N-terminal signal peptide is cleaved off by a peptidase (signal peptidase) (Weihofen et al., 2002) in the ER lumen and a variety of luminal chaperones assist the folding of the protein within the ER (Braakman and Bulleid, 2011).

Comparison between the Transport Routes

Altogether, the mechanisms of protein transport from the cytosol into peroxisomes, mitochondria, chloroplasts, and the ER differ remarkably, but the import can be initiated by targeting signals proximal to the N-terminus (PTS2 for peroxisomes), which become processed during or after the import. Peroxisomal protein import differs from other import mechanisms in several important aspects: (i) It acts on fully folded proteins, whereas the post-translational import routes and the import into the ER all translocate proteins in an unfolded state; (ii) Cytosolic chaperones are required for protein folding, but are not as essential for protein transfer to the peroxisomal membrane as they are for post-translational import into mitochondria, chloroplasts or the ER; (iii) The peroxisomal receptor proteins (Pex5 and Pex7) are predominantly soluble like the SRP, whereas the other receptor proteins (Tom20 and Toc34) are membrane bound and receive the majority of proteins via chaperone assisted transfer. (iv) The peroxisomal import of folded proteins needs a flexible pore with large diameter, which is provided by the dynamic cooperation of Pex14 with the receptor Pex5, whereas the translocon structures of mitochondria (Tom40), chloroplasts (Toc34), or the ER (Sec61α) have a small, but defined diameter and permit the channeling of unfolded linear proteins across the membrane; (v) Cargo-loaded peroxisomal receptor proteins (Pex5 and Pex7) integrate into the membrane and reach into the organellar lumen to release their cargo proteins inside peroxisomes, which requires an energy-consuming extraction of the receptor to recycle it to the cytosol; (vi) ATP hydrolysis for receptor extraction is the sole nucleotide triphosphate-consuming step of peroxisomal import, whereas the transfer of unfolded proteins through the translocons of mitochondria, chloroplasts, and the ER requires intraorganellar ATP hydrolysis by chaperones to pull the preproteins into the organelle. In addition, the forward motion of translation that is driven by GTP hydrolysis provides energy for the co-translational import into the ER. These processes are distinct from the energy consumption for the pathfinding of N-terminal signals, which involves GTP hydrolysis for the signal peptides (ER) and transit peptides (chloroplasts). (vii) As peroxisomal proteins are imported in a folded state, they do not require extensive folding inside the organelle, whereas all other organelles have an elaborate folding machinery inside.

Targeting Signals and Their Receptors

Targeting signals have been described as amino acid sequences necessary and sufficient for the proper localization of a protein, which emphasizes the functional properties of these sequence elements. Alternatively, targeting signals could be defined by their ability to mediate an interaction between the protein harboring the signal and a receptor protein, which is required to initiate protein transport across a specific organellar membrane. The majority of soluble proteins enclosed in mitochondria, chloroplasts or the ER harbor targeting signals that are all encoded within the N-terminal region of the protein. Within the target organelle, a short N-terminal fragment including the targeting signal is cleaved off the protein. The type 2 peroxisomal targeting signal (PTS2) was known to resemble these targeting signals with regard to its position within the primary sequence of the protein and to the intraperoxisomal processing. However, recent investigations elucidated the structural properties of the PTS2 and its binding mode to its receptor, which revealed further similarities to other N-terminal targeting signals. In contrast, the PTS1 resides at the extreme C-terminus and is recognized by another receptor protein. Thus, we mainly compare the properties of the N-terminal targeting signals and only briefly

touch on PTS1, because the comparison of the two peroxisomal targeting signals will be required in later chapters.

Peroxisomal Targeting Signals

Although the two *peroxisomal targeting signals* (PTS1 and PTS2) have been amply described in various organisms and their receptor proteins have been identified, individual soluble peroxisomal proteins have been identified that do not encode any of these signals. This has originally been attributed to a potential third type of peroxisomal targeting signals (PTS3), but this signal has never been characterized and the import either depends on a PTS-independent interaction with a receptor protein (Klein et al., 2002) or on co-import of proteins (piggy-back), which is a specific property of peroxisomal import (Yang et al., 2001; Subramani, 2002; van der Klei and Veenhuis, 2006; Islinger et al., 2009).

PTS2 and Its Interaction with the Receptor Protein Pex7

The observation that a peroxisomal targeting signal is encoded in proximity to the N-terminus of the rat peroxisomal enzyme thiolase led to the identification of the PTS2 (Osumi et al., 1991; Swinkels et al., 1991), which was later also identified in yeast and plants (Gietl et al., 1994; Glover et al., 1994). The consensus sequence has originally been described as (R/K)-(L/V/I)-X_5-(Q/H)-(L/A)[4] (**Figure 2A**) highlighting two conserved dipeptide motifs separated by five arbitrary amino acids, which are sensitive to different point mutations (Glover et al., 1994; Tsukamoto et al., 1994). Later on, this motif was extended to R-(L/V/I/Q)-*X*-*X*-(L/V/I/H)-(L/S/G/A)-*X*-(H/Q)-(L/A) based on a compilation of the most common PTS2 variants (Petriv et al., 2004). This suggested a previously unrecognized conservation at the central amino acid X_3, which was consistently found to present with large and hydrophobic properties (Petriv et al., 2002; Reumann, 2004; Kunze et al., 2011). In a reporter construct harboring the N-terminus of rat thiolase, the functionality of the PTS2 was destroyed by a substitution of residue X_3 with a negatively or positively charged amino acid (Kunze et al., 2011). Based on the sequence of charged/polar and hydrophobic residues, an α-helical structure with two turns was suggested, which orients all key residues of the consensus sequence toward one side of this helix (**Figure 2E**). Moreover, PTS2 motifs are highly enriched in amino acids overrepresented in helical structures and the introduction of the helix-breaking amino acid proline at the least conserved position of a prototypical PTS2 abrogated its functionality (Kunze et al., 2011). This was in line with previous suggestions of a helical structure for PTS2 motifs based on the paucity of proline residues within PTS2 motifs (Reumann, 2004) and the observation that a PTS2-destroying point mutation in the rat thiolase N-terminus generated a mitochondrial targeting signal *de novo* (Osumi et al., 1992). Finally, this suggestion was confirmed by the elucidation of the 3D structure of the N-terminus of the yeast ortholog of thiolase (Fox3) in a receptor bound state, in which the PTS2 non-apeptide presented as α-helix (Pan et al., 2013). Altogether, the linear PTS2 non-apeptide corresponds to an α-helix, in which one flank is occupied by the key residues that align amino acids of the same property. When comparing the N-terminal sequences of PTS2-containing proteins, the region upstream of the PTS2 was found enriched in acidic residues (Reumann, 2004; Kunze et al., 2011), whereas the region downstream of the PTS2 contains many amino acids, which are typical for unstructured stretches (Kunze et al., 2011). The latter probably reflects a linker domain, which serves the exposure of the PTS2 helix from the fully folded core protein. Accordingly, a similar linker domain has been described next to the PTS1 (Neuberger et al., 2003c), but was not observed in proteins that are imported in an unfolded state into other organelles. In addition, the flexible linker domain of PTS2-carrying proteins could also be necessary for the exposition of the processing site toward the peptidase inside peroxisomes.

The PTS2 receptor Pex7 has been identified in various organisms as a protein essential for the import of PTS2-encoding proteins (Marzioch et al., 1994; Braverman et al., 1997; Woodward and Bartel, 2005). It belongs to the family of WD40 domain proteins sharing a cone-like shape (Stirnimann et al., 2010). Thus, the structure of the human PEX7 protein has been predicted several times by independent groups (Braverman et al., 2002; Stanley and Wilmanns, 2006; Kunze et al., 2011), although early predictions were hampered by the lack of closely related template structures. However, the identification of the PTS2 binding site within these structures was difficult, until the pattern of evolutionary highly conserved surface residues was taken into account to identify the most important areas of the protein. This allowed the identification of a groove on top of the cone structure of human PEX7, which is covered with residues that are suitable for an interaction with the conserved side of a PTS2 helix (**Figure 2I**) (Kunze et al., 2011). This prediction was verified experimentally (Kunze et al., 2011) and the elucidation of the 3D structure of yeast Pex7 together with the N-terminus of thiolase confirmed the suggested model (Pan et al., 2013). Thus, the α-helix is located horizontally in a shallow groove on the top side of the Pex7 cone burying about half of the helix. The interaction obtains energetic contributions from several hydrophobic, but also from ionic and hydrogen bonds, which is conserved across evolution from yeast to man. However, this interaction appears to be weak until co-receptor binding transforms the cargo-bound receptor into a stable trimeric complex (Mukai and Fujiki, 2006; Pan et al., 2013; Kunze et al., 2015). This has been originally attributed to a conformational change in the receptor (Mukai and Fujiki, 2006), but the structural resolution of the yeast trimeric complex consisting of Pex7, Fox3, and a part of the yeast co-receptor Pex21 indicated that the co-receptor directly interacts with residues of the PTS2 helix (**Figure 2I**) (Pan et al., 2013). Furthermore, the interaction of Pex7 and the co-receptor is dependent on the presence of a cargo protein (Grunau et al., 2009; Kunze et al., 2015) and on the ability of Pex7 to bind the cargo protein (Kunze et al., 2015). This suggests that the co-receptor is able to discriminate PTS2-like motifs, which are bound to Pex7, but expose residues with different physical properties from the Pex7 averted side of the helix. Accordingly, Pex7 and its

[4]For the PTS2 consensus sequence we introduced a nomenclature that indicates all residues independently, but highlights the key residues with S and the arbitrary residues with X: S1-S2-X1-X2-X3-X4-X5-S3-S4 (Kunze et al., 2011).

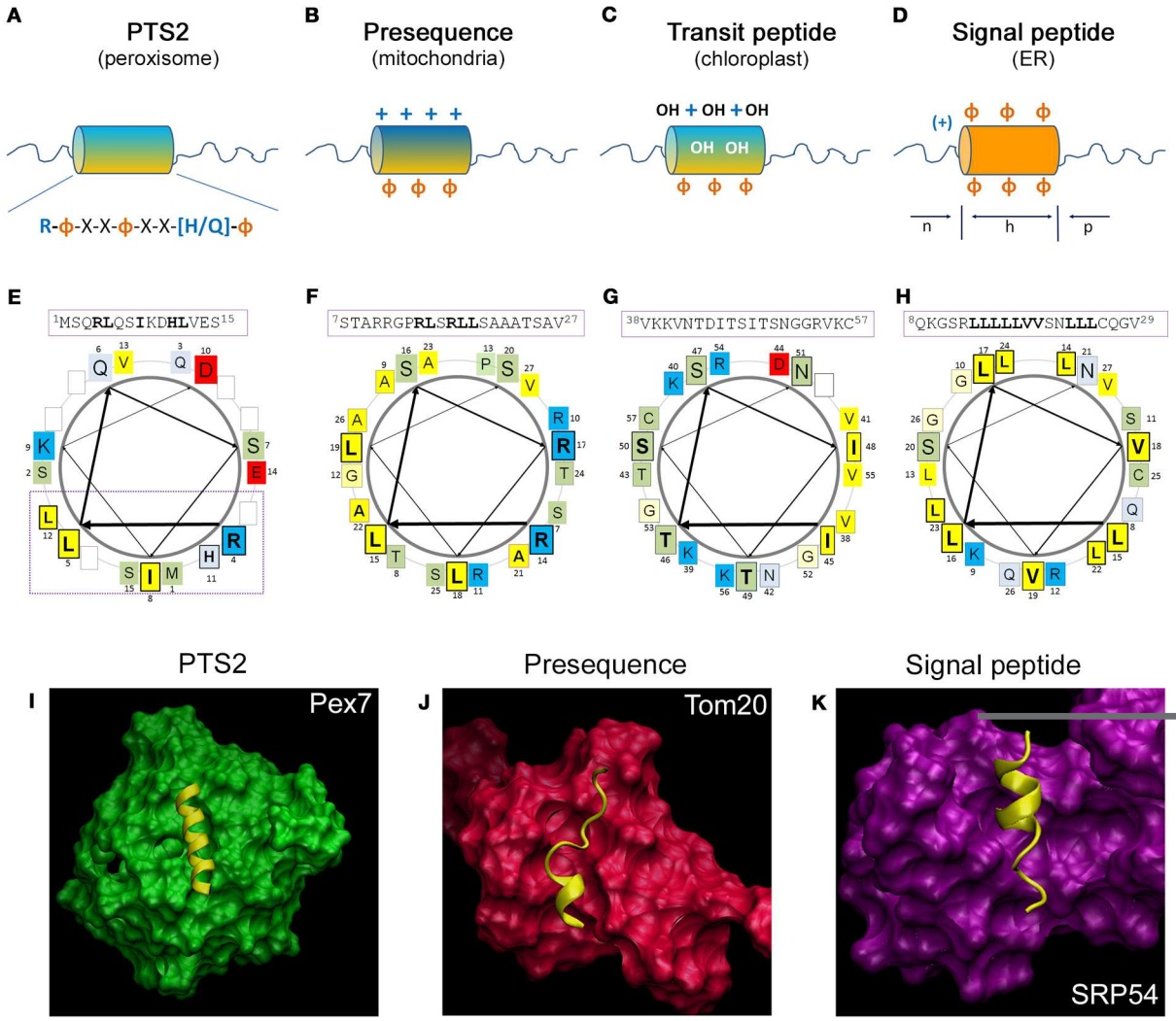

FIGURE 2 | Comparison of the structural properties of N-terminal targeting signals and their interaction with the receptor proteins. (A–D) *Schematic representation of the N-terminal amino acid sequences encoding different targeting signals*: **(A)** the peroxisomal PTS2 forming an α-helical domain encoding the consensus sequence, which is followed by an unstructured sequence element; **(B)** the mitochondrial presequence is enriched for positive charges and forms an amphipathic α-helical domain, **(C)** the chloroplast transit peptide sequence is enriched in hydroxylated amino acids; and **(D)** the signal peptide for the ER is composed of a positively charged (n)-domain, a hydrophobic (h)-domain, and a polar (p)-domain. **+,** positive charges; OH, hydroxylated residues; Φ, hydrophobic residues; *orange,* hydrophobic side; *blue,* hydrophilic side of the helix. **(E–H)** *Helical wheel depiction of typical N-terminal targeting signals*: **(E)** the PTS2 of yeast thiolase (*Sc*Fox3), **(F)** the presequence of rat aldehyde dehydrogenase (*Rn*Aldh2), **(G)** the transit peptide of pea ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*Ps*prSSU), and **(H)** the signal peptide of bovine preprolactin (*Bt*PRL). The amino acid sequences depicted in the α-helical wheel projections are indicated above using the numbering of the primary sequence; amino acids of the central turn are indicated by larger letters; residues of the PTS2 consensus sequence, residues of the presequence interacting with Tom20, the hydroxylated residues of the transit peptide and the hydrophobic patch of the signal sequence are indicated bold and boxed. The color code for the physical properties of the residues is as follows: *acidic* red, *basic* blue, *hydrophobic* yellow, *polar basic* bluish gray and *polar neutral* green. The arrows indicate the progression of the amino acid sequence within the α-helical wheel. **(I–K)** *3D structure of the receptor protein and the α-helix of the targeting signal:* **(I)** the N-terminus of yeast Fox3 involving a PTS2 (yellow) together with the receptor protein Pex7 (green), **(J)** the presequence of rat Aldh2 (yellow) together with the soluble domain of Tom20 (red), **(K)** the leader peptide of yeast dipeptidylpeptidase B (yellow) together with the cargo binding domain of archeal Srp54. The structures have been generated by the program *visual molecular dynamics* (VMD) (Humphrey et al., 1996) based on the datasets PDB:3W15 (Pan et al., 2013) (I), PDB:1OM2 (Abe et al., 2000) (J) and PDB:3KL4 (Janda et al., 2010) **(K)**.

co-receptor could be considered as bipartite receptor, in which Pex7 exerts a preselection of putative cargo proteins, which are subsequently approved by the binding of the co-receptor. Such mechanism would enlarge the area of the receptor that scans a PTS2 motif and increases the number of residues encoding targeting information.

PTS1 and Its Interaction with the Receptor Protein Pex5

The PTS1 has been identified as peroxisomal targeting signal mediating the import of firefly luciferase into peroxisomes of monkey cells (Gould et al., 1987). The signal is located at the extreme C-terminus of the protein (Gould et al., 1988) and the

minimal targeting signal has been narrowed down to a tripeptide consisting of serine, lysine, and leucine (–SKL) or conserved variants thereof (Gould et al., 1989; Swinkels et al., 1992). Later on, it was found that the interaction with the receptor protein Pex5 is also severely affected by the preceding sequence (Lametschwandtner et al., 1998) that mediates flexibility for a proper exposure of the PTS1 from the folded core protein (Neuberger et al., 2003a; Brocard and Hartig, 2006).

Proteins encoding a PTS1 interact with the receptor protein Pex5 (Van der Leij et al., 1993; Dodt et al., 1995; Wiemer et al., 1995; Kragler et al., 1998) via a tetratricopeptide repeat (TPR) domain covering the C-terminal half of the receptor (Brocard et al., 1994; Dodt et al., 1995). The structure of the TPR resembles a bent half-pipe (Gatto et al., 2000), into which the last three amino acids of the cargo proteins insert and thereby induce a conformational change (Stanley et al., 2006; Fodor et al., 2015).

Mitochondrial Targeting Signals

Research on the N-terminal part of mitochondrial preprotein sequences (*presequence*) encoding the mitochondrial targeting signal revealed that these sequences do not present with a conservation pattern at the level of the primary amino acid sequence, which could be converted into a consensus sequence. However, these sequences share preferences in physicochemical properties and the frequency of individual amino acids such as an overrepresentation of positively charged residues and, more specifically, of arginine, whereas negatively charged residues are nearly absent (**Figure 2B**) (von Heijne et al., 1989; Huang et al., 2009). Accordingly, mitochondrial targeting signals can be generated quite easily *de novo* by mutations (Vassarotti et al., 1987) or insertion of arbitrary amino acid sequences at the N-terminus of a protein (Lemire et al., 1989). Moreover, these sequences contain elements with a high propensity to form α-helices with amphipathic properties, in which hydrophobic residues cover one side and positively charged residues the other side of the helix (Roise et al., 1986; von Heijne, 1986). The α-helical element of the rat aldehyde dehydrogenase (Aldh2) presequence, which binds the cytosolic part of the receptor Tom20, consists of a six amino acid core element (^{14}RLSRLL^{19}) (Abe et al., 2000; Muto et al., 2001) (**Figure 2F**). Comparison of mitochondrial presequences revealed the conserved pattern φχχφφ, in which φ represents a bulky hydrophobic residue and χ indicates any amino acid (Obita et al., 2003), although substitutions of φ by alanine are partially tolerated (Mukhopadhyay et al., 2006).

The elucidation of the 3D structure of cargo-loaded Tom20 (Abe et al., 2000; Saitoh et al., 2007) revealed that the amphipathic helix of the presequence lays within a broad, shallow binding groove consisting of a four helix bundle (**Figure 2J**). The hydrophobic residues comprising one side of the amphipathic helix reach into the hydrophobic binding groove of Tom20, whereas the positive charges of the presequence interact with negatively charged residues at the border of the binding groove (Abe et al., 2000). Thus, the interaction between signal and receptor is mediated by hydrophobic and ionic interactions, although it appears insensitive to the salt concentration (Brix et al., 1997). Interestingly, a peptide can interact with Tom20 in more than one binding state, which fits with a certain degree of mobility of the peptide within the binding groove and the acceptance of divergent peptides as interaction partners (Saitoh et al., 2007, 2011).

Chloroplast Targeting Signals

The N-terminal sequences of soluble chloroplast proteins, called *transit peptides* (Bruce, 2000), encode targeting information, which involves binding motifs for receptor proteins of the Toc34 and the Toc159 family and binding sites for Hsp70 (Rial et al., 2000; Zhang and Glaser, 2002) and Hsp90 proteins (Qbadou et al., 2006). Moreover, specific sites within transit peptides facilitate their phosphorylation, which has not been observed in mitochondrial presequences (Waegemann and Soll, 1996; May and Soll, 2000), but is required for the interaction with 14-3-3 proteins (May and Soll, 2000). Transit peptides show a characteristic amino acid distribution, but a consensus sequence cannot be delineated from primary sequences of naturally occurring transit peptides (Bruce, 2001). This is in line with a high promiscuity of the import system for arbitrary N-terminal peptides. Naturally occurring transit peptides are rich in hydroxylated amino acids (von Heijne et al., 1989), whereas negative charges are underrepresented and, in contrast mitochondrial presequences, arginines are not overrepresented (von Heijne et al., 1989) (**Figure 2C**). On a helical wheel prediction, typical transit peptides encode a domain, which shows amphipathic properties due to a hydrophobic and a positively charged hydrophilic patch on opposite sides of the α-helix, but between these elements polar wedges of hydroxylated residues and occasionally negativly charged residues seem to be present (Bruce, 2000) (**Figure 2G**). The structure of the transit peptide of ribulose bisphosphate carboxylase (Rubico) activase from the green algae *Chlamydomonas reinhardii* has been resolved confirming the α-helical domain (Krimm et al., 1999). However, transit peptides are predominantly unstructured in aqueous environment (Bruce, 1998; Krimm et al., 1999), which fits to their amino acid distribution (von Heijne and Nishikawa, 1991), but in hydrophobic environment the fraction of α-helical elements increases (Endo et al., 1992; Bruce, 1998; Krimm et al., 1999). However, these common properties of all transit peptides are complemented by more specific ones, which allow the discrimination of transit peptides by different members of the Toc159 receptor family (Jelic et al., 2003; Demarsy et al., 2014; Dutta et al., 2014). This is compatible with the observation that within a transit peptide the binding sites for Toc34 and Toc159 are only partially overlapping leaving space for receptor discrimination. Thus, the relative affinity of a transit peptide to different receptor proteins determines the transport route of the encoding protein into different types of plastids.

At the chloroplast surface, transit peptides interact with the receptor Toc34 in a first step and, subsequently, with different members of the Toc159 receptor family. The first resolution of the 3D structure of pea Toc34 identified the GTP binding domain within the overall structure of the receptor (Sun et al., 2002), whereas more recent investigation studied the monomeric and dimeric state of the receptor (Koenig et al., 2008). In the latter study, a groove was identified in proximity to the GTP

binding site, which has been proposed as transit peptide binding site (Koenig et al., 2008). However, a 3D structure of Toc34 together with a transit peptide is not available and, thus, cannot be presented here (**Figure 2**).

Targeting Signals for the ER

The signal determining a protein for the import into the ER/secretory apparatus has been already described in 1981 (Kreil, 1981). Detailed analysis of available signal sequence revealed that signal peptides are usually rich in hydrophobic residues with a core element composed of a positively charged domain, a hydrophobic domain of 8–12 amino acids and a polar C-terminal region, which have been denominated as [n]-domain, [h]-domain, and [c]-domain (Briggs and Gierasch, 1984; von Heijne, 1985; Gierasch, 1989) (**Figure 2D**). Individual changes in the charge pattern of the [n]-domain or of the [c]-domain had little effect, whereas a shortening of the [h]-domain had severe consequences for the import of a reporter protein (Nilsson et al., 2015) and the presence of several positive charges in the [c]-domain was also detrimental (Fujita et al., 2011). According to their hydrophobic character signal peptides are often not soluble in water, but form α-helical domains in hydrophobic environment (Briggs and Gierasch, 1984; Yamamoto et al., 1990), which can be depicted on a helical wheel projection for a typical signal peptide (**Figure 2H**). The importance of the hydrophobic helical element is further supported by detrimental effects of a single charged and helix breaking residue within the [h]-domain (Bruch et al., 1989; McKnight et al., 1989; Rothe and Lehle, 1998). However, in contrast to previous assumptions (Bird et al., 1987), the hydrophobic properties alone are not directly correlated with the quality of the signal peptides, and an excess of hydrophobic residues was found detrimental for signal peptides (Huber et al., 2005). A comparison of naturally occurring signal peptides could not delineate a conservation pattern that allows the definition of a consensus sequence. Accordingly, the signal sequences are often resistant to mutations (Gierasch, 1989) and many arbitrarily generated N-terminal sequences can act as signal peptides (Kaiser et al., 1987) similar to the signals recognized by the chloroplast and mitochondrial import systems.

The 3D structure of the ligand-binding domain of the SRP has been first resolved for Srp54 of *Thermus aquaticus* (Keenan et al., 1998), but later on also the M-domain of the human Srp54 protein has been resolved (Clemons et al., 1999). Moreover, these complexes were analyzed together with the nascent chain bound ribosome and the SRP receptor (Halic et al., 2004, 2006). However, the interaction between the SRP and a signal peptide has only been elucidated with high resolution for archaeal SRPs (Janda et al., 2010; Hainzl et al., 2011). The binding site for the signal peptide is composed of four helices that form a groove, which is limited on one side by the finger domain of the RNA. The binding groove is covered with hydrophobic residues with mobile side chains, especially methionines, supporting the flexibility in cargo selection (Bernstein et al., 1989). Moreover, more than one binding mode for signal peptides have been obtained in archeal Srp54 proteins (Janda et al., 2010; Hainzl et al., 2011). We depict the archaeal Srp54 structure together with a signal peptide (**Figure 2K**) (Janda et al., 2010) in spite of the evolutionary distance between archaea and eukaryotes, because the structural conservation between the protein complexes (RNC-SRP-SR) has recently been demonstrated (Halic et al., 2006) and the eubacterial Srp54 homolog can even be functionally integrated into the mammalian SRP (Bernstein et al., 1993). Illustratively, this depiction demonstrates the similarity of the binding mode of a signal peptide to Srp54 proteins with that of other targeting signals their receptor proteins.

However, co-translational protein import is only one path into the ER, whereas post-translational import is independent of the recognition of a signal peptide by the SRP. Thus, the existence of two alternative pathways suggests that certain properties of the signal peptides specify them for one of these transport routes, although all N-terminal amino acid sequences that successfully mediate the import of the encoding protein into the ER are considered signal peptides. The co-translational transport route requires the early recognition of the signal peptide upon its appearance at the ribosomal exit site, whereas the post-translational transport route skips this recognition, but the protein needs to remain unfolded. Accordingly, in yeast the hydrophobicity of the signal peptides was suggested as primary determinant favoring co-translational protein import (Ng et al., 1996), whereas in multicellular animals the post-translational protein import appears restricted to small proteins (Johnson et al., 2012, 2013a).

Comparative Summary

The PTS2 and targeting signals for soluble proteins of mitochondria, chloroplast or the ER share their position within an N-terminal sequence element that is cleaved upon import into the target organelle and the involvement of an α-helical domain that mediates the interaction with the receptor protein. However, the targeting signals for mitochondria, chloroplasts, and the ER are highly diverse and relatively robust against single amino acid substitutions. Moreover, these signals can be easily generated *de novo*, whereas the PTS2 has a clear consensus sequence consisting of five key positions which are sensitive to amino acid substitutions. The composition of the complete N-terminal sequences shows characteristic patterns for each organelle, but in case of the PTS2 the unstructured domain following the consensus sequence appears most obvious. The α-helical elements of the signals bind to the receptor proteins in a similar mode with one side of the helix embedded into a binding groove on the receptor surface (**Figures 2I–K**). However, in Pex7 the binding groove is narrower compared to the other receptors, which is in agreement with its binding of peptides with a well-defined consensus sequence, whereas Tom20 and Srp54 require more flexibility to enable binding of peptides with variable primary sequence. Moreover, the helical element encoding the PTS2 (I) appears longer when compared to that encoding the presequence (J) or the signal peptide (K), although on average the α-helical elements should have comparable length (Gierasch, 1989; Moberg et al., 2004; Kunze et al., 2011; Nilsson et al., 2015). However, this might be due to the tight cargo binding of Pex7 in the presence of the co-receptor (not shown), which forces the peptide into a well-defined structure. In contrast, only a short sequence element of the presequence or of the signal

peptide has to be in a helical conformation, whereas the larger binding groove of Tom20 or Srp54 might be compatible with other forms of cargo binding. In all cases, the major fraction of the interaction area between α-helix and receptor protein is covered by hydrophobic residues, whereas ionic interactions are restricted to the edges of the binding groove. A characteristic of the PTS2-Pex7 interaction is the contribution of the co-receptor protein that enlarges the interaction area and increases the affinity.

Similarity of Targeting Signals and the Specificity of Protein Transport

Although the N-terminal targeting signals for mitochondria, chloroplasts, the ER and also for peroxisomes (PTS2) are structurally similar, the accurate distribution of proteins between different subcellular compartments demonstrates that protein transport is highly specific. At first glance, the N-terminal amino acid sequence of a newly synthesized protein is concomitantly exposed to all available receptor proteins, which compete for the N-terminal sequence (**Figure 3A**). Accordingly, the specificity of protein transport can only be achieved by promoting the interaction between an N-terminal amino acid sequence and its appropriate receptor protein, whereas interactions with undesired receptor proteins that would induce mistargeting must be avoided. However, in reality the different receptor proteins scan an N-terminal amino acid sequence during distinct phases of protein formation, because of the different mechanisms of protein import (**Figure 3B**). The recognition of a signal can occur either directly upon its appearance at the exit site of the ribosome (signal peptide), or after translation, when the unfolded protein reaches the organellar membrane (presequence, transit peptide, signal peptide) or after completion of protein folding (peroxisomal targeting signal). This implicates that an early decision in favor of one transport route might exclude other routes that are initiated by receptor interactions at a later stage of protein formation. Thus, the properties of the protein import machineries modulate the specificity of protein transport, although the relative affinity of an N-terminal amino acid sequence to different receptor proteins remains a crucial determinant for the choice of the transport route.

Relative Affinity of Targeting Signals to Different Receptor Proteins

The effectivity of the interaction between an amino acid sequence and a receptor protein should correlate with the quality of this sequence as targeting signal. This gets even more important under conditions, when different receptor proteins compete for the same amino acid sequence and, thus, the relative affinity of this sequence for diverse receptor proteins appears as key determinant for targeting specificity. In this case, the fitting between a targeting signal and the signal binding domain of its cognate receptor protein should be much better than with any other receptor protein, which favors the formation of the desired receptor-cargo interaction (*positive discrimination*). However, the idea of tight fitting is inconsistent with the conspicuous degeneration of targeting signals and the high portion of

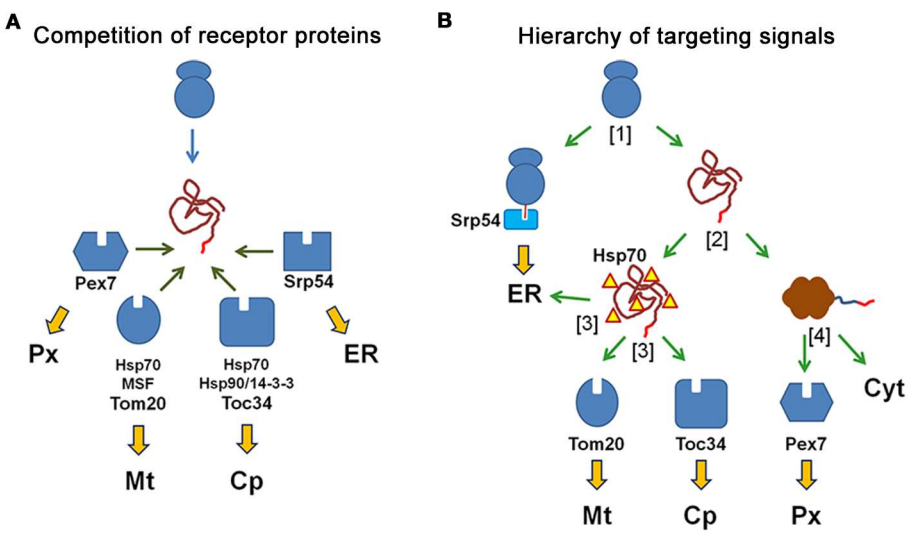

FIGURE 3 | N-terminal targeting signals determine the transport route of proteins by the interaction with the receptor proteins. (A) *Competition of receptor proteins*: the N-terminal amino acid sequence of a newly synthesized protein can interact with all receptor proteins, which compete for the peptide sequence (peroxisomal Pex7, mitochondrial Tom20, chloroplast Toc34, and Srp54 for the ER) and with additional cytosolic proteins that might affect these interactions (Hsp70, Hsp90, 14-3-3 proteins). The choice of the transport route is based on the relative affinity of the peptide sequence to different receptor proteins. **(B)** *Different import mechanisms generate a hierarchy of targeting signals*: An N-terminal amino acid sequence is sequentially scanned by diverse receptor proteins, because these interactions occur at different time points during the production and folding of the protein. A newly synthesized protein either binds to the SRP to become translated into ER or it finishes translation in the cytosol [1] Next, the protein either becomes folded or remains unfolded due to its interaction with chaperones, [2] Unfolded proteins can interact with the mitochondrial receptor Tom20, the chloroplast receptor Toc34 or the Sec61 complex of the ER (translocon), [3] Finally, folded proteins can either interact with the soluble receptor protein Pex7, which initiates their transport into peroxisomes, or they remain in the cytosol [4].

hydrophobic residues in the interaction domains, which render a specific interaction less plausible. Moreover, the basic interaction strength between a mitochondrial presequence and Tom20 (Abe et al., 2000) or between the N-terminus of a PTS2-carrying cargo protein and PEX7 is surprisingly weak (Mukai and Fujiki, 2006; Pan et al., 2013; Kunze et al., 2015), which is in good agreement with the low number of amino acids involved in this interaction. In contrast, the interaction strength between phosphorylated transit peptides and the chloroplast receptor Toc34 is drastically higher (Sveshnikova et al., 2000).

Alternatively, the specificity for a single binding partner could originate from the existence of individual residues within targeting signals that exclude an interaction with competing receptor proteins by their physico-chemical properties (*negative discrimination*). Such a mechanism could account for the specificity of PTS2 motifs, because individual point mutations in a prototypical PTS2, which retain peroxisomal targeting, allow concomitant alternative targeting (Kunze et al., 2011). Moreover, the interaction of an amino acid sequence with a receptor could also be modulated by sequences or residues in proximity to the direct binding site, which might exert additional stabilizing or repulsive effects. Altogether, the necessary difference in the affinity to different receptor proteins can originate either from specifically strengthening the desired interaction or from disfavoring the interaction with other receptor proteins. However, the discriminatory power is most probably the product of a co-evolution of targeting signals and available receptor proteins, which is supported by the observation that a plant chloroplast protein is targeted to mitochondria, when ectopically expressed in yeast cells (Hurt et al., 1986).

Focusing on the short amino acid segments directly interacting with the receptor proteins might cause a disregard of the surrounding amino acid sequences that are also part of the processed N-terminal sequence. As these sequences are cleaved off, they do not contribute to protein function and should be flexible for adaptation processes. Moreover, these sequences encode information for the binding of chaperones in mitochondrial presequences and chloroplast transit peptides (Zhang and Glaser, 2002) or for phosphorylation sites within transit peptides that mediate the interaction with 14-3-3 proteins (Waegemann and Soll, 1996; May and Soll, 2000). Therefore, it is remarkable that the sequence preceding the PTS2 motif was found enriched in negative charges (Reumann, 2004), whereas mitochondrial presequences are rich in positive charges and depleted of negative ones (Pujol et al., 2007). The similarity of the targeting signals for mitochondria and for chloroplasts have been long recognized starting with glutathione reductase from pea (Creissen et al., 1995) and has been amply investigated since then. A direct comparison of presequences and transit peptides revealed an overrepresentation of positive charges in presequences and of hydroxylated residues in transit peptides (Jarvis and Robinson, 2004). This study was extended by a combination of bioinformatic and mutational approaches (Pujol et al., 2007) and even a restraint to the residues at the extreme N-terminus of the proteins showed characteristic differences (Bhushan et al., 2006). This suggests that general properties of the whole N-terminal sequence (presequence, transit peptide or signal peptide) influence the quality of a targeting signal specified by the domain directly interacting with the receptor protein. These properties are probably shaped by evolutionary processes and can be used by prediction algorithms that successfully discriminate N-terminal targeting signals (Emanuelsson et al., 2007; Mitschke et al., 2009).

Coupling of Independent Recognition Steps

Although the direct interaction between the targeting signal and the receptor protein is a key step in the initiation of protein import, the implementation of an additional recognition event, which secondarily scans already chosen targeting signals, can provide a selectivity filter function to improve specificity. Such additional evaluation of a targeting signal is compatible with the formation of a trimeric complex consisting of targeting signal, receptor, and a third protein as well as with a hand-over mechanism, in which the cargo protein is further processed by a second protein. However, both mechanisms benefit from the involvement of additional sequence motifs within or in proximity to the targeting signal, which do not participate in the primary binding of the receptor protein. Accordingly, sequence elements that are not directly involved in receptor binding should be able to modulate the import efficiency of a protein. Exemplarily, the co-receptor protein for the PTS2 receptor Pex7 drastically stabilizes the interaction between this receptor and its cargo (Mukai and Fujiki, 2006; Pan et al., 2013; Kunze et al., 2015) and the 3D structure of the yeast trimeric complex (Pex7, Pex21, N-terminus of Fox3) indicates a direct interaction between residues of the co-receptor and of the PTS2 (Pan et al., 2013). This contribution of the co-receptor increases the area of the PTS2 helix, which is available for the recognition of a PTS2 by the receptor/co-receptor complex. However, the sequential assembly of the trimeric complex (Kunze et al., 2015) suggests that the co-receptor interacts with a preformed PEX7-cargo dimer and, thus, the binding of the co-receptor acts as independent quality control of the preformed dimeric complex. At the mitochondrial membrane, the presequence not only interacts with Tom20, but also with the second receptor protein Tom22 (Brix et al., 1997). However, the presequence binds Tom20 predominantly via hydrophobic interactions, whereas the interaction with Tom22 is mainly dependent on ionic interactions (for discussion see, Endo and Kohda, 2002). On the chloroplast surface, members of the Toc159 family bind to a sequence element of the transit peptide, which only partially overlaps with the Toc34 binding site, and thereby independently evaluate transit peptides after their primary recognition by Toc34. Moreover, phosphorylation is a frequently observed property of transit peptides that increases their affinity for the chloroplast receptor Toc34 (Sveshnikova et al., 2000), although the lack of phosphorylation sites did not change the specificity of targeting (Nakrieko et al., 2004). However, this phosphorylation also allows the interaction with proteins of the 14-3-3 family, which support the transport of the preprotein to the chloroplast surface together with Hsp70 proteins (May and Soll, 2000). Finally, the binding of a signal peptide to the bacterial homolog of SRP induces a conformational change within this protein, but the quality of the signal peptide markedly correlates with the velocity, at which the first

intermediate state is reached (Zhang et al., 2010). Furthermore, a good signal peptide delays GTP hydrolysis by the GTPase activity of the SRP, which extends the time window during which the trimeric complex of nascent chain, ribosome, and SRP can reach the ER membrane (Zhang et al., 2010). Altogether, the different import routes all involve mechanisms that add such selectivity filters.

Differences in the Import Mechanism Pose a Hierarchy of Targeting Signals

In contrast to the mechanisms listed above, which improve the fidelity of transport route selection by the choice of the appropriate receptor protein(s), the transport routes themselves are also ranked by the distinct phases of protein formation, during which a particular receptor scans the N-terminal amino acid sequence of a newly generated protein. This is equally important for the choice of the transport route, because it renders the alternative import mechanisms unequal (**Figure 3B**). The entrance into the ER is triggered by signal peptides directly after the appearance of the nascent chain at the exit site of the ribosome. These peptides are recognized by the SRP, which acts as soluble receptor. However, this interaction also induces translational stalling, which prevents the synthesis of the residual protein until the SRP-bound signal sequence has been transferred to the translocon (Sec61 complex). Thus, the newly translated protein is directly guided into the ER, whereas other targeting signals that might be also encoded within the protein sequence are never accessible in the cytosol. This renders co-translational import into the ER dominant over all other transport routes (**Figure 3B**; [1]). The translation of other proteins, which have not been sequestered by the SRP, is completed in the cytosol generating folding competent polypeptides. However, only a fraction of these polypeptides is actually folded, whereas proteins encoding a mitochondrial presequence, a chloroplast transit peptide or a signal peptide for post-translational ER import remain in an unfolded state due to binding of various chaperones of the Hsp70 family (**Figure 3B**; [2]). These unfolded proteins can interact with membrane-bound receptors on the surface of mitochondria, chloroplasts, or the ER and the transfer to the organellar membrane can be accelerated by cytosolic factors such as mitochondrial import stimulatory factor (MSF) (Hachiya et al., 1993) or 14-3-3 proteins for chloroplasts (May and Soll, 2000). Properties that distinguish mitochondrial and chloroplast preproteins have been elucidated (Huang et al., 2009), but the import mechanisms do not suggest a hierarchical relation between these targeting signals (**Figure 3B**; [3]). In contrast, peroxisomal, nuclear, and cytosolic proteins are folded in the cytoplasm with the help of folding chaperones. However, proteins exposing a peroxisomal targeting signal either at their N-terminus (PTS2) or its C-terminus (PTS2) bind to cytosolic receptor proteins and become imported into peroxisomes (**Figure 3B**; [4]).

Altogether, in this concept the choice of an import route is the consequence of temporarily distinct decisions, in which the different receptor proteins interfere with one step in the production of a folded protein. Accordingly, an early route decision can exclude a protein from all transport pathways that are chosen at a later stage, which implements a hierarchy of transport routes reflected by the hierarchy of targeting signals (Neuberger et al., 2004). This idea is supported by the analysis of naturally occurring proteins encoding a functional PTS1, which revealed that various proteins located exclusively in mitochondria or the ER sometimes encode a functional PTS1 that is not utilized (Neuberger et al., 2004). This suggests that in these cases, an evolutionary selection preventing undesired peroxisomal targeting is not required, whereas cytosolic proteins are sensitive to the addition of PTS1 motifs. Moreover, *de novo* generated mitochondrial targeting signals can suppress naturally occurring PTS1. This is exemplified in the human enzyme alanine:glyoxylate aminotransferase (AGXT) involved in peroxisomal glyoxylate detoxification. A mutation generating a mitochondrial tartgeting signal causes the mistargeting of an otherwise intact enzyme from peroxisomes to mitochondria, which is sufficient to cause a clinical picture of primary hyperoxaluria type 1 (OMIM #259900) similar to the loss of enzymatic activity (Danpure, 2006). This highlights the clinical importance of the hierarchical ranking of targeting signals. Moreover, the importance of the folding state for the choice of the import route was investigated by the use of a reporter protein (dihydrofolate reductase, DHFR), which can be forced into a folded state by a pharmaceutical compound (methotrexate). When this reporter protein was equipped with a mitochondrial targeting signal and a PTS1, it is exclusively found in mitochondria. However, when protein folding was favored by the addition of methotrexate, this led to peroxisomal targeting of the reporter protein corroborating the concept of a hierarchy of targeting signals (Mukhopadhyay et al., 2004). It remains to be clarified, whether the dominance of the mitochondrial targeting signal is solely caused by an efficient avoidance of protein folding or whether the late exposure of the PTS1 during translation also contributes to the subordination of the PTS1. In the latter case, the N-terminal position of the PTS2 might offer a possibility to (partially) overcome the hierarchy of targeting signals.

Importantly, this hierarchy of targeting signals implicates that the specificity of protein import is primarily dependent on the ability to make certain crucial decisions during protein formation, which are only partially determined by the relative affinity of different receptor proteins to the same amino acid sequence.

Additional Levels of Regulation

In addition to the mechanisms that support a high specificity of protein transport at the level of cargo recognition, further cell biological processes might support this specificity. One promising candidate is the enrichment of mRNA encoding organellar proteins in proximity to these organelles. Such mRNA enrichment has been described for fractions containing predominantly peroxisomes (Zipor et al., 2009), mitochondria (Kaltimbacher et al., 2006; Eliyahu et al., 2010), chloroplasts (Weis et al., 2013), or the ER (Reid and Nicchitta, 2015), but only the latter was independent of translation (Pyhtila et al., 2008; Jagannathan et al., 2014).

In summary, several mechanisms supposedly act in concert to facilitate the specificity of transport processes in spite of the similarity of N-terminal targeting signals.

Dual Targeting and Bilocalization of Proteins

As specific targeting signals initiate the transport of proteins to distinct subcellular compartments, a tight relation between the primary sequence and the subcellular location of proteins was supposed, which resulted in the assumption of a predefined distribution of all proteins. Any deviation from a discrete location such as the occurrence of minor protein fractions in other compartments was attributed either to contaminations indicating the imperfectness of the isolation procedure (e.g., density gradient centrifugation) or to insufficiencies of the detection tool (e.g., low of antibody specificity). This assumption was corroborated by the observation that the concurrent presence of the same enzymatic activity in different subcellular compartments is often achieved by the existence of homologous proteins (isoenzymes), which encode different targeting signals.

However, more recently, the number of reports describing real *bilocalization* of individual proteins by *dual targeting* has been steadily increasing, which has been summarized for proteins localized in peroxisomes and other organelles (Ast et al., 2013), mitochondria and chloroplasts (Small et al., 1998; Carrie and Small, 2013), or secretory proteins and other organelles (Porter et al., 2015). These observations were sometimes made by accident, but more often were facilitated by modern techniques such as the detailed analysis of subcellular fractionation by advanced mass spectrometric methods (e.g., protein correlation profiling), which allows a better discrimination of organellar constituents from contaminants (Andersen and Mann, 2006; Foster et al., 2006; Wiese et al., 2007), or by the systematic investigation of EGFP-fusion proteins (Li et al., 2006; Carrie et al., 2009). An obvious biological advantage of such bilocalization of a single protein is genomic efficiency, because the number of genes that are required to supply different organelles with the same protein function is reduced. This is most obvious when considering the dual targeting of about 100 proteins to mitochondria and chloroplasts, many of which are involved in organellar DNA replication and protein synthesis (Carrie and Small, 2013). However, the savings due to bilocalization of proteins probably require a complex arrangement of targeting information, because the presence of two targeting signals alone might not be sufficient for dual targeting. Many targeting signals are positioned within the N-terminal part of the encoding proteins, which share organelle specific properties. Whereas, these differences support specificity of targeting signals by interfering with competing transport routes, they might pose a problem for the performance of dual targeting. Moreover, the hierarchy of targeting signals can also prevent dual targeting of proteins that encode two targeting signals, because even targeting signals that are positioned at different ends of a protein can negatively affect each other, such as the dominance of N-terminal targeting signals over the C-terminal PTS1.

The concurrent presence of a protein function or protein activity within different subcellular compartments can be achieved by various means (**Figure 4**). In the traditional concept, the bilocalization of a protein function is realized by *independently encoded homologous proteins* that are equipped with different targeting signals (**Figure 4A**). These signals can either be both located at the N-termini of the proteins (*upper part*) or at opposite ends (*lower part*). Alternatively, the cell can produce different protein variants (isoforms) derived from one gene that share the core domain, but differ slightly in their primary sequence, which is sufficient to exchange targeting signals (**Figure 4B**). In this process, either variants with alternative N-terminal amino acid sequences are generated that differ by the encoded targeting signal (*upper part*) or variants are produced that share a C-terminal PTS1, but encode or lack an additional N-terminal targeting signal (*lower part*). Protein variants with alternative N-terminal sequences (*upper panel*) can be generated from a single gene by the production of different mRNAs that are obtained either by alternative splicing of the same pre-mRNA or by alternative transcription initiation based on different promoters that generate different pre-mRNAs (Mueller et al., 2004; Yogev and Pines, 2011). Protein variants that encode targeting signals at the opposite ends of the protein probably necessitate the omission of the N-terminal targeting signal to disclose a functional PTS1 (*lower panel*). Thus, the two protein variants should differ in the absence or presence of the N-terminal targeting signal, which can be achieved by the omission of the N-terminal part of the protein sequence either by alternative translation initiation or leaky ribosome scanning (Elgersma et al., 1995; Wamboldt et al., 2009), next to the abovementioned mechanisms of alternative splicing and alternative transcription initiation (Ast et al., 2013).

Finally, an increasing number of reports describe *dual targeting* of a protein, which means the transport of the identical protein into different subcellular compartments. These proteins harbor an *ambiguous targeting signal* (Small et al., 1998; Silva-Filho, 2003; Yogev and Pines, 2011) that induces the concomitant transport to alternative destinations by overlapping targeting signals (**Figure 4C**). Such targeting signals have been predominantly found in plant proteins bilocalized to mitochondria and chloroplasts (Carrie and Small, 2013; Baudisch et al., 2014) and use the traditional import pathways into these organelles (Langner et al., 2014). The amino acid composition of N-terminal sequences encoding ambiguous targeting signals show properties of both targeting signals, which emphasizes the intermediate state of such peptides (Pujol et al., 2007). However, it should be mentioned that protein transport into mitochondria and chloroplasts is especially suitable for such a mechanism, because the import route into these organelles is highly similar involving chaperones that keep the proteins in an unfolded state within the cytosol before the proteins bind to membrane bound receptors.

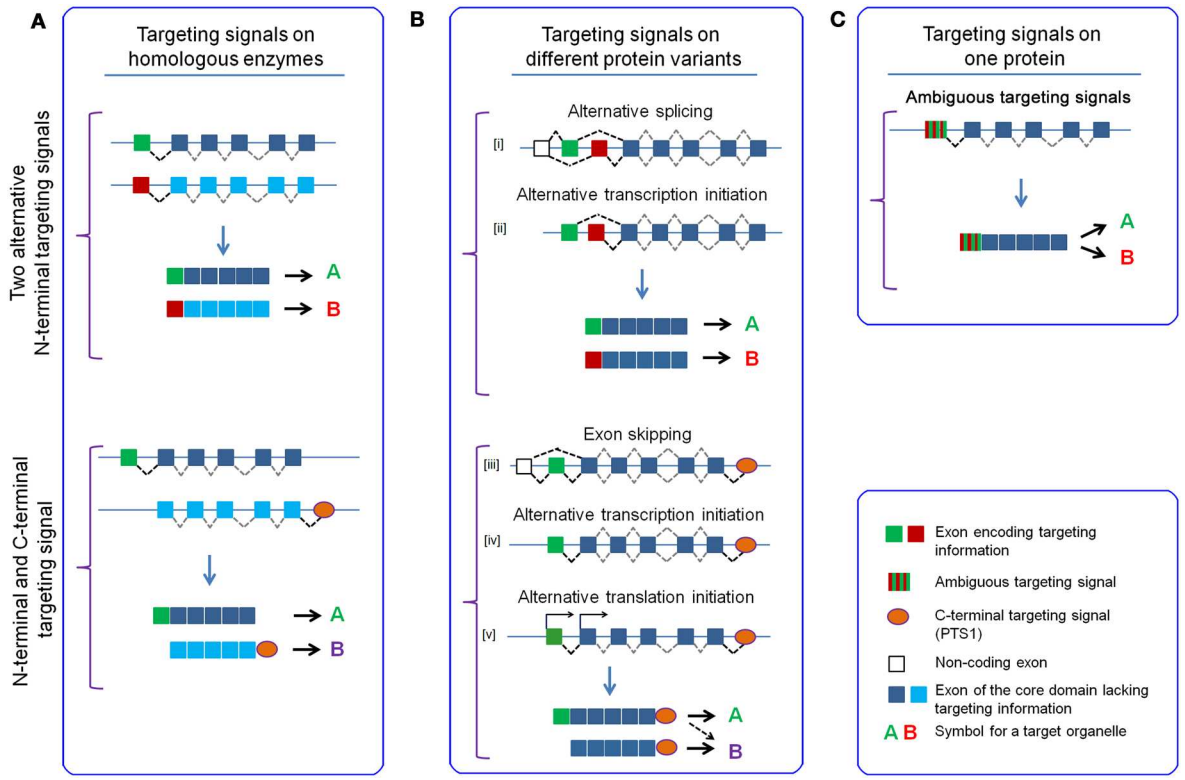

FIGURE 4 | Mechanisms to provide the same enzymatic activity or protein function within different subcellular compartments. Bilocalization of protein(s) requires the presence of two alternative targeting signals, which can be either encoded by alternative N-terminal sequences (*upper part*) or can be encoded by an N-terminal targeting signal and a C-terminal PTS1, respectively (*lower part*). **(A)** Two independent genes code for proteins with the same enzymatic activity of function (isoenzymes/homologoues), which harbor different targeting signals. **(B)** Two variants of the same protein are generated from a single gene, which share the core domain(s), but differ in the encoded targeting signals; variants encoding alternative N-terminal sequences can be obtained by [i] alternative splicing from the same pre-mRNA with a non-coding first exon or [ii] by alternative transcription initiation generating alternative first exons, which use to the same splice acceptor site of the second exon. Variants with and without N-terminal targeting signal, but sharing a C-terminal PTS1 can be generated by [iii] exon skipping behind the first non-coding exon, which omits the second exon encoding the N-terminal targeting signal, [iv] alternative transcription initiation ablating the first exon and [v] alternative translation initiation at different start codons within the same mRNA. **(C)** One protein is equipped with an ambiguous targeting signal, which is sufficient to mediate the concomitant targeting of the protein to more than one organelle.

Two Independent Peroxisomal Targeting Signals as Evolutionary Advantage

The import machinery of peroxisomes for soluble proteins can accept two completely independent types of targeting signal due to two receptor proteins with specific cargo binding domains, although the transport routes converge at an early stage of the import process. This could be an evolutionary heritage tracing back to ancient developments of eukaryotic cells, but, surprisingly, some organisms lack the whole PTS2 mediated import pathway (Motley et al., 2000; Gonzalez et al., 2011; Faust et al., 2012). However, the possibility to encode targeting signals at different termini of a protein could also pose an advantage under specific conditions. Especially, those properties of one targeting signal, which allow the performance of an irreplaceable function might account for the increased fitness of organisms that have two targeting signals at their disposal. In this context, the position of the PTS2 next to the N-terminus and its structural similarity with other N-terminal targeting signals might confer a functional distinction between the two types of peroxisomal targeting signals.

The appearance of a second targeting signal for peroxisomes could have been relevant during a specific phase of evolution, in which novel N-terminal targeting signals occurred, such as the era after the endosymbiontic uptake of purple bacteria and cyanobacteria as protomitochondria and protoplastid, which later developed to mitochondria and chloroplasts, respectively (Dyall et al., 2004). In this time period, many genes were relocated from the organellar genome to the nucleus, which required the establishment of novel protein import machineries for the endosymbiontic organelles, because the proteins, now encoded by nuclear genes, were produced in the cytosol and had to be imported into mitochondria and chloroplasts. This included the creation of receptor proteins accepting a plethora of targeting signals with variable similarity, which can be easily generated *de novo* and suffice to initiate the translocation of proteins across the organellar membranes. However, these novel transport routes could easily act as competitors for the peroxisomal

protein import machinery, particularly when considering that the mechanistic differences render the latter subordinate to the mitochondrial or chloroplast import pathways. This could have caused a detrimental relocation of some peroxisomal proteins comparable to the mislocalization of alanine:glyoxylate aminotransferase (AGXT) in human patients (Section Additional Levels of Regulation) unless the cells were able to reestablish the specificity of protein transport. Under these conditions, the genesis of a second peroxisomal targeting signal could have been a countermeasure in a competitive situation originating from novel import systems utilizing N-terminal targeting signals. Different evolutionary processes are conceivable within such a scenario. The PTS1-mediated import system could have existed before the endosymbiontic events, but might have been overruled and functionally disabled by the dominance of newly generated import systems utilizing N-terminal targeting signals. In such a scenario, the development of an independent peroxisomal targeting signal that is also encoded close to the N-terminus (PTS2) could have been required to perpetuate peroxisomal protein import unless further adaptations enabled the continuation of the original transport route. Alternatively, the PTS2 mediated import pathway could have been the original one, but when this targeting signal was recognized by the receptors of the protein import machineries of mitochondria or chloroplasts, a novel targeting signal close to the C-terminus (PTS1) could have facilitated the abrogation of undesired N-terminal targeting signals without affecting targeting to peroxisomes. Both models suppose the existence of the peroxisomal import system before the appearance of competing import machinery. Alternatively, the co-existence of two independent peroxisomal targeting signals could also present a continuous advantage during evolution. Provided that the similarity between the PTS2 and other N-terminal targeting signals allows the generation of ambiguous targeting signals, which is hardly conceivable for the PTS1, this should allow the bilocalization of the encoding protein. Such ambiguous targeting signals have previously been discussed in the context of dual targeting of proteins to mitochondria and chloroplasts, but could also involve protein transport to peroxisomes and other organelles. This could be an important intermediate step during the change of protein compartmentation, because peroxisomal protein import via the PTS1 is notoriously subordinate to other protein transport routes. Thus, any *de novo* generation of an alternative targeting signal at the N-terminus of a soluble peroxisomal protein encoding a PTS1 should abrogate peroxisomal transport and prohibit bilocalization. Similarly, the *de novo* generation of a PTS1 at the C-terminus of a mitochondrial or chloroplast protein should remain free of consequences, because in this context the novel PTS1 cannot initiate peroxisomal import due to the hierarchy of targeting signals. In contrast, an ambiguous targeting signal that concurrently destines the protein for peroxisomes and another organelle by the same N-terminal amino acid sequence could allow the bilocalization of this protein, which would be an important intermediate step in the exchange of a targeting signals.

Changes of Targeting Signals and the Subcellular Localization in an Evolutionary Context

In contrast to the presentation in many textbooks, the compartmentation of enzymatic reactions and even of whole metabolic pathways can differ between evolutionary distant organisms. A well-known example is the degradation of the most abundant fatty acids in mitochondria of chordates, which contrasts the exclusively peroxisomal degradation of these fatty acids in yeast and plant species (Poirier et al., 2006; Houten and Wanders, 2010). Less prominent examples are changes in the compartmentation of an individual enzyme, which can occur within relatively short time scales such as the relocation of the glyoxylate-degrading enzyme alanine:glyoxylate aminotransferase (AGXT) (Danpure, 2006). This enzyme has been found exclusively in mitochondria, exclusively in peroxisomes or bilocalized in different mammalian species (Birdsey et al., 2004) and even within the family of bats (*chiroptera*), the localization of the protein differs between species (Liu et al., 2012). The importance of proper targeting of this enzyme for mammalian physiology is highlighted by the inherited human disease hyperoxaluria (type 1), which can originate either from a loss of the enzyme activity (Salido et al., 2012) or from a mistargeting of an otherwise intact enzyme from peroxisomes to mitochondria (Purdue et al., 1990).

Certainly, the presently observable differences in the enzymatic compartmentation between organisms are the product of evolutionary processes, based on which the subcellular distribution of an enzyme has changed over time. This relocation of a protein had to be achieved by an exchange of targeting signals, which is based on stepwise alteration in the primary sequence. Importantly, all intermediate steps of such a development had to be compatible with the functioning of the affected metabolic pathway(s) to fulfill the demands of the organism. Thus, a gradual change of a protein's subcellular location is highly desirable to facilitate concomitant adaptation processes, which is another important application of dual targeting. However, a gradual exchange of targeting signals has to cope with the hierarchy of targeting signals, which might prohibit dual targeting in spite of the presence of two independent targeting signals.

As many targeting signals are encoded close to the N-terminus (PTS2, presequences, transit peptides, and signal peptides), whereas the PTS1 resides at the extreme C-terminus, an exchange of targeting signals either involves two different N-terminal targeting signals or the replacement of an N-terminal targeting signal by a C-terminal one or of a C-terminal targeting signal by an N-terminal one.

The substitution of N-terminal targeting signals can be achieved either by the gradual substitution of single amino acids to convert one targeting signal into another one, or by the replacement of a complete N-terminal sequence module by an amino acid stretch that is encoded by an independent DNA sequence. The latter requires the invention of a novel DNA element encoding an independent amino acid sequence, which

has to be integrated into the transcriptional and translational unit of the gene. In the transitional phase the concomitant production of the old and the new protein variant and their transport into different organelles should be important and can be achieved by diverse mechanisms comparable to the examples described above (**Figure 4B**). In contrast, a process involving the gradual substitution of amino acids offers an ambiguous targeting signal as a suggestive intermediate (**Figure 4C**). The observation that the N-termini of dually targeted proteins (mitochondria and chloroplasts) unite properties of presequences and transit peptides (Pujol et al., 2007) suggests the feasibility of a gradual change. Whether similar processes are feasible for PTS2 motifs has not been studied yet.

In contrast, the exchange of an N-terminal targeting signal for a PTS1 or *vice versa* within a naturally occurring protein requires independent mechanisms for the generation or inactivation of each of these targeting signals. A *de novo* generation of an N-terminal targeting signal for mitochondria, chloroplasts or the ER can be obtained by an elongation of the protein at its N-terminus using various mechanisms such as the introduction of a start codon in the 5′-UTR or of an alternative transcription initiation site, which all benefit from the high degeneracy of these targeting signals and the efficiency of this process has been described (Kaiser et al., 1987; Vassarotti et al., 1987; Lemire et al., 1989). It should be stressed that such newly generated N-terminal extensions have to encode more than just the minimal receptor binding site, because the N-terminal sequences of naturally occurring preproteins present with additional properties that are characteristic for the organelle or with the ability to interact with cytosolic chaperones. However, most probably these properties need not be perfectly realized in the beginning. The ablation of an existing N-terminal targeting signal can be exerted by the inverse mechanisms such as the inactivation of the first start codon, alternative splicing that skips the exon encoding the start codon together with a part of the N-terminal sequence or the generation of an alternative transcription initiation site.

The position of the PTS1 at the extreme C-terminus renders it suitable for an easy ablation of this signal, but certain properties of this signal facilitate its spontaneous formation as well. The first description of the PTS1 as C-terminal tripeptide in its most prominent form (-SKL) (Gould et al., 1987) revealed the involvement of two amino acids encoded by six different codons (serine and leucine), which renders its *de novo* generation by a statistical event rather probable. Moreover, the apparent degeneracy of the PTS1 (Lametschwandtner et al., 1998; Brocard and Hartig, 2006) further extends the number of arbitrary tripeptides functioning as weak PTS1, which further increases the probability of spontaneous formation. Thus, a novel PTS1 could easily be generated by point mutations within the original protein, but the finding that an unstructured linker domain between the core protein and the PTS1 is important for its functionality (Neuberger et al., 2003a) took this simple model into question. Thus, an alternative mechanism appears more promising, which permits the elongation of the protein by a (partial) read-through of the endogenous stop codon (Freitag et al., 2012; Schueren et al., 2014; Stiebler et al., 2014). This mechanism also generates a novel C-terminal ending and benefits from the relatively high propensity to obtain a PTS1-like sequence by such arbitrary extension. Furthermore, it introduces a short amino acid sequence that can serve as favorable linker domain in front of the PTS1. Conversely, the ablation of a functional PTS1 can easily be accomplished by point mutations or the introduction of a premature stop codon within the linker domain, because this sequence should not contribute to the structure of the core protein. However, the exchange of targeting signals involving a PTS1 is prone to detrimental effects caused by the hierarchy of targeting signals, because the PTS1 is subordinate to N-terminal targeting signals. Accordingly, the *de novo* generation of an N-terminal targeting signal should abrogate the peroxisomal targeting mediated by the original PTS1 and, thus, should prevent bilocalization. Reciprocally, the spontaneous generation of a PTS1 alone is not sufficient to induce peroxisomal targeting of a protein encoding an alternative targeting signal at its N-terminus, which excludes a beneficial effect of the novel PTS1. In this context, the similarity of the PTS2 and other N-terminal targeting signals might represent a functional distinction between the PTS1 and the PTS2, because it is conceivable that PTS2 motifs can be part of an ambiguous targeting signal that concomitantly targets a protein into peroxisomes and another organelle.

Summary

A specific and efficient transport of proteins from the cytosol into various compartments is a prerequisite for the beneficial effects of sequestering proteins and metabolites into membrane-bound subdomains. The mechanisms of protein import across the confining single or double membrane differ remarkably in the timing of receptor binding, the folding status of the transported protein, the function of the energy consuming steps or the requirement for intraorganellar folding. However, all transport routes are accessible by N-terminal targeting signals that involve an α-helical domain, which interact with the appropriate receptor protein to initiate translocation. In spite of the structural similarity between these N-terminal targeting signals the distribution of the majority of cellular proteins is well-defined, highlighting the specificity of the transport processes. This specificity is enhanced by unique properties of the targeting signals, which render them suitable for a classification into a type of targeting signal (PTS2, presequence, transit peptide, signal peptide), although these targeting signals are not highly conserved, but rather degenerate. These properties are sufficient to discriminate between receptor proteins and thus to select the appropriate transport route. However, the different mechanisms of protein import implicate that the different receptor proteins do not simply compete for the N-terminal sequence of a newly generated protein, but individual receptors can interact solely within a certain time frame during the formation of a fully folded protein. This can be either during translation (ER: co-translational) or after translation, but also before folding starts (mitochondria, chloroplasts, ER: post-translational) or after the folding of the protein (peroxisome). The chronological order of peptide scanning by different receptor proteins is reflected by the hierarchy of targeting signals, because an early decision

for one transport route (e.g., mitochondria) excludes the later choice for another organelle (e.g., peroxisomes), which depends on the interaction with another receptor at a later stage. However, the specificity of protein transport does not preclude a bilocalization of proteins by dual targeting, which necessitates the concomitant presence of more than one targeting signal. Such bilocalization increases genetic efficiency, because only one gene can supply protein function within diverse cellular compartments. However, bilocalization can also serve as an important intermediate step during evolutionary adaptation processes involving a redistribution of proteins, because during a transitional phase a continuation of a process at its original location is as important for survival as its invention and optimization at a novel place. In this context, the hierarchy of targeting signals is important, because the presence of two targeting signals is not sufficient if one route is subordinate to the other one.

Interestingly, two functionally equivalent targeting signals can initiate the transport of a soluble protein into peroxisomes (PTS1 and PTS2), which differ by their relation to other targeting signals. The PTS1 is encoded at the extreme C-terminus and appears late during translation, which renders the PTS1 clearly subordinate to the N-terminal targeting signals. In contrast, the PTS2 is structurally similar to other N-terminal targeting signals, which might enable the generation of ambiguous targeting signals. We suggest that this difference might be a crucial advantage for the organism, which favors the coexistence of two peroxisomal targeting signals. The PTS2 is probably more compatible with a bilocalization of the encoding protein, but might be more prone to mislocalization due to its similarity to other targeting signals.

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Metabolic Interplay between Peroxisomes and Other Subcellular Organelles Including Mitochondria and the Endoplasmic Reticulum

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Peroxisomes are unique subcellular organelles which play an indispensable role in several key metabolic pathways which include: (1.) etherphospholipid biosynthesis; (2.) fatty acid beta-oxidation; (3.) bile acid synthesis; (4.) docosahexaenoic acid (DHA) synthesis; (5.) fatty acid alpha-oxidation; (6.) glyoxylate metabolism; (7.) amino acid degradation, and (8.) ROS/RNS metabolism. The importance of peroxisomes for human health and development is exemplified by the existence of a large number of inborn errors of peroxisome metabolism in which there is an impairment in one or more of the metabolic functions of peroxisomes. Although the clinical signs and symptoms of affected patients differ depending upon the enzyme which is deficient and the extent of the deficiency, the disorders involved are usually (very) severe diseases with neurological dysfunction and early death in many of them. With respect to the role of peroxisomes in metabolism it is clear that peroxisomes are dependent on the functional interplay with other subcellular organelles to sustain their role in metabolism. Indeed, whereas mitochondria can oxidize fatty acids all the way to CO_2 and H_2O, peroxisomes are only able to chain-shorten fatty acids and the end products of peroxisomal beta-oxidation need to be shuttled to mitochondria for full oxidation to CO_2 and H_2O. Furthermore, NADH is generated during beta-oxidation in peroxisomes and beta-oxidation can only continue if peroxisomes are equipped with a mechanism to reoxidize NADH back to NAD^+, which is now known to be mediated by specific NAD(H)-redox shuttles. In this paper we describe the current state of knowledge about the functional interplay between peroxisomes and other subcellular compartments notably the mitochondria and endoplasmic reticulum for each of the metabolic pathways in which peroxisomes are involved.

Keywords: peroxisomes, mitochondria, endoplasmic reticulum, fatty acids, etherphospholipids, genetic diseases, peroxisomal diseases, metabolism

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INTRODUCTION

Eukaryotic cells contain a variety of different subcellular compartments, which differ from one another in multiple aspects including their biogenesis, enzyme content, and role in metabolism. Lysosomes, for instance, are primarily involved in the breakdown of macromolecules, whereas mitochondria are the ultimate site of aerobic metabolism. Peroxisomes perform both catabolic as

well as anabolic functions. The role of peroxisomes in human metabolism and their importance for human health and development has largely been elucidated thanks to the detailed work on a rare genetic human disease called the cerebro-hepato-renal syndrome, or Zellweger syndrome (ZS). In patients with the classical form of ZS, functional peroxisomes are lacking due to a genetically determined defect in the biogenesis of peroxisomes. ZS is genetically heterogeneous, which is explained by the fact that peroxisome biogenesis involves the obligatory participation of multiple proteins required for the formation, proliferation, and maintenance of these organelles (Mast et al., 2010; Waterham and Ebberink, 2012; Hasan et al., 2013; Fujiki et al., 2014). The proteins involved are called peroxins and are encoded by *PEX* genes. Clinically, ZS patients show multiple abnormalities including cranial facial, neurological, hepatic, and other aberrations, and usually die early in life. Work on ZS has resulted in the discovery of the various unique metabolic functions exerted by peroxisomes which include: (A) etherphospholipid biosynthesis; (B) fatty acid beta-oxidation; (C) docosahexaenoic acid synthesis; (D) bile acid synthesis; (E) fatty acid alpha-oxidation; (F) glyoxylate detoxification; (G) amino acid metabolism, and (H) ROS/RNS-metabolism.

The essential role of peroxisomes in each of these metabolic pathways is emphasized by the fact that a variety of genetic diseases in man has been identified, usually with severe clinical signs and symptoms, that are caused by mutations in genes coding for peroxisomal enzymes involved in each of these metabolic pathways. **Table 1** lists the single peroxisomal enzyme deficiencies identified so far including two recently identified peroxisomal disorders including fatty acyl-CoA reductase 1 (FAR1) deficiency (Buchert et al., 2014) and Peroxisomal Membrane Protein 70 (PMP70/ABCD3) deficiency (Ferdinandusse et al., 2015).

To fulfill their role in metabolism peroxisomes rely very much on the interaction with other subcellular organelles. For instance, beta-oxidation of fatty acids (FAs) in peroxisomes generates NADH from NAD^+. Reoxidation of NADH back to NAD^+, however, relies on the interaction with the cytosol and subsequently the mitochondrion (see **Figure 1**). Furthermore, peroxisomes produce a range of chain-shortened acyl-CoAs including acetyl-CoA, which can only be fully oxidized to CO_2 and H_2O in mitochondria and not in peroxisomes. This is due to the fact that mitochondria contain the citric acid (KREBS) cycle, which catalyses the degradation of acetyl-CoA to CO_2 and H_2O (**Figure 1**). Also for other metabolic functions, peroxisomes depend on the interaction with other subcellular organelles. In this paper we will describe the interactions between peroxisomes and other subcellular organelles for each of the metabolic functions of peroxisomes in humans.

(A) Etherphospholipid Biosynthesis

The first major function of peroxisomes in metabolism involves the synthesis of ether-linked phospholipids (EPLs). In humans most EPLs occur in their plasmalogen form (Brites et al., 2004). Peroxisomes are indispensable for etherphospholipid synthesis, which is due to the fact that the enzyme responsible for the generation of the ether-bond in EPLs is strictly peroxisomal. The enzyme involved is alkyl-glyceronephosphate synthase [AGPS; formally named alkyldihydroxyacetonephosphate synthase (ADHAPS)] which catalyses the exchange of the acyl-group in acyl-DHAP for a long-chain alcohol via an ingenious mechanism to ensure formation of the ether-bond. The two substrates for AGPS include acyl-DHAP and a long-chain alcohol which are both synthesized by peroxisomal enzymes including glycerone-phosphate O-acyltransferase [GNPAT; formally called dihydroxyacetonephosphate acyltransferase (DHAPAT)] and one of two peroxisomal acyl-CoA reductases named FAR1 and FAR2 (Cheng and Russell, 2004). Both AGPS/ADHAPS as well as GNPAT/DHAPAT are true intra-peroxisomal enzymes targeted to peroxisomes via the PTS1- and PTS2-import pathways respectively (Mast et al., 2010; Waterham and Ebberink, 2012; Hasan et al., 2013; Fujiki et al., 2014; **Figure 2A**). Both enzymes are bound to the inner site of the peroxisomal membrane and form a functional complex (see **Figure 2B**). On the other hand, earlier work by Hajra and coworkers (Burdett et al., 1991) had shown that the situation is different with respect to the third peroxisomal enzyme involved in etherphospholipid biosynthesis which is the enzyme acyl-CoA reductase. This enzyme catalyzes the synthesis of the long-chain alcohol from the corresponding acyl-CoA ester using NADPH as reductant (Bishop and Hajra, 1981). The enzyme from rat brain (Bishop and Hajra, 1981) showed high specificity for palmitoyl-CoA (C16:0-CoA), stearoyl-CoA (C18:0-CoA), and oleoyl-CoA (C18:1-CoA). Importantly, this acyl-CoA reductase activity was found at the outer aspect of the peroxisomal membrane, which implies that the substrates including the acyl-CoA ester plus NADPH are in the cytosol and not localized in the peroxisomal matrix (see **Figure 2**). Most likely, the long-chain alcohol produced in the acyl-CoA reductase reaction is also released at the cytosolic face of the peroxisomal membrane (see **Figure 2B**; Honsho et al., 2013).

Work by the group of Russell has led to the identification and characterization of two acyl-CoA reductase isozymes named FAR1 and FAR2 (Cheng and Russell, 2004). The two FAR isozymes are ∼58% identical in sequence and are encoded by two distinct genes with similar exon/intron structures located on different chromosomes. FAR1 has a broad tissue distribution and acts on acyl-CoAs that vary in size and saturation suggesting that this isozyme plays a general role in the synthesis of fatty alcohols. In contrast, the more narrow tissue distribution

Abbreviations: ACAA1, peroxisomal 3-ketoacyl-CoA-thiolase1; ACBP, acyl-CoA binding protein; ACOX, acyl-CoA oxidase; ADHAPS, alkyl-dihydroxyacetonephosphate synthase; AGPS, alkyl-glyceronephosphate synthase; AMACR, 2-methyl-acyl-CoA racemase; BAAT, bile acid-CoA: amino acid *N*-acyltransferase; BSEP, bile salt export pump; CACT, carnitine/acylcarnitine translocase; CrAT, carnitineacetyltransferase; CrOT, carnitine octanoyltransferase; DHA, docosahexaenoic acid; DHAP, dihydroxyacetone phosphate; DHAPAT, dihydroxyacetonephosphate acyltransferase; DHCA, dihydroxycholestanoic acid; EPL, etherphospholipid; ER, endoplasmic reticulum; ETF, Electron-Transfer-Flavoprotein; FA, fatty acid; FAR1, fatty acyl-CoA reductase 1; FAR2, fatty acyl-CoA reductase 2; G3PDH, GPD1, glycerol-3-phosphate dehydrogenase; G3P, glycerol-3-phosphate; GNPAT, glycerone-phosphate O-acyltransferase; IDH, isocitrate dehydrogenase; MUFA/PUFA, mono/poly-unsaturated FA; PMP70, peroxisomal membrane protein 70; ROS, reactive oxygen species; RNS, reactive nitrogen species; THCA, trihydroxycholestanoic acid; VLCFA, very long-chain fatty acid; ZS, Zellweger syndrome.

TABLE 1 | The single peroxisomal enzyme deficiencies and their underlying enzyme and gene defects.

Metabolic pathway involved	Peroxisomal disorders	Enzyme deficiencies	Mutant gene
PEROXISOMAL BETA-OXIDATION			
	• X-linked adrenoleukodystrophy	ALDP	*ABCD1*
	• Acyl-CoA oxidase deficiency	ACOX1	*ACOX1*
	• D-bifunctional protein deficiency	DBP, MFE2, MFP2, D-PBE	*HSD17B4*
	• SCPx-deficiency	SCPx	*SCP2*
	• AMACR deficiency	AMACR	*AMACR*
	• PMP70-deficiency	PMP70	*ABCD3*
PLASMALOGEN BIOSYNTHESIS			
	• RCDP Type 2	DHAPAT/GNPAT	*GNPAT*
	• RCDP Type 3	ADHAPS/AGPS	*AGPS*
	• RCDP Type 4	Acyl-CoA reductase 1	*FAR1*
	• RCDP Type 5	PEX5L	*PEX5*
FATTY ACID ALPHA-OXIDATION			
	• Refsum disease	Phytanoyl-CoA hydroxylase	*PHYH*
BILE ACID SYNTHESIS			
	• BAAT-deficiency	BAAT	*BAAT*
GLYOXYLATE METABOLISM			
	• Hyperoxaluria Type 1	AGT	*AGXT*
ROS/RNS METABOLISM			
	• Acatalasaemia	Catalase	*CAT*

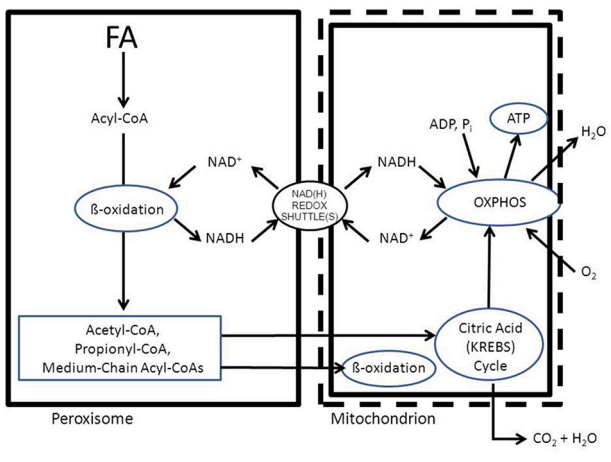

FIGURE 1 | Schematic diagram showing the functional interplay between peroxisomes and mitochondria in the beta-oxidation of fatty acids in peroxisomes. See text for detailed discussion.

and substrate preference of the FAR2 isozyme are indicative of a more specialized function. Recent work by Fujiki and coworkers (Honsho et al., 2010, 2013) has revealed that FAR1 is a peroxisomal tail-anchored protein targeted to peroxisomes via PEX19 (Honsho et al., 2013). Furthermore, the expression of FAR1 but not FAR2 is posttranslationally regulated. Indeed, FAR1 but not FAR2 is preferentially degraded in response to the cellular level of plasmalogens (Honsho et al., 2010, 2013). Peroxisomes have also been found to contain alkyl-DHAP reductase activity although the bulk of activity is in the endoplasmic reticulum.

The indispensable role of the peroxisomal enzymes GNPAT (Wanders et al., 1992), AGPS (Wanders et al., 1994), and FAR1 (Buchert et al., 2014) in the formation of etherphospholipids has become clear from the identification of patients with genetically determined deficiencies of these three enzymes. In such patients EPL-synthesis is severely deficient as concluded from the markedly reduced plasmalogen levels in erythrocytes from patients (for reviews see Brites et al., 2004; Braverman and Moser, 2012; Malheiro et al., 2015).

Etherphospholipid Biosynthesis and the Interplay with Other Organelles

GNPAT and AGPS are true intraperoxisomal enzymes targeted to peroxisomes via the PTS1- and PTS2-pathways respectively. Inspection of the two reactions catalyzed by GNPAT and AGPS reveals that the acyl-CoA ester used by GNPAT to produce acyl-DHAP, is released in its free acid form in the AGPS-catalyzed reaction, whereas the CoA unit is released in the GNPAT catalyzed reaction. Taken together it would make sense to regenerate the acyl-CoA ester within the peroxisome via an acyl-CoA synthetase. Peroxisomes contain at least one truly intraperoxisomal acyl-CoA synthetase called ACSVL1 (SLC27A2), which is a peripheral membrane protein facing the interior of the peroxisome. The same enzyme also occurs in the endoplasmic reticulum (ER; Steinberg et al., 1999). If such a scenario would be true, the formation of alkyl-DHAP would be as depicted in **Figure 2B**, the net equation being: DHAP + long-chain alcohol ⟶ alkyl-DHAP (see **Figure 2C**). The implication of this would be that DHAP and the long-chain alcohol need to be transported from outside the peroxisome, whereas alkyl-DHAP needs to be exported from the inside of the

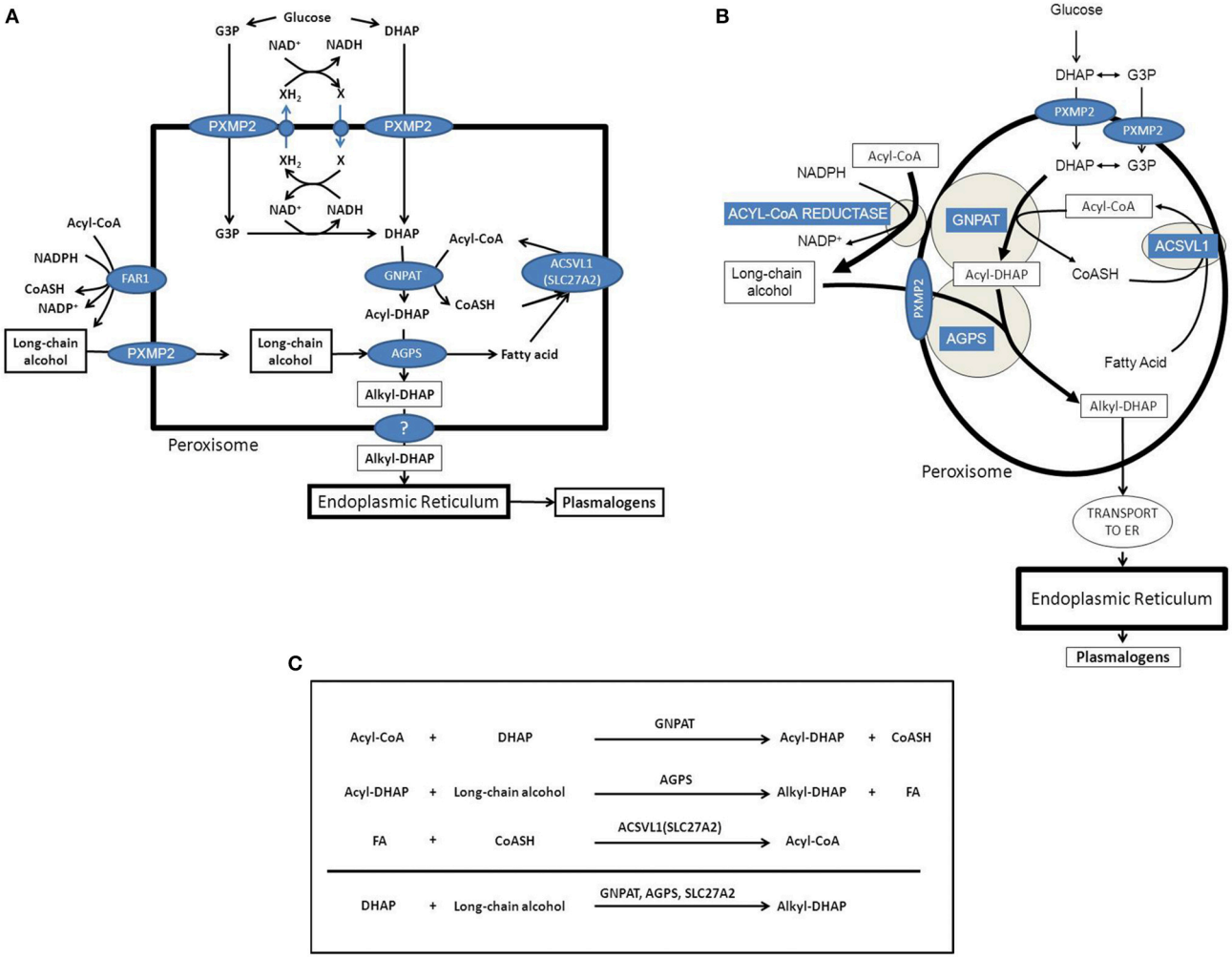

FIGURE 2 | Etherphospolipid biosynthesis and the important role of peroxisomes and the endoplasmic reticulum. (A) Schematic representation of the peroxisome and the enzymes and transporters involved in the peroxisomal synthesis of alkyl-DHAP. **(B)** Schematic representation of the peroxisome and the enzymes and transporters involved in the peroxisomal synthesis of alkyl-DHAP with special emphasis on the sub-peroxisomal localization of GNPAT and AGPS as peripheral peroxisomal membrane proteins joined together in a functional complex. **(C)** Overview of the reactions catalyzed by the three intraperoxisomal enzymes involved in etherphospholipid synthesis including GNPAT, AGPS, and ACSVL1 (SLC27A2). G3P, glycerol-3-phosphate; DHAP, dihydroxyacetonephosphate; GNPAT, glyceronephosphate O-acyltransferase; AGPS, alkyl-glyceronephosphate synthase; FAR1, fatty acyl-CoA reductase 1.

peroxisome to the outside. DHAP synthesized from glucose in the cytosol can move into the peroxisome interior without any problem if it is true that small solutes up to an $M_w$of 300 Da, can move across the peroxisomal membrane freely via PXMP2 (Rokka et al., 2009). However, it has also been claimed that DHAP is synthesized endogenously from glycerol-3-phosphate (G3P) as catalyzed by the enzyme glycerol-3-phosphate dehydrogenase (G3PDH/GPD1). Interestingly, earlier work from Antonenkov (Antonenkov et al., 1985) has shown that rat liver peroxisomes do contain G3PDH activity although the true identity of this enzyme activity has never been resolved definitively. In this respect it is important to mention the work of Jung et al. in yeast which revealed dynamic changes in the subcellular distribution of G3PDH ranging from peroxisomal to cytosolic depending on the metabolic status of the cells (Jung et al., 2010). Either way, both G3P and DHAP would qualify for transport across the peroxisomal membrane via the peroxisomal porine PXMP2 (Rokka et al., 2009).

Since the contribution of peroxisomes to EPL-biosynthesis is restricted to the formation of alkyl-DHAP, or—at best—alkyl-G3P, completion of EPL-biosynthesis is very much dependent upon the interaction with the rest of the cell. In fact, all subsequent steps in EPL-biosynthesis are catalyzed by ER-bound enzymes (see **Figure 2**).

It remains to be established whether the transfer of alkyl-DHAP and/or alkyl-G3P occurs via the cytosol with the possible involvement of a binding protein analagous to the binding of acyl-CoAs by acyl-CoA binding protein (ACBP), or whether this is mediated via direct interorganellar contact sites between peroxisomes and the endoplasmic reticulum. Ultrastructural

studies in the early 1960s had already demonstrated a close proximity between the smooth ER and peroxisomes (Novikoff and Shin, 1964). Indeed, transmission electron microscopy analysis revealed that both organelles appear to be interconnected by electron-dense intermembrane cross-bridges spanning some 10–15 nm. Additional evidence in favor of the existence of such cross-bridges came from biochemical studies in which peroxisomes were isolated from bovine kidney (Zaar et al., 1987). The identity of the proteins mediating the physical interaction between peroxisomes and the ER remains to be identified.

(B) Fatty Acid Beta-Oxidation

Like mitochondria, peroxisomes contain a fatty acid beta-oxidation machinery, which catalyses the stepwise shortening of acyl-CoAs to produce acetyl-CoA in case of straight-chain acyl-CoAs and propionyl-CoA when a 2-methyl-branched-chain acyl-CoA is oxidized. Although the beta-oxidation systems in peroxisomes and mitochondria are basically identical in chemical terms and involve four sequential steps of dehydrogenation, hydration, dehydrogenation again, and thiolytic cleavage, there are major differences between the two systems which include: (a.) the four reactions of the mitochondrial and peroxisomal beta-oxidation pathways are catalyzed by different enzymes each encoded by a distinct gene; (b.) the mitochondrial enzymes catalyzing the first step of beta-oxidation are FAD-dependent dehydrogenases, which feed their electrons into the respiratory chain via the Electron-Transfer-Flavoprotein (ETF) cycle, whereas the corresponding peroxisomal enzymes are FAD-dependent acyl-CoA oxidases donating their electrons directly to molecular oxygen (O_2); (c.) fatty acids are transported across the peroxisomal membrane as acyl-CoAs, or as free fatty acids, whereas fatty acids are transported across the mitochondrial membrane as acylcarnitine esters mediated by the carnitine cycle via the concerted action of carnitine palmitoyl transferase 1 (CPT1), carnitine acylcarnitine translocase (CACT), and carnitine palmitoyl transferase 2 (CPT2); (d.) carnitine does not play a role in the uptake of fatty acids into peroxisomes but is required for the transport of the end products of peroxisomal beta-oxidation to mitochondria for full oxidation to CO_2 and H_2O, which requires the active participation of the citric acid cycle and the mitochondrial oxidative phosphorylation system (respiratory chain); (e.) mitochondria are able to degrade FAs to CO_2 and H_2O, whereas peroxisomes can only chain-shorten fatty acids to acetyl-CoA, propionyl-CoA, and different medium-chain acyl-CoAs, which all need to be transferred to mitochondria for full oxidation to CO_2 and H_2O, and (f.) both peroxisomes and mitochondria are equipped with auxiliary enzymes for the oxidation of unsaturated fatty acids and *2R*-methyl branched chain FAs.

Peroxisomal beta-oxidation of the various acyl-CoA esters is mediated by two acyl-CoA oxidases, two bifunctional proteins, and two thiolases (**Figure 3A**). Much of our knowledge about the physiological roles of the different enzymes involved has come from studies in human patients in whom one of the peroxisomal beta-oxidation enzymes is deficient due to mutations in the structural genes encoding these proteins (see **Figure 3B**, **Table 1**) as well as in mutant mice. Taken all data together, current knowledge holds that acyl-CoA oxidase 1 (ACOX1) is the main enzyme involved in the oxidation of VLCFAs and dicarboxylic acids (DCAs), whereas ACOX2, also called branched-chain acyl-CoA oxidase (BCOX), is the prime oxidase handling the CoA esters of pristanic acid and di- and trihydroxycholestanoic acid (DHCA/THCA) (Vanhove et al., 1993). The second and third steps of beta-oxidation in peroxisomes are catalyzed by two multifunctional proteins alternatively named L- and D-bifunctional protein (LBP and DBP), peroxisomal multifunctional enzyme type 1 and 2 (MFE1 and MFE2), multifunctional protein 1 and 2 (MFP1 and MFP2), and L- and D-peroxisomal bifunctional enzyme (L-PBE and D-PBE) which have been purified, characterized and cloned from various sources (Dieuaide et al., 1996; Leenders et al., 1996; Dieuaide-Noubhani et al., 1997; Jiang et al., 1997; Qin et al., 1997a,b). With respect to their physiological role, it is now clear that the D-specific enzyme as encoded by *HSD17B4* catalyzes the hydration and subsequent dehydrogenation of most peroxisomal beta-oxidation substrates including the enoyl-CoA esters of VLCFAs, pristanic acid and DHCA and THCA, whereas the L-specific enzyme appears to handle the dicarboxylic enoyl-CoA esters specifically (see **Figure 4**; Houten et al., 2012). Finally, with respect to the two thiolases, work on the isolated enzymes (Antonenkov et al., 1997; Wanders et al., 1997) as well as Sterol Carrier Protein X (SCPx)-deficient patients (Ferdinandusse et al., 2006) and mutant mice (Seedorf et al., 1998; Kannenberg et al., 1999) has shown that both peroxisomal 3-ketoacyl-CoA-thiolase 1 (ACAA1; pTH1) and SCPx (pTH2) encoded by *ACAA1* and *SCP2*, respectively, are involved in the oxidation of VLCFAs, whereas the 3-ketoacyl-CoA esters of pristanic acid, DHCA and THCA are solely cleaved by SCPx. The redundancy of the two thiolases with respect the oxidation of VLCFAs may explain why peroxisomal 3-ketoacyl-CoA-thiolase1 (ACAA1)-deficiency has not been identified sofar.

Peroxisomes are not only able to oxidize saturated FAs but also catalyze the oxidation of certain mono- and polyunsaturated FAs as well as 2-(*R*)-methyl branched-chain FAs and 2-hydroxy-FAs (see Wanders and Waterham, 2006; Van Veldhoven, 2010 for reviews). To this end, peroxisomes contain a set of so-called auxiliary enzymes including enoyl-CoA isomerase(s) and 2,4-dienoyl-CoA reductase(s) to remove the double bond present in mono- and polyunsaturated acyl-CoAs, respectively. It should be noted that it is currently unknown which mono- and/or polyunsaturated FAs (MUFA/PUFA) are solely oxidized in peroxisomes except from tetracosahexaenoic acid (C24:6 n-3) as discussed under (C.).

The peroxisomal branched-chain acyl-CoA oxidase ACOX2 only reacts with 2-methyl branched-chain acyl-CoAs if the methyl-group is in the 2(*S*)-position (Van Veldhoven et al., 1996). For DHCA and THCA, which are produced from cholesterol in the liver, this is a potential problem because the methyl-group in the carboxy-terminal side chain of DHCA and THCA has the 2(*R*)- rather than 2(*S*)- configuration which thus prohibits beta-oxidation. This problem is resolved by an enzyme called 2-methyl-acyl-CoA racemase (AMACR), located both in peroxisomes and mitochondria, which is able to convert 2(*R*)-acyl-CoAs into 2(*S*)-acyl-CoAs (Schmitz et al., 1997; Amery

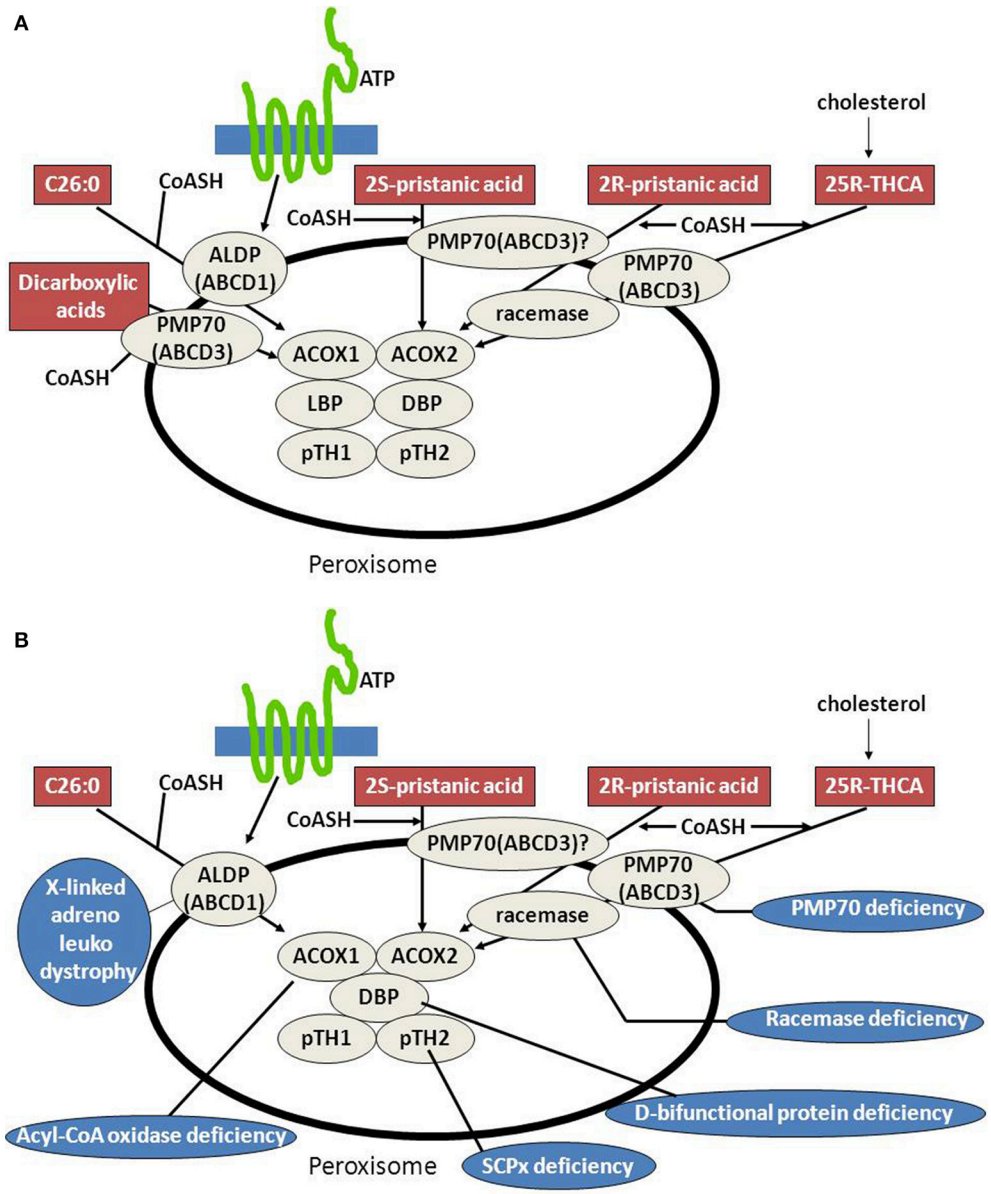

FIGURE 3 | Peroxisomes, fatty acid beta-oxidation and the human deficiencies of peroxisomal beta-oxidation. (A) Schematic diagram depicting the substrates known to be oxidized in peroxisomes exclusively and the transporters and enzymes involved in their degradation (see text for detailed information). **(B)** Schematic diagram depicting the substrates known to be oxidized in peroxisomes exclusively and the transporters and enzymes involved in their degradation and the human deficiencies in the peroxisomal beta-oxidation pathway so far identified (see text for more information).

et al., 2000; Ferdinandusse et al., 2000; Kotti et al., 2000). The same enzyme is also required to convert the 2(*R*)-methyl-group in pristanic acid (2,10,14,16-tetramethylpentadecanoic acid) as derived from phytanic acid into the 2(*S*)-position (**Figure 3A**).

Peroxisomal Beta-Oxidation and the Interplay with Mitochondria

As already eluded to before, peroxisomes are dependent on other organelles for several of their metabolic functions. The interplay with mitochondria is especially important for further metabolism of the end-products of beta-oxidation in peroxisomes including: (1.) NADH; (2.) acetyl-CoA; (3.) propionyl-CoA, and (4.) a variety of acyl-CoAs chain-shortened in peroxisomes.

- *NADH reoxidation*: beta-oxidation in peroxisomes can only continue if the NADH formed in peroxisomes is reoxidized to NAD^+. Ideally, a carrier system in the peroxisomal membrane catalyzing the exchange between NADH in the peroxisome and NAD^+ in the cytosol would serve this purpose. However, since such a system appears to be lacking at least in higher eukaryotes, the existence of

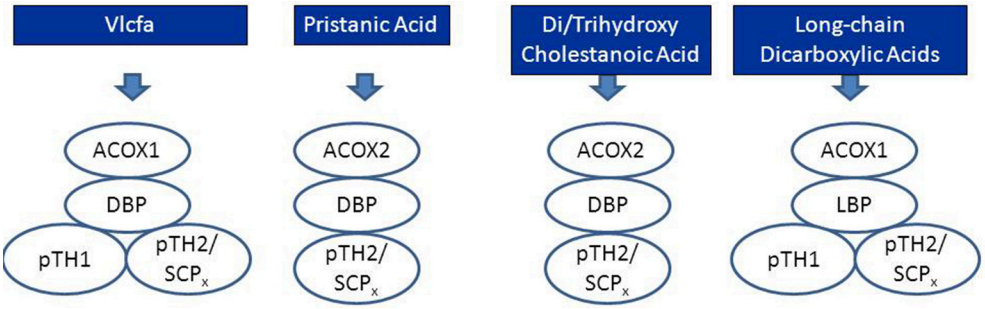

FIGURE 4 | Schematic diagram depicting the peroxisomal enzymes involved in the degradation of very long-chain fatty acids (VLCFAs), pristanic acid, di- and trihydroxycholestanoic acid, and long-chain dicarboxylic acids. See text for detailed information.

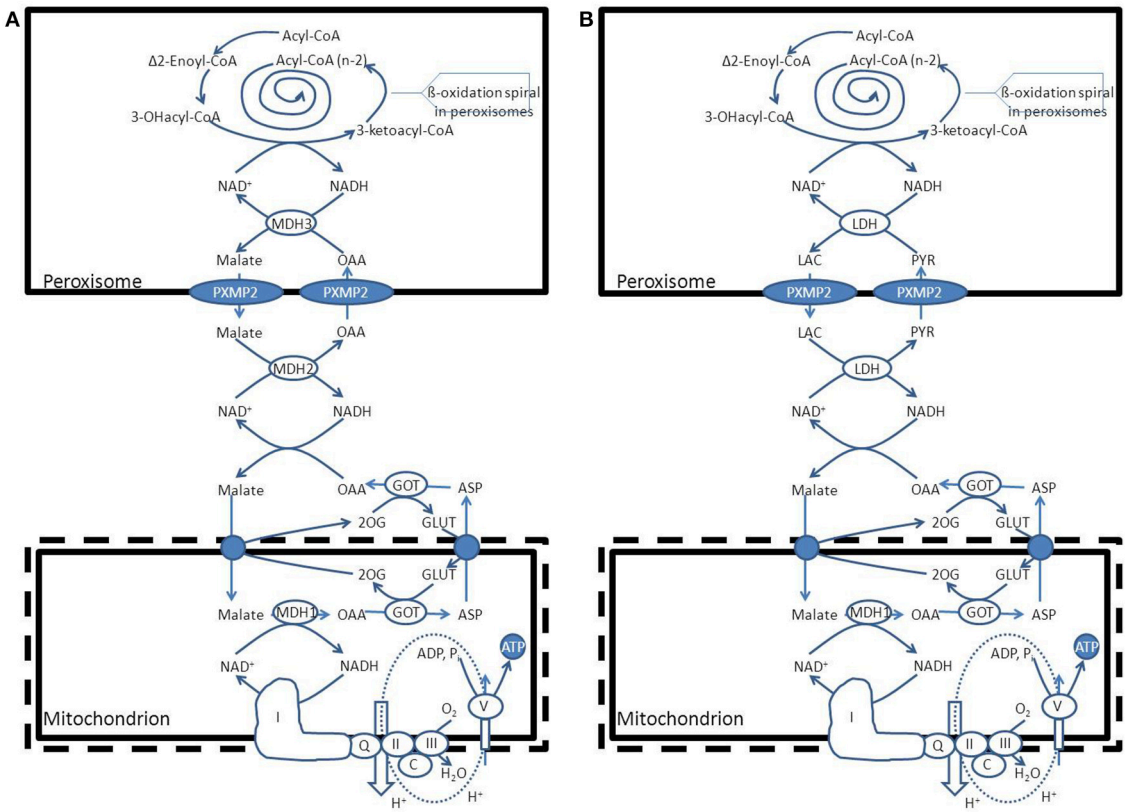

FIGURE 5 | Functional interplay between peroxisomes and mitochondria with respect to the reoxidation of NADH produced within the peroxisomal beta-oxidation system. (A) *S. cerevisiae* (see text for detailed information). **(B)**: *H. sapiens*. PYR, pyruvate; LAC, lactic acid; LDH, lactate dehydrogenase; GOT, glutamate oxaloacetate transaminase; ASP, L-aspartate, GLUT, L-glutamate; 2OG, 2-oxoglutarate; MDH1, mitochondrial malate dehydrogenase.

metabolite-based redox shuttles has been proposed analogous to the well-known malate/aspartate shuttle in mitochondria (see **Figure 1**). In baker's yeast (*S. cerevisiae*) the existence of a peroxisomal NAD(H)-redox shuttle has been demonstrated and involves a malate/oxaloacetate based redox shuttle for reoxidation of peroxisomal NADH (**Figure 5A**). In higher eukaryotes, however, including humans, the identity of the peroxisomal NAD(H)-redox shuttle has not been resolved definitively although evidence in favor of the existence of a lactate/pyruvate-based redox shuttle has been provided by Baumgart et al. (1996), at least in rat liver peroxisomes (see **Figure 5B**). Whatever the precise identity of the peroxisomal NAD(H)-redox shuttle, ultimate reoxidation of peroxisomal NADH back into NAD^+, can only be achieved in mitochondria. The same is true for the NADH produced in the cytosol from glucose upon its conversion into pyruvate during glycolysis. Reoxidation of cytosolic NADH is mediated by one of two NAD(H)-redox shuttles including the malate/aspartate and glycerol-3-phosphate/dihydroxyacetonephosphate redox cycles.

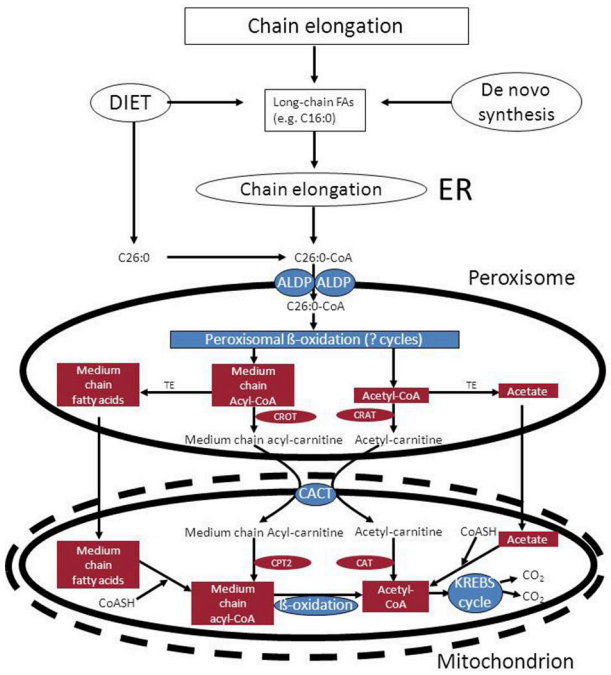

FIGURE 6 | Functional interplay between peroxisomes and mitochondria in the oxidation of C26:0. See text for detailed information. ALDP, adrenoleukodystrophy protein; TE, acyl-CoA thioesterase; CrOT, carnitine octanoyltransferase; CrAT, carnitine acetyltransferase; CACT, mitochondrial carnitine: acylcarnitine translocase.

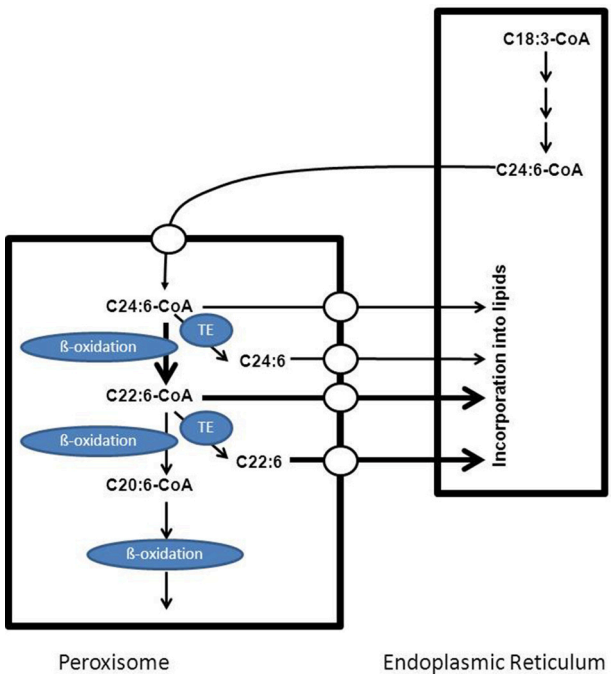

FIGURE 7 | Schematic diagram showing the key role of peroxisomes in the formation of C22:6 n-3. Doxosahexaenoic acid is synthesized from C18:3 n-3 which first undergoes a number of elongation and desaturation steps in the endoplasmic reticulum to produce C24:6-CoA which is then transported to the peroxisome and imported via a mechanism not yet resolved. Within peroxisomes C24:6-CoA undergoes one cycle of beta-oxidation to produce the corresponding C22:6-CoA which can then be exported out of the peroxisome for subsequent incorporation into lipids in the endoplasmic reticulum or may undergo additional sequential rounds of oxidation in peroxisomes and mitochondria. See text for more detailed information.

- *Reduction of peroxisomal NADP back into NADPH*: as for the reoxidation of peroxisomal NADH, redox shuttles have been proposed also for the reduction of NADP as produced in the dienoyl-CoA reductase reaction back into NADPH. In the yeast *S. cerevisiae* there is strong evidence in favor of the existence of a peroxisomal 2-oxoglutarate/(iso) citrate NADP(H)-redox shuttle (van Roermund et al., 1998) next to a similar shuttle identified earlier in mitochondria. These two shuttle systems require the active participation of NADP-linked isocitrate dehydrogenases (IDHs) in mitochondria, peroxisomes and the cytosol respectively. These three activities are catalyzed by three distinct enzymes each encoded by a different gene, at least in *S. cerevisiae*. In higher eukaryotes, however, it appears that there are only two genes coding for NADP-linked IDHs, one in mitochondria, and the other one in peroxisomes, and the cytosol. Detailed work by Yoshihara and coworkers has shown that the bulk of the peroxisomal/cytosolic IDH-activity is actually peroxisomal (>90%), at least in hepatocytes (Yoshihara et al., 2001). As shown by Geisbrecht and Gould, the peroxisomal/cytosolic NADP-linked IDH is equipped with a true PTS1 sequence (Geisbrecht et al., 1999).
- *Mitochondria as ultimate site of oxidation of the different acyl-CoA esters produced in peroxisomes*: the acetyl-CoA, propionyl-CoA, and medium-chain acyl-CoAs produced in peroxisomes, ultimately require the mitochondrial citric acid cycle and oxidative phosphorylation system for full oxidation to CO_2 and H_2O. Shuttling of the different CoA-esters can occur via two mechanisms including: (1.) the carnitine-mediated pathway, and (2.) the free-acid pathway. The first pathway requires conversion of the different acyl-CoA species into acylcarnitines in peroxisomes. To this end, peroxisomes contain two distinct carnitine-acyltransferases, named carnitine-acetyltransferase (CrAT) and carnitine-octanoyltransferase (CrOT) reactive with short and medium-chain acyl-CoAs respectively. The different acylcarnitines are then exported out of the peroxisome via some unknown mechanism, and enter the mitochondrion via the mitochondrial carnitine/acylcarnitine translocase (CACT) followed by the reconversion of the acylcarnitines esters back into the corresponding acyl-CoAs followed by further degradation to CO_2 and H_2O. The alternative, free-acid route first involves cleavage of the acyl-CoA esters by one of a variety of different thioesterases that have been identified in peroxisomes (see Hunt et al., 2012 for review). Next, the free acids move out of the peroxisome probably through the porine (PXMP2) identified by Hiltunen and coworkers (see Rokka et al., 2009; Antonenkov and Hiltunen, 2012 for review) and then enter

the mitochondrion most likely in their protonated form. Mitochondria contain both short-chain as well as medium-chain acyl-CoA synthetase activities to reactivate the free acids back into the corresponding acyl-CoAs followed by oxidation either directly (acetyl-CoA) or indirectly (propionyl-CoA and medium-chain acyl-CoAs). **Figure 6** shows how this works out for C26:0. It remains to be established whether the transfer of metabolites from peroxisomes to mitochondria occurs via simple diffusion through the cytosol, or whether this is mediated through direct interorganellar contacts between peroxisomes and mitochondria. Close proximity between peroxisomes and mitochondria has been observed in intrastructural studies already many years ago (Hicks and Fahimi, 1977). In addition, there is biochemical evidence for direct peroxisome-mitochondrion interactions from density gradient centrifugation analyses (Islinger et al., 2006). Very recently, a genome-wide localization study of peroxisome-mitochondria interactions in yeast has led to the identification of a direct interaction between Pex11, a membrane-bound peroxin involved in peroxisome division and proliferation, and the mitochondrial ERMES complex. Interestingly, PEX11 was found to physically interact with Mdm34 to establish the contact between peroxisomes and mitochondria (see Schrader et al., 2015 for recent review). Tethering of both organelles is supposed to enhance metabolism by reducing the distance for efficient transport of metabolites from the peroxisome to the mitochondrion. Mammalian cells, however, lack ERMES so that another tethering complex is supposed to perform a similar function in higher eukaryotes, including humans.

(C) Docosahexaenoic Acid (C22:6 n-3) Synthesis

DHA (C22:6 n-3) is the most important n-3 PUFA and is the major PUFA in adult mammalian brain and retina. A deficiency of DHA can lead to memory loss, learning disabilities and impaired visual acuity (Jump, 2002). DHA is synthesized from dietary linolenic acid (C18:3 n-3) in the endoplasmic reticulum via a series of elongation and desaturation reactions. This pathway requires that C22:5 n-3 would be desaturated at position 4 by an acyl-CoA-dependent delta4-desaturase to form C22:6 n-3. Several studies have shown that mammals do not possess such a delta4-desaturase. Instead, a 24-carbon n-3 fatty acid is first synthesized which is then desaturated at position six to produce C24:6 n-3 followed by one round of beta-oxidation in the peroxisome with C22:6 n-3 as final product (see **Figure 7**). Interestingly, some of the enzymes involved in the beta-oxidation of C24:6 n-3-CoA have been identified and include the straight-chain acyl-CoA oxidase (ACOX1) and D-bifunctional protein (Su et al., 2001; Ferdinandusse et al., 2003). The C22:6-CoA produced in peroxisomes may either undergo continued beta-oxidation in peroxisomes and subsequently in mitochondria or be exported out of the peroxisome for incorporation into lipids in the endoplasmic reticulum. The exact mechanism by which DHA is exported from the peroxisomes either as coenzyme A ester or as free acid has not been deduced sofar.

(D) Bile Acid Synthesis

Peroxisomes also play an indispensable role in the biosynthesis of the primary bile acids cholic acid and chenodeoxycholic acid. The underlying basis for the obligatory role of peroxisomes in bile acid formation, resides in the fact that the two bile acid intermediates, i.e., 3alpha, 7alpha-dihydroxy-5beta-cholestanoic acid (DHCA) and 3alpha, 7alpha, 12alpha-trihydroxy-5beta-cholestanoic acid (THCA) undergo beta-oxidative chain shortening in peroxisomes with propionyl-CoA as one product and chenodeoxycholoyl-CoA and choloyl-CoA as the respective other products of beta-oxidation (see **Figure 8**). The enzymes catalyzing the formation of DHCA and THCA respectively are localized in different subcellular compartments including the cytosol, endoplasmic reticulum, and mitochondrion (CYP27A1) (see Russell, 2003 for review). Activation of DHCA and THCA produced by the mitochondrial enzyme CYP27A1 most likely occurs by the ER enzyme bile acid-CoA ligase (BACL) encoded by SLC27A5 (Wheeler et al., 1997; Falany et al., 2002) after which the two CoA-esters of DHCA and THCA enter the peroxisome. The recent identification of PMP70 deficiency in a patient with markedly elevated DHCA and THCA levels in plasma supported by additional studies in the *Pmp70*(-/-/) mouse has led to the conclusion that the peroxisomal half-ABC-transporter PMP70 (ABCD3) catalyzes the import of these acyl-CoAs into peroxisomes (Ferdinandusse et al., 2015). The actual beta-oxidation of DHC-CoA and THC-CoA is catalyzed by the enzymes ACOX2, D-bifunctional protein, and peroxisomal thiolase-2 (SCPx) (see **Figure 4**). Subsequently, the two acyl-CoAs are converted into the corresponding taurine and/or glycine esters by the enzyme bile acid-CoA: amino acid *N*-acyltransferase (BAAT). Work by Faber and coworkers has shown that BAAT is a strictly peroxisomal enzyme indicating that tauro/glycocholate and tauro/glycochenodeoxycholate are the true end products of peroxisomal bile acid metabolism (Pellicoro et al., 2007). The tauro/glycoconjugates of cholic acid and chenodeoxycholic acid are then exported from the peroxisome interior via some unknown mechanism into the cytosol followed by the rapid excretion from the hepatocytes into bile via BSEP (ABCB11) localized in the canulicular membrane (**Figure 8**).

(E) Phytanic Acid Alpha-Oxidation

3-methyl-FAs cannot be beta-oxidized right away simply because the methyl-group at the 3-position prohibits beta-oxidation. In order to allow oxidation of 3-methyl-FAs, these FAs first need to undergo one cycle of alpha-oxidation thereby converting the 3-methyl-FA into a 2-methyl-FA which can then be beta-oxidized (Wanders et al., 2011b). Alternatively, 3-methyl-FAs may be oxidized via the omega end so that phytanic acid is actually chain-shortened from the omega-end (see Wanders et al., 2011a for review). The best known FA undergoing alpha-oxidation, is phytanic acid (3,7,11,14-tetramethylhexadecanoic acid) as concluded from observations on a rare disease called Refsum disease in which alpha-oxidation is blocked due to a genetic deficiency of the enzyme phytanoyl-CoA 2-hydroxylase encoded by *PHYH*. Phytanic acid is strictly derived from

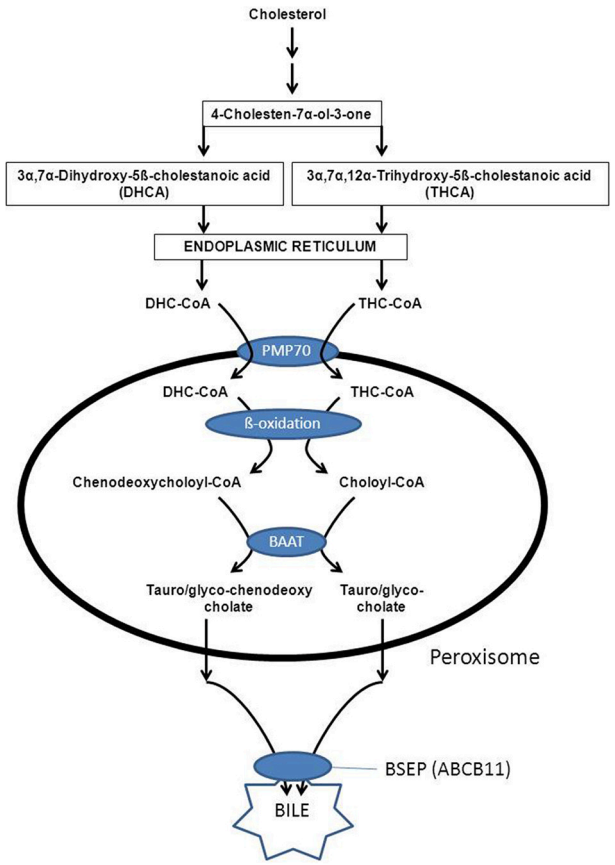

FIGURE 8 | Schematic diagram showing the unique and important role of peroxisomes in the formation of the primary bile acids cholic acid and deoxycholic acid. See text for detailed information.

dietary sources and cannot be synthesized *de novo*. Although not well-studied, the general notion is that phytanic acid is transported throughout the body via the blood in its free as well as esterified form. Indeed, in plasma phytanic acid has been identified in triglycerides but also in other lipid species. According to Wierzbicki et al. (1999) most of the phytanic acid in Refsum disease patients is present in the LDL-fraction. Hydrolysis of LDL-particles after receptor mediated uptake into cells within lysosomes would then release the phytanic acid into the cytosol. The fact that there are multiple acyl-CoA synthetases able to convert phytanic acid into phytanoyl-CoA (see Wanders et al., 2011b for review) would ensure rapid formation of phytanoyl-CoA in the extra-peroxisomal space. This implies that phytanoyl-CoA is the most likely substrate to be transported across the peroxisomal membrane. Based on our own recent work in a patient with a genetic defect in *ABCD3* coding for PMP70 as well as studies in a mutant *Abcd3(-/-)* mouse model we conclude that PMP70 (ABCD3) catalyzes the uptake of phytanoyl-CoA into peroxisomes (Ferdinandusse et al., 2015; see **Figure 9**).

Once inside the peroxisome interior, phytanoyl-CoA is hydroxylated by the enzyme phytanoyl-CoA 2-hydroxylase first identified by Mihalik et al. (1995) to produce 2-hydroxyphytanoyl-CoA. The enzyme involved belongs to the group of 2-oxoglutarate-dependent dioxygenases and the hydroxylation of the substrate is driven by 2-oxoglutarate and molecular oxygen with succinate and CO_2 as products (Mukherji et al., 2003). Subsequently, the enzyme 2-hydroxyacyl-CoA lyase (HACL) cleaves 2-hydroxyphytanoyl-CoA, and a range of other 2-hydroxy acyl-CoAs in fact, between the first and second carbon atom to produce formyl-CoA plus the aldehyde pristanal in case of phytanic acid alpha-oxidation (Foulon et al., 2005). This aldehyde is then converted into the corresponding acid (pristanic acid). Available evidence holds that peroxisomes do contain aldehyde dehydrogenase activity as shown for pristanal by Jansen et al. (2001). The true identity of this enzyme activity has not been settled definitively. For humans it has been suggested that FALDH-V, a truncated splice product produced from the *ALDH3A2* gene, is directed to peroxisomes (Ashibe et al., 2007). The bulk of FALDH-activity produced from the *ALDH3A2* gene, however, is located in the endoplasmic reticulum (ER). Whether FALDH-V is truly the enzyme responsible for the pristanal dehydrogenase activity in peroxisomes remains doubtful, however, for various reasons including the fact that FALDH-V appears to be a membrane-bound enzyme with its catalytic domain exposed to the cytosol, whereas the peroxisomal pristanal dehydrogenase activity is catalyzed by a soluble matrix enzyme (Jansen et al., 2001). Furthermore, phytanic acid alpha oxidation is fully normal in patients suffering from Sjögren Larsson syndrome (SLS) caused by mutations in the *ALDH3A2* gene. In line with these results, phytanic acid does not accumulate in SLS-patients. **Figure 9** depicts the organization of the alpha-oxidation system in peroxisomes as envisaged right now with phytanoyl-CoA 2-hydroxylase, HACL, and also the putative pristanal dehydrogenase all localized in the matrix of the peroxisome. The alpha-oxidation machinery can only work efficiently if the NADH produced in the aldehyde dehydrogenase reaction is reoxidized back to NAD^+ as described above. Furthermore, constant supply of 2-oxoglutarate is required, coupled to the removal of succinate. Both 2-oxoglutarate and succinate can probably move freely through the peroxisomal membrane via the peroxisomal porine PXMP2. Since succinate is a 4-carbon molecule, whereas 2-oxoglutarate has 5-carbon atoms, reconversion of succinate back into 2-oxoglutarate can only be achieved via one of the carboxylases or some other mechanism. A possible role for one of the four known carboxylases, including pyruvate carboxylase, acetyl-CoA carboxylase, propionyl-CoA carboxylase, and methylmalonyl-CoA carboxylase is hard to envisage. However, conversion of succinate back into 2-oxoglutarate can also occur in the mitochondrion by using part of the citric acid cycle and in particular the citrate synthase reaction which can turn a C4-molecule like oxaloacetate into the 6-carbon molecule citrate. The mechanism would then be that succinate enters the mitochondrion via the mitochondrial dicarboxylate carrier and is converted back into 2-oxoglutarate via the concerted action of succinate dehydrogenase, fumarase, malate dehydrogenase, citrate synthase, and NAD-linked isocitrate dehydrogenase followed by export of 2-oxoglutarate via the mitochondrial carrier specific for 2-oxoglutarate (see **Figure 9**).

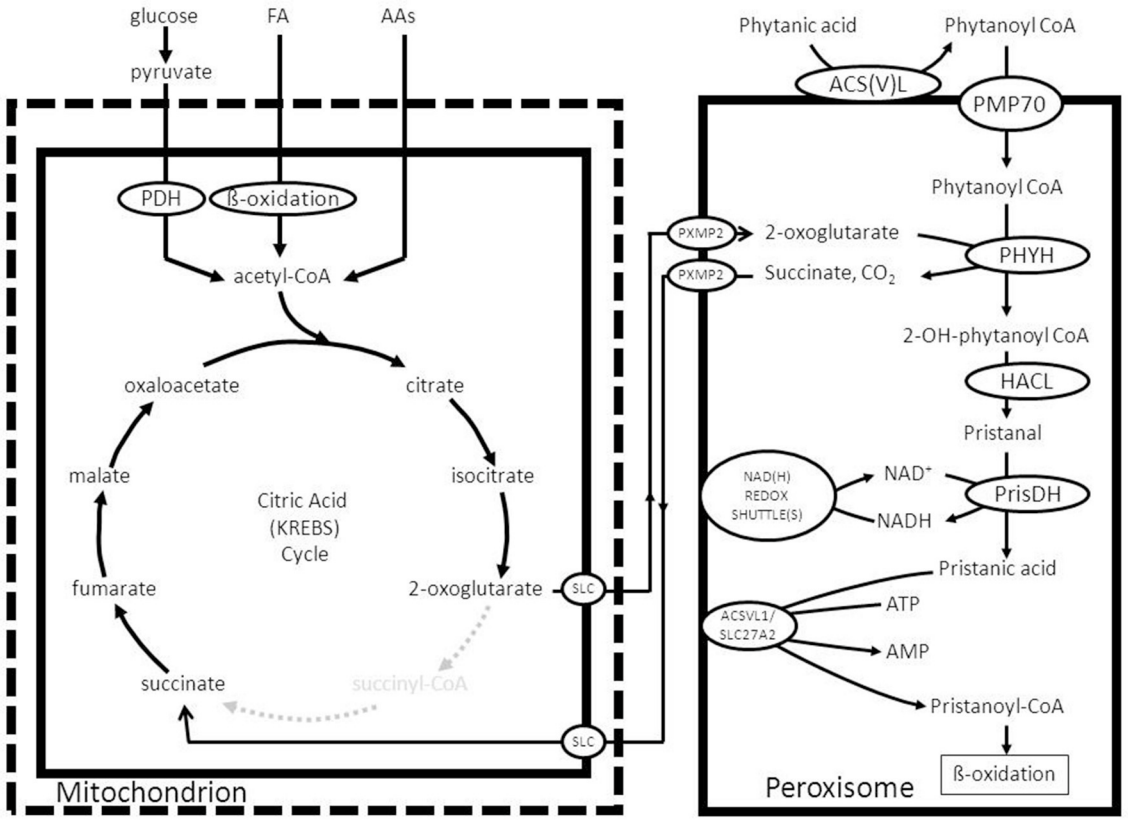

FIGURE 9 | Functional interplay between mitochondria and peroxisomes in the alpha-oxidation of phytanic acid. See text for detailed information.

With respect to one of the other products of alpha-oxidation, i.e., formyl-CoA, the current notion holds that formyl-CoA is rapidly hydrolyzed spontaneously to produce free CoASH and formic acid (Croes et al., 1997). Formic acid can be degraded via two pathways including: (1.) a catalase mediated pathway in which catalase operates in the peroxidative mode and (2.) via the folate-dependent pathway (Tephly, 1991). Finally, the CoA released from formyl-CoA could be used to convert pristanic acid to pristanoyl-CoA as described above. In fact, peroxisomes do contain pristanoyl-CoA synthetase activity which is probably catalyzed by the enzyme ACSVL1 (SLC27A2) as already mentioned above. **Figure 9** shows the final scheme in which the considerations above have been incorporated and used to construct a feasible model.

(F) Glyoxylate Detoxification

In humans the enzyme alanine glyoxylate aminotransferase (AGXT) is the principal enzyme involved in the detoxification of glyoxylate, is strictly peroxisomal in human liver (Danpure and Jennings, 1986) and a deficiency of this enzyme causes hyperoxaluria type 1 (Danpure et al., 1987) which in its extreme form may be lethal due to the accumulation of calcium oxalate in multiple tissues including the kidneys, liver, and heart (see Salido et al., 2012 for review). The product of the AGXT reaction in peroxisomes is pyruvate which needs to be reconverted into alanine via different transaminases localized in the cytosol or degraded in the mitochondrion via the enzyme pyruvate dehydrogenase, which again shows the interaction of peroxisomes with multiple subcellular compartments including the cytosol and the mitochondrion in the case of glyoxylate metabolism (**Figure 10**; Salido et al., 2012). Glycine is further metabolized in mitochondria and broken down via the glycine cleavage enzyme which is made up of four different proteins, named P-, T-, H-, and L-protein (see **Figure 10**; Kikuchi et al., 2008 for review).

It should be noted that much remains to be learned about the metabolic precursors of glyoxylate although glycolate is definitely one of the major sources of glyoxylate with the peroxisomal enzyme 2-hydroxy acid oxidase (HAO1; alternative named: glycolate oxidase) as the enzyme responsible for the conversion of glycolate to glyoxylate (Vignaud et al., 2007). Other known sources of glyoxylate are hydroxyproline (Knight et al., 2006) and glycine.

(G) Amino Acid Metabolism

Peroxisomes also play an indispensable role in the degradation of a range of amino acids, notably the D-amino acids. Indeed, mammalian tissues contain at least two different degradative enzymes that are stereospecific for D-amino acids including D-amino acid oxidase (DAO; also known as DAAO) and D-aspartate oxidase (DDO; also known as DASPO). Both DAO

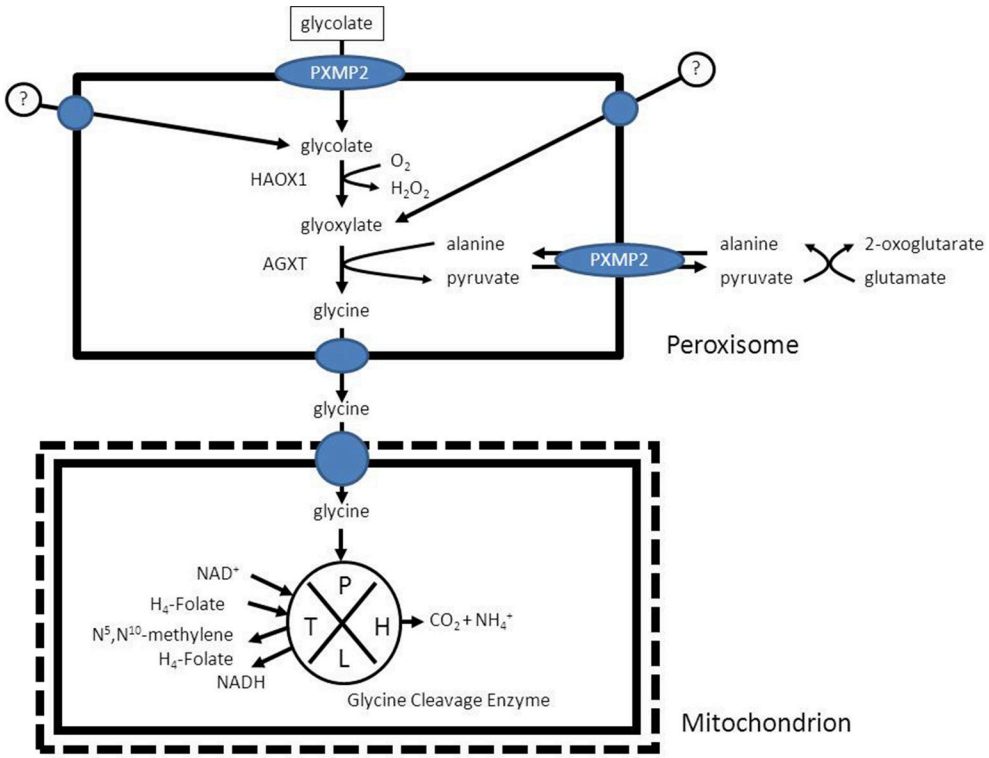

FIGURE 10 | The detoxification of glyoxylate in peroxisomes as catalyzed by the enzyme alanine glyoxylate aminotransferase (AGXT).

and DDO are FAD-linked flavoproteins able to catalyze the oxidative deamination of D-amino acids to produce H_2O_2, ammonia, and the corresponding 2-oxoacid. The *DAO* gene product displays a broad substrate specificity and reacts with a range of neutral and basic D-amino acids including D-serine, D-alanine, and others (Krebs, 1935; Dixon and Kleppe, 1965). The other oxidase (DDO) is highly specific for acidic D-amino acids such as D-aspartate and D-glutamate but also reacts with N-methyl-D-aspartate acid. Mammalian DAO and DDO are presumed to regulate the levels of several endogenous and exogenous D-amino acids including D-serine and D-aspartate in various organs notably the brain. D-serine for instance binds to the glycine binding site of the N-methyl-D-aspartate (NMDA) receptor and potentiates glutamatergic neurotransmission in the central nervous system. Several lines of evidence suggest that D-serine plays an important role in the regulation of brain functions by acting as co-agonist for the NMDA receptor and perturbations in D-serine in the nervous system have recently been implicated in the pathophysiology of various neuropsychiatric disorders (see Katane et al., 2015 for references). Recent studies have also shown that D-aspartate acts as signaling molecule in nervous and neuroendocrine systems at least in part by binding to the NMDA receptor and, thus plays an important role in the regulation of brain function (Katane and Homma, 2011; Errico et al., 2012; Ota et al., 2012). Furthermore, peroxisomes, at least in humans, are the sole site of L-pipecolic acid oxidase activity which is a metabolite derived from lysine (Wanders et al., 1988; Mihalik et al., 1989). There is currently very little information in literature on the functional interplay between peroxisomes and other subcellular compartments in the oxidation of the various amino acids in humans is concerned.

(H) ROS/RNS-Metabolism

In line with its name, the peroxisome also plays a major role in cellular ROS/RNS-metabolism. Indeed, peroxisomes contain a large number of ROS-producing enzymes of which the acyl-CoA oxidases are the most abundant being present in virtually all peroxisomes independent of the tissue and cell type involved. Other H_2O_2 producing oxidases include D-amino acid oxidase (DAO), D-aspartate oxidase (DDO), L-pipecolate oxidase (PIPOX), 2-hydroxy acid oxidases (HAO), polyamine oxidase, and xanthine oxidase. Furthermore, the inducible form of NOS (NOS2) is localized in peroxisomes (for review see Schrader and Fahimi, 2006; Antonenkov et al., 2010; Fransen et al., 2012; Nordgren and Fransen, 2014; Lismont et al., 2015). In addition, peroxisomes contain a large network of enzymatic and also non-enzymatic antioxidants that protect the organelle from oxidative damage. The main antioxidant enzymes include thioredoxin 2(TRX2), thioredoxin reductase (TXNRD2), the glutaredoxins 2 (GLRX2), and 5 (GLRX5), the peroxiredoxins 3 (PRDX3), and 5 (PRDX5), GSH peroxidase 1 (GPX1), oxidized glutathione (GSSG) reductase (GSR), and the copper/zinc (SOD1)- and manganese (SOD2)-containing SODs (see Lismont et al., 2015 for review). Recent work by Fransen and coworkers (Wang et al., 2013)

has shown that also with respect to ROS/RNS-metabolism there is marked functional interplay between peroxisomes and other subcellular organelles, notably mitochondria (Wang et al., 2013).

CONCLUDING REMARKS

Peroxisomes play a crucial role in cellular metabolism as exemplified by the different inborn errors of metabolism caused by a deficiency of one of the peroxisomal enzymes (**Table 1**) as reviewed in this paper. It is also fully clear that the metabolic capabilities of peroxisomes are very much dependent on the functional interplay with other organelles, notably the mitochondrion and endoplasmic reticulum. Although much has been learned about the functional organization of the peroxisome in terms of the enzymes involved and the end-products of peroxisomal metabolism, there are still substantial gaps in our knowledge about peroxisome metabolism. These include the question how substrates and products of peroxisome metabolism are transported across the peroxisomal membrane, especially since PXMP2 only allows passage of small molecules with an M_w < 300 (Antonenkov and Hiltunen, 2012). One other area which has remained relatively unexplored involves the mechanism of transfer of the end products of peroxisome metabolism from the peroxisome to other organelles like the mitochondrion and ER. It is gratifying to see that several recent reports are beginning to shed light on the mechanisms involved in the physical interaction between individual organelles and the proteins involved. This is especially true for the peroxisome-mitochondrion association with the identification of PEX11 and its interaction with the ERMES complex (Mattiazzi Usaj et al., 2015; see Schrader et al., 2015 for review).

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REVIEW
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The lipid droplet—a well-connected organelle

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Our knowledge of inter-organellar communication has grown exponentially in recent years. This review focuses on the interactions that cytoplasmic lipid droplets have with other organelles. Twenty-five years ago droplets were considered simply particles of coalesced fat. Ten years ago there were hints from proteomics studies that droplets might interact with other structures to share lipids and proteins. Now it is clear that the droplets interact with many if not most cellular structures to maintain cellular homeostasis and to buffer against insults such as starvation. The evidence for this statement, as well as probes to understand the nature and results of droplet interactions, are presented.

Keywords: lipid droplet, organelle junction, protein trafficking, endoplasmic reticulum, mitochondria

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Introduction

Cytoplasmic lipid droplets (usually shortened to "droplets" hereafter) are virtually ubiquitous in eukaryotic cells and exist even in prokaryotes (Alvarez and Steinbüchel, 2002; Chapman et al., 2012; Walther and Farese, 2012). They dominate the cytoplasm of certain normal cells, such as those of plant oil seeds, fungal cells growing on lipid sources, and adipocytes and cells of the fat body in animals. Lipid droplet-packed cells are the hallmarks of two common human diseases: foam cells in atherosclerotic plaques, and hepatic parenchymal cells in fatty liver (Yuan et al., 2012; Sahini and Borlak, 2014). Although in the light microscope one observes apparently free-standing coalescent spherical units of translucent material that stain with lipid dyes such as Oil Red O, early ultrastructural studies revealed a thin phospholipid membrane encircling the lipid core that further analyses indicated was a single phospholipid leaflet (Tauchi-Sato et al., 2002). Moreover, subjecting the "fat cake" formed from centrifuging adipose tissue homogenates to SDS gels revealed a protein component of droplets (Greenberg et al., 1991). Early proteomic studies of isolated droplets (Athenstaedt et al., 1999; Brasaemle et al., 2004) confirmed a rich assortment of droplet-associated proteins, many of which were already known to play roles in generation and breakdown of neutral lipids (reviewed in Yang et al., 2012).

These proteomic studies also revealed highly specific markers of other organelles, such as ER luminal chaperones and components of mitochondrial oxidative phosphorylation. That ER and mitochondrial proteins were so frequently associated with lipid droplets, which are typically purified through several rounds of flotation in aqueous buffers under conditions in which other organelles pellet, suggested that they represented more than contaminants adventitiously adhering to droplets during fractionation.

Inter-organellar junctions are proving to be the rule, not the exception, in cell biology. Examples of stable or dynamic associations include junctions between the ER and several organelles including mitochondria, plasma membranes, vacuoles/lysosomes, Golgi, and endosomes (reviewed in Helle et al., 2013) Peroxisomes, components of which form at the ER, have contacts with lysosomes, which may be of fundamental importance in cholesterol transport (Chu et al., 2015). Lipid

droplets also form associations with these organelles (with the possible exceptions of Golgi and plasma membrane), the subject of this review (**Figure 1**). Whether these physical connections between organelles have physiological importance is now a tractable question as proteins specific to junctions are being identified, and reverse genetics used to probe their function by observing phenotypes in their absence.

Droplets form stable associations of demonstrated physiological value at least with the ER and mitochondria, and the nature of these connections are a large part of this review. However, droplets also appear to bind to other organelles such as the inner nuclear envelope, lysosomes/vacuoles, and endosomes. Evidence for these connections are also presented here with speculation (from us and others) about their relevance.

Relationship with the ER

Introduction

Cytoplasmic lipid droplets, as well as secreted lipoproteins, originate in the ER. But the relationship does not end there. Associations between droplets and the ER, first observed by electron microscopy 35 years ago (Novikoff et al., 1980), remain. In yeast, droplets do not appear to ever dissociate from the ER (Szymanski et al., 2007), although in mammals there may be two distinct populations, one attached to the ER, the other not (Wilfling et al., 2013). Membrane bridges between ER and droplets were also observed in this work, although the molecular composition of the bridge remains to be determined. Formation of such contact zones is hypothesized to involve the Arf1-COPI complex (see below; **Table 1** lists proteins that play important roles in the interactions of droplets with organelles).

Besides its involvement in droplet assembly, the functions of ER–droplet connections that explain their stable nature likely include protein and lipid trafficking, response to ER stress, and a role in ER-associated degradation (ERAD).

Droplet Assembly

There are several recent reviews on lipid droplet formation (Gross and Silver, 2014; Pol et al., 2014; Wilfling et al., 2014a; Hashemi and Goodman, 2015). Neutral lipids are initially generated by enzymes in the ER. As droplets form, some of these proteins such as isozymes of glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) partially or fully transfer to the droplet surface (Jacquier et al., 2011; Wilfling et al., 2013). Time-lapse images showed droplets emanating from the perinuclear ER ring in yeast, a process catalyzed by seipin (Cartwright et al., 2015). How seipin initiates droplet formation is still obscure, although it may serve as a scaffold for enzymes in the pathway of neutral lipid synthesis, such as the phosphatidate hydrolase lipin (Sim et al., 2012). FIT2, an ER protein that binds triacylglycerols, likely also contributes to droplet formation (Gross et al., 2011; Miranda et al., 2014). Cytoplasmic proteins such as PLIN3 (perilipin 3/Tip47), may facilitate membrane curvature that must accompany droplet formation (Skinner et al., 2009). How these factors, and those still-to-be identified, coordinate their function, is still unknown, although seipin may act as a binding scaffold (Talukder et al., 2015).

Droplets do not always form *de novo*. Pre-lipid droplets on the ER exist on starved mammalian cells that are the loci for new droplet assembly when incubated with fatty acids (Kassan et al., 2013). Droplets may also form by fission, as documented in *Schizosaccharomcyes pombe* (Long et al., 2012). Care was taken to rule out z-section artifact in this study, which showed a small droplet emanating from a larger one. Interestingly, the young droplet was not adjacent to the ER during this process suggesting that it may begin its life independent of the ER. Small droplets also appear to form from a large one during acute lipolysis (Marcinkiewicz et al., 2006), although these new organelles are likely a product of *de novo* synthesis rather than fragmentation, based on evidence from time-lapse microscopy (Paar et al., 2012).

ER to Droplet Trafficking

A subset of proteins traffic to droplets via the ER, as covered in a recent review (Walther and Farese, 2012). Although we are not aware of any study showing trafficking of endogenous proteins in cells at steady state (i.e., without induction of droplet synthesis or overexpression of cargo), the evidence is strong that this pathway exists. Several proteins such as caveolins have distinct ER and droplet targeting domains (Ingelmo-Torres et al., 2009); When separate, deletion of the droplet-targeting domain results in ER localization. Deletion of the ER localization domain results either in failure to target to either organelle, or targeting to droplets via the cytosol. Ancient Ubiquitous Protein 1 (AUP1) is an exception with overlapping ER and droplet targeting domains (Stevanovic and Thiele, 2013). Another line of evidence is in systems in which droplet formation is stimulated, by incubation of cells in oleic acid or induction of a neutral lipid-synthesizing enzyme. Before stimulation, several droplet proteins have been shown to accumulate in the ER. They then migrate to droplets upon induction (Jacquier et al., 2011; Thiel et al., 2013; Wilfling et al., 2013). Two motives for droplet targeting have been established: amphipathic helices and hydrophobic hairpins (Thiam et al., 2013b), although other motifs can target as well (Murugesan et al., 2013). Some of these may simply bind other resident droplet proteins. It has been difficult to derive the rules for a prototypic "lipid droplet targeting motif." There is even less known about how this signal (singular or plural) is/are specifically recognized at the droplet, and whether this recognition has its origin in a classical receptor/docking complex or is a product of the physicochemical nature of the droplet phospholipid monolayer and underlying neutral lipid core. Whatever the rules, this process is conserved: mammalian and plant droplet proteins can target efficiently in yeast and even induce droplet formation (Jacquier et al., 2013). There is evidence supporting the role of phospholipid density and surface tension on droplets controlling trafficking of proteins (Thiam et al., 2013b). Related questions remain: How do droplet proteins initially enter the ER? Thus far there are no examples to our knowledge of endogenous droplet proteins containing traditional signal peptides that are cleaved during initial translocation across the ER membrane. It is reasonable that droplet proteins that originate in the ER bypass the SEC61/signal peptidase

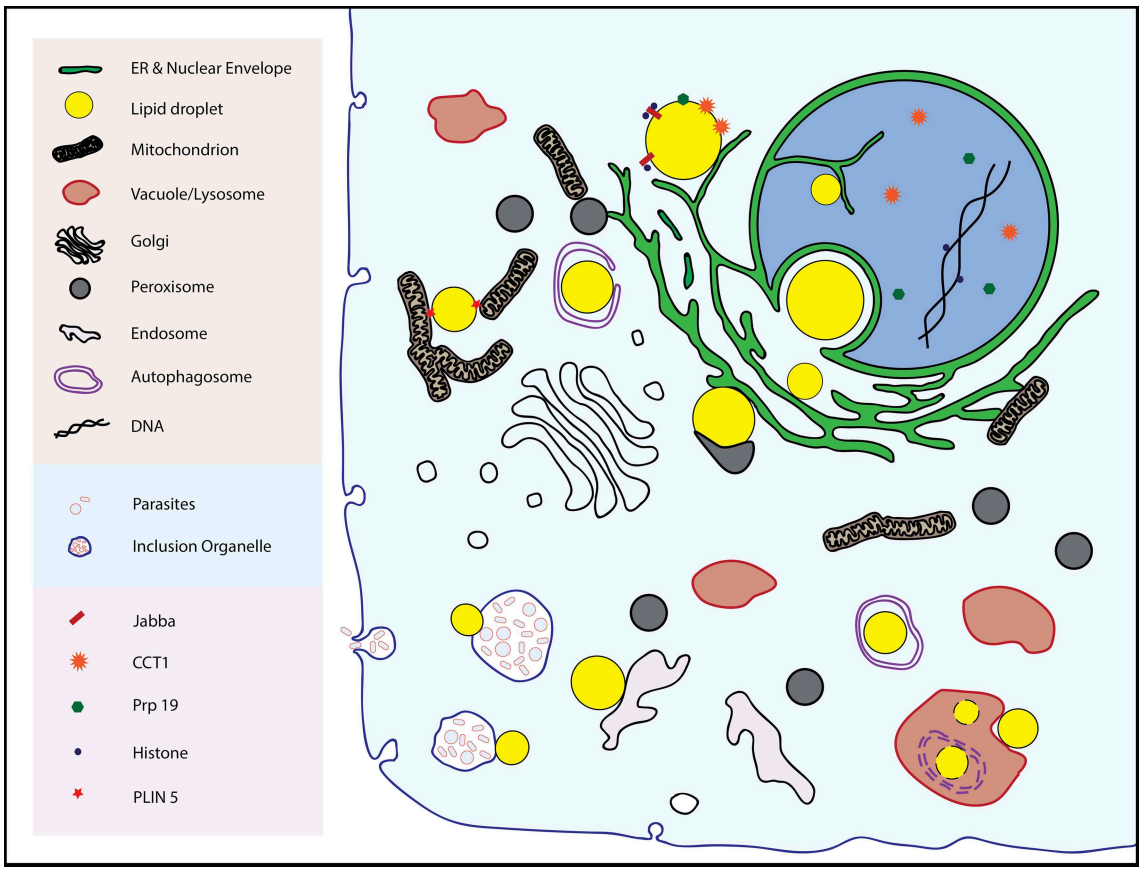

FIGURE 1 | The multitude of inter-organellar interactions involving lipid droplets are shown. See text for details.

system, which is designed for secreted or transmembrane (with hydrophilic domains on both sides) proteins, both incompatible with the droplet topology. Is there a unique targeting pathway into the ER for droplet proteins? Trafficking from ER to droplets occurs over several minutes (Jacquier et al., 2011), suggesting that there may be more involved than simple lateral diffusion. What is the rate-limiting step in trafficking? No doubt studies in the near future will address these issues.

A layer of regulation in ER–droplet protein trafficking has been discovered in yeast in which the growth phase affects the partitioning of the diacylglycerol (DAG) acyltransferase, Dga1p, between these two organelles. Targeting of Dga1p from ER to droplets was first described in a system in which droplet assembly was induced (Jacquier et al., 2011). In more recent work, the regulation was uncovered: In early log phase, when DAG is largely channeled into phospholipids, Dga1p is relatively inactive in the ER. As cells approach stationary phase, it is transported to lipid droplets for triacylglycerol synthesis. Return of Dga1p to the ER is promoted by Ice2p (previously known to be involved in the inheritance of ER), which also coordinates the use of DAG for phospholipid synthesis (Markgraf et al., 2014).

Besides the trafficking of endogenous proteins, trafficking of viral proteins from ER to droplets is required for the assembly of *Flaviviridae* family viruses, notably hepatitis C (HCV) and Dengue viruses, as well as for other viral families (Saka and Valdivia, 2012). Droplets promote the assembly of viral capsids by attracting amphipathic helices of the viral proteins to their surfaces. This has been best studied in HCV, in which the core protein first enters the ER via a cleaved signal sequence (presumably through SEC61!), before migrating to droplets, where it then attracts other viral proteins. One of these is NS4B, which contains both ER and lipid droplet targeting signals. Surprisingly, in the absence of the ER hydrophobic signal, the protein appears to go directly to droplets from the cytosol, as determined by fluorescence microscopy (Tanaka et al., 2013).

It should be noted that there are routes to droplets other than through the ER. Evidence supports a direct path from cytosol to droplets for CCT1 (phosphocholine cytidylyltransferase) which likely traffics there depending on the phospholipid density on the droplet surface (Krahmer et al., 2011) and the exchangeable perilipins (perlipins are a group of droplet-associated proteins that share a common domain) PLIN3, PLIN4, and PLIN5 (Wolins et al., 2006). The trafficking of the adipose triglyceride lipase, ATGL, to droplets depends on the COPI pathway of retrograde protein transport (Beller et al., 2008), but the mechanistic relationship (whether it is direct or indirect) is

TABLE 1 | Proteins implicated in lipid droplet interactions with other organelles.

Organelle	Protein	Description	References
Endoplasmic reticulum	Arf-COPI components	Its action may result in ER tethering	Wilfling et al., 2013
	Seipin	Important for droplet biogenesis	Cartwright et al., 2015
	FIT2	Important for TAG transfer to droplets	Miranda et al., 2014
	Lipin	May provide DAG for droplet assembly	Sim et al., 2012
	Lro1p	Produces TAG at the ER/droplet interface	Wang and Lee, 2012
	UBXD2, UBXD8/Ubx2p, p97/VCP, AUP1,	ERAD proteins often found on droplets, connecting function of the two organelles	Suzuki et al., 2012; Olzmann et al., 2013
Mitochondria	PLIN5	Mediates droplet–mitochondrial interactions, modulates droplet lipases	Mason and Watt, 2015
Peroxisomes	SDP1	Lipase in plants that traffics between peroxisomes and droplets	Thazar-Poulot et al., 2015
Nucleus	Histones, Jabba	Certain histones stored on droplets	Welte, 2015; Wang et al., 2012
	CIDE family proteins CCT1	Control transcription when not bound to ER or droplets Shuttles from nucleus to droplets to affect phospholipid synthesis	Guo et al., 2008; Krahmer et al., 2011
	Prp19	Found on LDs. In nucleus controls many processes	Cho et al., 2007
Lysosomes/yeast vacuoles	Core autophagy machinery	Mediates lipophagy	van Zutphen et al., 2014
Endosomes	RAB5	Mediates binding of droplets to endosomes *in vitro*	Liu et al., 2007
Parasitic vacuoles	No known factors		
Droplet (homotypic)	Fsp27	Mediates droplet fusion	Gong et al., 2011
	RAB8A	Mediates Fsp27 function	Wu et al., 2014

not clear. RAB18 presumably binds to droplets from the cytosol through its isoprenoid modification, similar to other Rab proteins. Finally, a triacylglycerol (TAG) lipase in *Arabidopsis thaliana*, SDP1, can transit to droplets from peroxisomes via a retromer complex (previously known to transport proteins from endosomes to the trans-Golgi network) during seed development (Thazar-Poulot et al., 2015).

Bridges between the ER and droplets should allow transfer of phospholipid and neutral lipids between these two compartments. Experiments are lacking to probe for barriers to lipid trafficking at ER/droplet junctions or the extent to which new lipid synthesis is concentrated at junctions. The yeast diacylglycerol acyltransferase Lro1p is localized to ER/droplet junctions, suggesting that synthesis from this source is indeed coupled to droplet expansion (Wang and Lee, 2012).

ER Stress and Droplets

There is a conserved correlation between ER stress and an increase in lipid droplets. In yeast, the knockout of genes involved in the protein glycosylation pathway or by administration of tunicamycin or brefeldin A (strong inducers of ER stress), resulted in an increase in the number of lipid droplets and often an increase in neutral lipids (Fei et al., 2009). These effects are not caused by the classical stress response pathway because they occur even in the absence of the conserved UPR initiator Ire1p. Most likely there is a rerouting of precursors such as phosphatidic acid and diacylglycerol from phospholipid to neutral lipid synthesis. Consistent with this, the anterograde inhibitor, brefeldin A, was found to cause an increase in lipid droplets at the expense of phospholipid synthesis (Gaspar et al., 2008). Brefeldin A treatment also resulted in an increase in TAG and lipid droplets in *Clamydomonas* and the related alga, *Chlorella vulgaris* (Kim et al., 2013).

In mammals, ER stress is linked to liver steatosis. Mice knocked out in a key ER stress component, ATF6α, were more prone to accumulation of liver lipid droplets, found to be caused by a combination of lower β-oxidation, less lipoprotein secretion, and an upregulation of adipogenic genes (Yamamoto et al., 2010).

The synthesis of droplets may be a protective mechanism to prevent aggregation of misfolded proteins as a result of ER stress (Welte, 2007). The fundamental question in all these systems is the mechanism by which lipids are re-routed from membrane synthesis to storage of neutral lipids, a question addressed by studies of ER-assisted degradation.

ER-assisted Degradation (ERAD)

ERAD is elicited by an accumulation of unfolded or misfolded proteins in the ER. There is growing evidence that ERAD is intimately tied to lipid droplets and control of neutral lipid accumulation.

Several proteins that function in ERAD to extract proteins from the ER are colocalized to ER and droplets. These include derlin-1, UBXD2, UBXD8, p97/VCP, and AUP1 (Suzuki et al., 2012; Olzmann et al., 2013; Stevanovic and Thiele, 2013). Evidence for colocalization is their appearance in proteomes of isolated droplets, and live and fixed cell fluorescence with antibodies or tagged proteins. Proteome evidence requires caution since classical luminal ER markers (such as Hsp70/BiP) often copurify with droplets and probably represent tightly-bound ER fragments. Yet fluorescence microscopy is compelling that ERAD proteins can localize around droplets. Ultrastructural studies usually do not accompany most of these reports, but it seems likely, based on a report with apolipoprotein B (see below) and the transmembrane topology of ERAD components that they are localized to a specialized region of the ER that surrounds droplets.

The trafficking of UBXD8 between bulk ER and droplets has been studied in detail (Olzmann et al., 2013). The ER protein UBAC2 retains UBXD8 in that compartment normally, but releases it to translocate to droplets upon the addition of oleate to the medium. Addition of oleate also causes trafficking of p97/VCP to the droplet, which depends on its direct binding to UBXD8.

Proteins destined for degradation by ERAD colocalize with droplets, notably HMG-CoA reductase and poorly lipidated apolipoprotein B-100 (Ohsaki et al., 2006; Hartman et al., 2010). For reductase, a small fraction of protein destined for degradation in the presence of cholesterol copurifies with isolated droplets (Hartman et al., 2010), although it is not clear if this fraction is a kinetic intermediate directly en route to degradation from the bulk ER. Apolipoprotein B accumulates around droplets if proteosomal degradation is blocked (Ohsaki et al., 2006), suggesting that this is the normal site for degradation. Ultrastructural studies indicate that the apolipoprotein accumulated with the block is contained within ER membranes and other structures that are tightly associated, but distinct from the droplet phospholipid monolayer (Suzuki et al., 2012).

To probe whether droplets are functionally important for ERAD, neutral lipid synthesis was inhibited by triacin C, a blocker of acyl-CoA synthetases. The number of droplets was reduced by 40% and was accompanied by a slower rate of degradation of three ERAD substrates, suggesting that droplets are important for ERAD. Surprisingly, knockdown of AUP1, a member of the ERAD complex, resulted in fewer lipid droplets, linking ERAD to droplet formation (Klemm et al., 2011). Conversely, trafficking of UBXD8 to droplets resulted in an increase in neutral lipid due to inhibition of the triglyceride lipase, ATGL, by promoting dissociation of its activator CGI-58 (Olzmann et al., 2013). Similarly, deletion of *UBX2* (yeast UBXD8) resulted in reduced levels of triacylglycerol (Wang and Lee, 2012).

These experiments show that ERAD and droplet lipid metabolism are intimately related. An early model hypothesized that droplets could be an escape hatch used by the ER through which unfolded proteins gain access to ERAD and degradation (Ploegh, 2007). However, in yeast, knocking out neutral lipid biosynthetic enzymes, and thereby eliminating visible droplets, had no effect on ERAD (Olzmann and Kopito, 2011). Although one can interpret these results as indicating that yeast and mammals have fundamentally different mechanisms for ERAD, it is more plausible that both systems share common droplet elements (for example, droplet-associated proteins that do not require droplets *per se*) that have not yet been elucidated.

Role of Arf1-COPI

A discussion of the Arf1-COPI complex is relevant since it can catalyze intra-organellar communication. The retrograde transport of cargo between Golgi stacks and from the cis-Golgi to the ER is mediated by Arf1-COPI machinery; details have been well worked out (Beck et al., 2009). An early report identified Arf1 (which is an adaptor for COPI coat binding to nascent vesicles) as a binding protein to PLIN2 and showed that a dominant negative mutant of Arf1, or the COPI poison brefeldin A, led to dissociation of PLIN2 from droplets (Nakamura et al., 2004).

More recently, components of COP1-mediated retrograde transport were found in an RNAi screen in *Drosophila* S2 cells for factors involved in droplet assembly or morphology. These included the Arf1 homolog, *Arf79f*, the Arf GEF (GTP exchange factor), *garz,* and several coat components. Cells from these knockdown strains contained larger and more dispersed droplets reflecting an increase of neutral lipid in these cells. In contrast, no gain-of-lipid phenotype was seen for RNAi knockdowns of COPII or clathrin coat subunits, suggesting an involvement of Arf1-COPI in the regulation of lipid droplet morphology and metabolism (Guo et al., 2008). The authors suggested possible functions in droplet budding (analogous to vesicle budding) or lipolysis. In an independent study, COPI was shown to be important for controlling neutral lipid levels in both mammalian and fly cell cultures (Beller et al., 2008). Knockdown of expression of COPI or Arf1 subunits, or administration of brefeldin A resulted in a large decrease in the droplet-associated lipase ATGL in this study. The effect is likely the cause of the larger droplets in COPI-knockdown cells since there was no further increase in neutral lipids if ATGL were knocked down in these cells. In addition, the authors found inappropriate colocalization of PLIN2 and PLIN3 on droplets. Normally, PLIN3 localizes only to smaller droplets while PLIN2 associates with larger ones. In a more recent study, GPAT4 was found to poorly localize to droplets in COPI-knockdown cells (Wilfling et al., 2014b).

A mechanistic explanation for the protein targeting defects in COPI-deficient cells was recently proposed (Thiam et al., 2013a). The authors developed an elegant inverted lipid droplet system in which phospholipid-lined aqueous droplets float in a sea of neutral lipids. The addition of GTP and Arf1-COPI components resulted in the budding of 60-nm nanodroplets into the aqueous phase, which led to an increase in the monolayer phospholipid surface tension, promoting fusion with other inverted droplets (Thiam et al., 2013a). The idea was further developed in intact *Drosophila* S2 cells (Wilfling et al., 2014b). In this study, depleting Arf1-COPI resulted in an increase in levels of phosphatidyl choline (PC) and phosphatidyl ethanolamine on the droplet surface, and the decrease in surface tension caused a delay in

the recruitment of the CTP:phosphocholine cytidylyltransferases, CCT1 and CCT2, to LDs. Furthermore, the work suggested that by raising surface tension by removing phospholipids, the Arf1-COPI machinery controlled not only protein targeting to droplets but the development of LD/ER bridges through which GPAT4 traffics (Wilfling et al., 2014b).

Droplets and Mitochondria

Direct and Indirect Communication

Close associations of lipid droplets with mitochondria are well known and seen in a variety of cell types including adipocytes, lactating cells, myotubes, and oocytes (summarized in Goodman, 2008). Junctions between these two organelles expand with an increased need for energy, for example, in exercising muscle (Tarnopolsky et al., 2007). It is logical to conclude that droplet/mitochondrial synapses allow the direct flow of fatty acids from neutral lipid stores to the mitochondrial matrix for β-oxidation to meet the cell's energy needs. However, since enzymes for reacylation of fatty acids are found in mitochondria (see Bosma et al., 2012), there may be two-way trafficking of lipids.

PLIN5 and Fatty Acid Flux

A key player in establishing the droplet mitochondrial junction is PLIN5. Expression of this protein drives mitochondria to lipid droplets (Wang et al., 2011). Mitochondrial binding depends on the C-terminus of the perilipin; ablation of the last 20 amino acids is sufficient to prevent mitochondrial aggregation onto droplets. The binding partner on the mitochondrial surface has not yet been determined.

There has been intense interest in the past few years regarding the role of PLIN5 in lipolysis of triacylglycerol from droplets (reviewed in Mason and Watt, 2015). The consensus is that PLIN5 normally serves as a barrier to lipolysis. PLIN5-knockout animals rapidly lose neutral lipid upon fasting, and PLIN5 overexpression results in larger triacylglycerol stores. However, PLIN5 is responsive to PKA stimulation: Upon PKA activation, lipolysis increases in heart tissue, and this is blocked by mutation of the consensus PKA phosphorylation site of the perilipin. PLIN5 can bind to both HSL (hormone-senstivie lipase) and ATGL as well as the ATGL activator, CGI-58, and phosphorylation likely displaces CGI-58 from PLIN5, allowing it to activate ATGL (Pollak et al., 2015).

Interestingly, overexpression of PLIN5 in skeletal muscle results in the protein localizing not only to droplets but also to the mitochondrial matrix (Bosma et al., 2012). Its function at that location is not known, and, as the protein does not have an obvious mitochondrial targeting signal, its import mechanism is unknown. More studies are needed to determine the physiological significance of its intra-mitochondrial localization.

The droplet–mitochondrial connection may be particularly important during starvation. The balance between lipophagy and cytoplasmic/droplet lipases in providing energy during starvation was probed in mouse embryonic fibroblasts (Rambold et al., 2015). The authors tracked the movement of the fluorescent fatty acid Red-C12 from lipid droplet to mitochondria (for oxidation) after incubating cells in Hank's Balanced Salt Solution without serum or energy source. In these conditions, Red-C12 appeared to move directly from droplet to mitochondria without significant involvement of the lysosome. Interestingly, attached mitochondria had fused, as if to promote fatty acid transfer into a large mitochondrial matrix space. Blocking mitochondrial fusion resulted in less efficient lipid-linked mitochondrial respiration.

Droplets and Peroxisomes

A close association of peroxisomes and lipid droplets was first noted in rabbit ovarian tissue nearly 50 years ago (Blanchette, 1966). Constellations of droplets with surrounding ER, mitochondria and microperoxisomes observed in differentiating 3T3-L1 cells led to the hypothesis that these organelles collaborate in lipid metabolism (Novikoff et al., 1980), an idea that was based in part on the recently discovered ability of mammalian peroxisomes to perform fatty acid β-oxidation [Lazarow, 1978; Lazarow, It had been known considerably earlier that plant glyoxysomes (specialized peroxisomes) could β-oxidize fatty acids (Cooper and Beevers, 1969)]. Studies on peroxisomal/droplet associations were extended to rat fat pads; while peroxisomes were observed close to droplets, direct contacts between the two organelles were not seen (Blanchette-Mackie et al., 1995). A more recent study using COS7 cells revealed a tubular-reticular cluster of peroxisomes, most of which were connected to droplets, especially at the tips of individual peroxisomes in the cluster (Schrader, 2001).

As noted above, the ability of plant peroxisomes to metabolize fatty acids has been known for many decades (Cooper and Beevers, 1969). Glyoxysomes, which metabolize fatty acids to succinate, and which will later transform to leaf peroxisomes, are abundant in oil seeds along with lipid droplets. In a fatty acid 3-ketothiolase mutant (i.e., deficient in fatty acid β-oxidation) in *Arabidopsis thaliana,* large electron-lucent structures are seen within glyoxysomes of etiolated cotyledons that appear to be invaginations from adjacent droplets, and these inclusions contain vesicles (Hayashi et al., 2001). The implication is that these inclusions represent TAG or fatty acids from the droplet that accumulates when β-oxidation is blocked; the structures may be too transient to easily see in wild-type plants.

In fact, neutral lipids as well as phospholipids can be transferred *in vitro* between isolated lipid droplets and peroxisomes, both organelles from cotton (Chapman and Trelease, 1991). The reaction, which involved incubation with radiolabeled lipid, required droplet membrane protein. Transfer of lipid from droplet to peroxisome was confirmed in intact cells by pulse-chase. The authors proposed that normal transfer of lipid from ER to growing peroxisomes involved a droplet intermediate. Droplet protein was necessary for this reaction, as reconstituted droplets devoid of protein were not active in this reaction.

In a recent follow up, a triglyceride lipase, SDP1, in *Arabidopsis* was shown to migrate from peroxisomes to lipid droplets during plant development. Trafficking depended on the retromer complex; mutants of retromer subunits resulted in altered droplet morphology (Thazar-Poulot et al., 2015).

In yeast growing on oleic acid, both peroxisomes and lipid droplets enlarge (Veenhuis et al., 1987). In this medium droplets and peroxisomes make extensive contacts with each other. Peroxisomes are observed that wrap around droplets and even insert processes (pexopodia) within them that are enriched in β-oxidation enzymes (Binns et al., 2006). The association of the two organelles is stable at least over several minutes, while it is much more transient in cells that do not rely on fatty acids for growth.

While there are clearly abundant examples of droplet–peroxisomal interactions, and some evidence for transfer of lipid from droplet to peroxisomes, the molecular and topological details remain murky. The lipase that releases fatty acids at the droplet/peroxisomal junction, let alone its regulation, is not known, nor is there any information regarding the mechanism of transfer of fatty acids between these compartments, or the relative significance of droplets compared to other sources of fatty acids for peroxisomal oxidation.

Associations with the Nucleus

Two types of interactions between lipid droplets and nucleus have been described, involving direct physical associations and indirect communication through bioactive molecules. The reader is referred to a recent reference where these interactions are discussed in detail (Welte, 2015).

Lipid droplets have been frequently observed surrounding nuclei in several types of cells and tissues (Blanchette-Mackie et al., 1995; Szymanski et al., 2007). Because droplets emerge from the ER, and the ER is contiguous with the nuclear envelope, such associations are not surprising. However, droplets have recently been observed within the nucleus. Intra-nuclear droplets (nLDs) were first reported by bright field and fluorescence microscopy in rat liver and HepG2 cells using lipophilic dyes (Layerenza et al., 2013). The nLDs were on average smaller than cytoplasmic droplets and apparently distributed randomly in the nuclear matrix. Isolated nLDs contained a higher ratio of free cholesterol and cholesteryl esters to triacylglycerols than cytoplasmic droplets. In an ultrastructural study of human livers taken at autopsy, most nLDs were observed as invaginations of the nuclear envelope, although the rare nLD (seen in about 1% of hepatocytes) was clearly separated from the envelope (Uzbekov and Roingeard, 2013). Nuclear droplets were also observed in yeast although only in cells with mutated or deleted seipin (Cartwright et al., 2015).

The presence, albeit rare, of nLDs should be viewed in the context of intranuclear lipids and lipid metabolism. Intranuclear phosphorylated inositol phospholipids (PIPs) have been known for years and their roles in chromatin remodeling is a subject of active research (Shah et al., 2013). Moreover, many (and maybe most) cell types have a nucleoplasmic reticulum (NR) (Malhas et al., 2011). The NR, derived from the nuclear envelope, may contain a lumen continuous with cytoplasm as well as one with the intermembrane space between the inner and outer nuclear envelope membranes. Thus, nLDs may directly face an intranuclear "cytoplasmic" compartment or actually bud into the nucleoplasm. Whether the nLDs service the nuclear pools of PIPs, or have any important nuclear function, is not known. Interestingly, the frequency of nuclear droplets can be vastly increased in the absence of seipin, suggesting that this protein ensures that newly formed droplets face into the cytoplasm (Cartwright et al., 2015).

Cytoplasmic lipid droplets may physically alter nuclear shape. HepG2 cells cultured with 1 mM fatty acid mixture for 24 h was found to increase the amount of lipid droplets in the perinuclear area, distorting nuclei (Anavi et al., 2015). Lipid peroxidation products, including hydroxyl-alkanals—alkenals, and alkadienals, likely caused by an excess of fatty acids above the amount that could be stored in droplets as well as increased ROS generation from mitochondrial oxidation, caused covalent modification of several nuclear proteins in this study.

Besides nLDs and physical association of cytoplasmic droplets with nuclei, lipid droplets can regulate nuclear events by storing histones, binding to transcription factors, and physically interacting with other proteins that shuttle to the surface of droplets (Welte, 2015). Droplets of *Drosophila* early embryos store certain histones through an interaction with the droplet surface protein Jabba. In the absence of Jabba, the histones are degraded (Cermelli et al., 2006; Li et al., 2012, 2014), suggesting that lipid droplets not only store lipid precursors but also can supply histones for rapid chromatin remodeling.

The CIDE (cell death inducing DFF45-like effector) family proteins, including Cidea, Cideb, and FSP27/Cidec are other examples of nuclear-droplet communication. These proteins colocalize on the ER and droplets (Puri et al., 2007; Liu et al., 2009; Konige et al., 2014). When not bound to droplets, they affect gene expression: Cidea binds to LXR in 3T3-L1 adipocytes (Kulyté et al., 2011), whereas Cidea and FSP27 interact with CCAAT/enhancer-binding protein β (C/EBPβ) in mammary glands and brown adipose tissue (Wang et al., 2012). Moreover, FSP27 on droplets can sequester NFAT5, which otherwise can respond to osmotic stress and regulate osmoprotective and inflammatory responses. The amino-terminal region of NFAT5 can directly interact with FSP27 on droplets, as determined by bimolecular fluorescence complementation, such that the overexpression of FSP27 inhibits the translocation and transcriptional activity of NFAT5 (Ueno et al., 2013). However, overexpression of FSP27 resulted in its accumulation in the cytosol. These data suggest that sequestration of NFAT5 on droplets plays a physiological role in its regulation.

Another protein that shuttles between nucleus and LDs is CCT1 in *Drosophila melanogaster* (Guo et al., 2008; Tilley et al., 2008; Krahmer et al., 2011). CCT1 is the rate-limiting enzyme in the biosynthesis of PC. Two genes encode this enzyme in the fly: CCT1 was originally found to be a nuclear protein, while CCT2 was cytoplasmic (Tilley et al., 2008). Both may play roles in PC biosynthesis or lipid signaling in their "home" compartments. After incubation in medium containing oleate, however, both forms shuttle to the surface of LDs. CCT1 returns to the nucleus upon removal of the fatty acid (Guo et al., 2008; Krahmer et al., 2011). Apparently, when cells are faced with fatty acid overload, they move to LDs to stimulate PC biosynthesis, allow expansion of LDs, and avoid fatty acid-induced lipotoxicity.

Finally, Prp19 colocalizes to the nucleus and lipid droplets. Prp19 (precursor RNA processing 19) is an essential member of

the Prp19 complex (PrpC, also known as NTC, or 19 Complex) which plays important roles in mRNA maturation (transcription elongation, splicing and export), genome stability, and protein degradation (Chanarat and SträssSer, 2013). Besides its nuclear localization, it was identified as a component of lipid droplets (Cho et al., 2007). It appears not to shuttle between nucleus and droplets as leptomycin, which blocks nuclear export, did not affect the distribution of PrpC. Knockdown of Prp19 resulted in lower expression of lipogenic enzymes in the 3T3L1 system (Cho et al., 2007). Because PrpC has many nuclear roles, the relationship of Prp19 binding to droplets to its role in lipogenesis has not yet been worked out.

Thus, there are several layers of interactions between nucleus and lipid droplets, and communication can flow in both directions. Future research will likely elucidate the physiological importance of morphological findings (such as nLDs) and work out downstream effects (such as metabolic signaling) of this rich collaboration between two cellular components.

Droplets and Lysosomes/vacuoles

Since autophagy of lipid droplets, lipophagy, was first reported in hepatocytes (Singh et al., 2009a), the interaction between lysosomes (vacuoles in yeast) and lipid droplets became a research highlight in the lipid metabolism area. Lipophagy, as an alternative to lipolysis by droplet or cytosolic lipases, has been reported in yeast, adipocytes, enterocytes, fibroblasts, neurons, and stellate cells (Singh et al., 2009b; Lettieri Barbato et al., 2013; Liu and Czaja, 2013; Khaldoun et al., 2014; van Zutphen et al., 2014; Wang et al., 2014). It can involve autophagosome formation (macrolipophagy), direct interaction of droplet with lysosome (microlipophagy), or chaperone-mediated autophagy (CMA).

Singh et al. found that the autophagy inhibitor 3-methyladenine (3MA), or a mutant *atg5* gene, resulted in an increase in the number and size of lipid droplets, TAG accumulation, and a decrease in fatty acid β-oxidation, suggesting that cells could break down the LDs through autophagy processes. Furthermore, the group showed that the processes needed ATG7-dependent conjugation for recruiting LC-3 to the LDs surface, an initial step of macroautophagy (Singh et al., 2009a). Regulation of lipophagy also requires the coordination of mTORC1 (the mammalian target of rapamycin complex 1) with nutrient-sensitive transcription factors TFEB, p53, and FOXOs, as described in an excellent recent review (Settembre and Ballabio, 2014). The system is controlled by the energy state within the lysosome, which signals across the membrane through the lysosomal acid lipase (LAL, itself controlled by FOXO1) and mTORC1 to transcriptional factors. In addition, dynamin 2 is important for the regeneration of nascent lysososomes by scission of the tubulated autolysosomes in macroautophagy in hepatocytes (Schulze et al., 2013).

To tease out the importance of lipophagy compared with lipolysis outside the lysosome, a study noted above (Rambold et al., 2015) found that cells starved of carbon source derived most of its fatty acids for mitochondrial oxidation from direct transfer from droplets, presumably from a droplet lipase. However, during serum-starvation the group found that lipophagy played a larger role. Precisely how these two pathways are coordinated will be fascinating to uncover.

In contrast to mammalian cells, lipophagy in yeast more closely resembles microautophagy and requires steps to modify the vacuolar membrane for engulfment of LDs (van Zutphen et al., 2014; Wang et al., 2014). All the core autophagy machinery associated genes are necessary for yeast lipophagy except *Shp1*, *Vps38*, *Nyv1*, *Atg11,* and *Atg20*. Tubulin and Vac8, which is involved in multiple vacuolar processes, are also important (van Zutphen et al., 2014). Wang et al. also reported that the existence of a sterol-enriched vacuolar microdomain is important for stationary phase yeast LDs translocation and hypothesized a feed-forward loop to promote stationary phase lipophagy (Wang et al., 2014).

CMA of droplet proteins is an alternative pathway for generating fatty acids. A recent report showed the involvement of PLIN2 and PLIN3 as substrates for CMA, a pathway that was stimulated during starvation (Kaushik and Cuervo, 2015).

Besides serving as a source of energy during starvation via lipophagy, droplets are also important to promote general autophagy of cellular contents. Thus, general autophagy in yeast was severely reduced in the absence of droplets (Li et al., 2015), and the level of autophagosome formation in HeLa cells was decreased in the absence of lipid droplets or the lipase PNPLA5 (Dupont et al., 2014).

Droplets, Endosomes, and Rab Proteins

No Rab protein is exclusively localized to droplets, but much of RAB18 is localized there, and its localization is regulated (Martin et al., 2005). Multiple Rab proteins, which catalyze and specify vesicular trafficking, have been identified as members of the lipid body proteome (Yang et al., 2012). Although their function at the droplet remain obscure (other than possibly a site for storage), some progress has been made. An early report demonstrated the ability of Rabs to reversibly traffic to droplets based on their guanine nucleotide-bound state, suggesting that the interaction is physiological (Liu et al., 2007). Moreover, in this report, RAB5 activation caused the binding of isolated droplets to purified early endosomes, and transfected activated RAB5 could also cause binding of these two organelles in cells. This work, although needing more development, suggests that droplet binding to endosomes is physiologically important and is mediated by a Rab protein.

Another Rab protein, RAB8A mediates droplet–droplet associations. Our group observed homotypic associations in yeast growing on oleic acid, in which multiple chains of droplets encircling the nucleus were found, linked to one another by nipple-like connections (Binns et al., 2006); the function of such junctions remains unknown, although they appear stable. Droplets of mammalian cells can contact each other leading to exchange of core lipids from the smaller to the larger droplet in a process that depends on Cidec/Fsp27 (Gong et al., 2011) and is regulated by RAB8A (Wu et al., 2014).

Droplets and Parasitic Vacuoles

Finally, there is dynamic interaction between lipid droplets and inclusion organelles (derived from plasma membrane) generated by unicellular parasites. The best studied is the interaction of droplets with the parasitopherous inclusion organelle formed upon entry of *Chlamydia trachomatis* (Cocchiaro et al., 2008). Droplets can be observed entering this structure from the cytosol. Other invaders use lipids derived from cytoplasmic lipid droplets for their nutrition (reviewed in Saka and Valdivia, 2012). Droplets are recruited to vacuoles containing *Mycobacterium leprae* in Schwann cells (Mattos et al., 2011). In addition fatty acids from lipid droplets are incorporated into neutral lipids in tubercle bacilli infecting lung macrophages, promoting the dormant state (Daniel et al., 2011).

Conclusion

It is apparent that lipid droplets are well connected to many other cellular compartments, and in some cases (notably ER and mitochondrial contacts) molecules have been identified that are important to initiate or maintain the connections. It is assumed that many of these contacts result in transfer of lipids between compartments, and those droplets serve as the source of lipids for membrane expansion, energy production, and signaling. However, the mechanism and extent of activation of lipases by contact sites, and the mode of fatty acid transfer between organelles, remain obscure. The basic mechanisms of droplet initiation and maintenance by the ER are no longer totally obscure but still lack much basic information. The role, if any, for provision of lipids by droplets within the nucleus is a fascinating issue that requires attention, as is the role that droplets perform in exocytic and endocytic trafficking. Finally, the regulation of energy release from droplets during starvation among pathways—general and specific autophagy and *in situ* lipolysis on droplets in the cytosol—is a research area that should provide answers in the near future. The intricate and intimate connections among cellular organelles form the basis of cell function and continue to provide inspiration to those of us working in this area of biology.

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Mitochondrial Dynamics and the ER: The Plant Perspective

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Whereas contact sites between mitochondria and the ER have been in the focus of animal and fungal research for several years, the importance of this organellar interface and the molecular effectors are largely unknown for plants. This work gives an introduction into known evolutionary differences of molecular effectors of mitochondrial dynamics and interactions between animals, fungi, and plants. Using the model plant *Physcomitrella patens*, we provide microscopic evidence for the existence of mitochondria-ER interactions in plants and their correlation with mitochondrial constriction and fission. We further investigate a previously identified protein of unknown function (MELL1), and show that it modulates the amount of mitochondrial association to the ER, as well as mitochondrial shape and number.

Keywords: organelle dynamics, *Physcomitrella patens*, fission, fusion, *Arabidopsis thaliana*

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INTRODUCTION

Subcellular compartmentation has enabled eukaryotes to simultaneously establish distinct reaction compartments with discrete protein content that need to be coordinated by interorganellar communication. Compartments are linked by signaling pathways and transport processes of different types of molecules such as proteins, lipids, and carbohydrates. Increasing evidence suggests that these processes are coordinated at specific contact interfaces (Prinz, 2014) which are either modulated by proteins or even membrane hemifusions (Mehrshahi et al., 2013, 2014). Multiple effectors of membrane contact sites (MCS) were identified linking the omnipresent ER to most other cell compartments, as e.g., the plasma membrane, lysosomes, vacuoles, and to mitochondria in mammals and yeast (for review, see Prinz, 2014). Identified functions of MCS include the transfer of lipids and the regulation of intracellular Ca^{2+} in animals and fungi (Prinz, 2014), and the accessibility to nonpolar metabolites between plant ER and plastids (Mehrshahi et al., 2014). In particular, the interactions between mitochondria and ER became a focus of research during the last decade in animals and fungi, linking ER-mitochondria contacts additionally to mitochondrial dynamics and quality control (Rowland and Voeltz, 2012; Kornmann, 2013; Lackner, 2014).

Mitochondria of a single cell have been described as a discontinuous whole (Logan, 2006), as they undergo frequent fusion and fission in animals, fungi, and plants (Arimura et al., 2004; Labbé et al., 2014), and thus maintain a certain rate of content exchange. This process was recently shown to be important for fatty acid metabolism in mammalian cells under starvation (Rambold et al., 2015), but is best known for its pivotal role in mitochondrial quality control (Twig et al., 2008b). Notably, mitochondrial fusion can either be transient ("kiss-and-run") while retaining mitochondrial identities, or of longer duration with increased exchange of matrix and also membrane content (Liu et al., 2009).

A model for the mixing and unmixing of mitochondrial content was proposed, describing a separation of dysfunctional mitochondria from the pool of fusing mitochondria, and their targeting to autophagosomes (Twig et al., 2008b). In mammalian cells mitochondrial fusion triggers fission which in turn is followed by selective fusion (Twig et al., 2008b): differences in membrane potential become evident in daughter mitochondria after a fission event (Twig et al., 2008a). As membrane potential and import capacity are linked, the PINK/Parkin pathway subsequently regulates the exclusion of dysfunctional mitochondria via degradation of components of the fusion machinery in mammals (Narendra et al., 2012). A loss of this quality control system can in turn disturb stem cell fate in mammals (Katajisto et al., 2015) and leads to the decrease or the total loss of mitochondrial genomes in yeast and mammals (Labbé et al., 2014).

Although it was known for some time that mitochondrial form and function are linked, i.e., that changes in mitochondrial morphology and/or dynamics often are the first marker for cell stress in mammals, fungi, and plants (Scott and Logan, 2008; Welchen et al., 2014), the identity of several molecular effectors was only discovered in recent years. Thus, several components of the fission machinery are evolutionary conserved, such as dynamin-related GTPases (yeast Dnm1p, mammals Drp1, and *A. thaliana* DRP3A/DRP3B) and FIS-type proteins (*FISSION*, also called *BIGYIN* in plants; Scott and Logan, 2011). Notably, in both yeast and mammalian cells ER-mitochondrial contacts contribute to mitochondrial fission, supposedly either by the physical constriction of mitochondria by ER tubules, or as platforms for recruitment of the fission machinery (Friedman et al., 2011). In yeast, ER-mitochondrial interactions are mediated by the ERMES [ER-Mitochondrial Encounter Structure (Kornmann, 2013)] complex which has no known homologs in mammals or plants (Duncan et al., 2013; Kornmann, 2013).

The fusion machinery of mitochondria is largely conserved between mammals and yeast and involves the dynamin-related GTPases homologous to the FUZZY ONIONS (Fzo) protein from *Drosophila melanogaster*: Fzo1p in yeast and mitofusins (Mfn1, Mfn2) in mammals. These GTPases contain two C-terminal transmembrane domains and mediate tethering of neighboring organelles and outer membrane fusion (Labbé et al., 2014). In mammalian cells Mfn2/Mfn1 interactions additionally regulate mitochondrial/ER tethering and Ca^{2+} uptake (de Brito and Scorrano, 2008). In contrast, in land plants the closest homologs of this protein family localize to chloroplasts and mediate thylakoid architecture (Gao et al., 2006), raising the question how plant mitochondria fuse (Arimura et al., 2004; Scott and Logan, 2011).

Although evidence for links between plant mitochondrial form, function, and dynamics exist, the molecular identity of interaction sites is mostly unclear and modulators and effectors known from animal systems, such as Bcl2-like proteins, mitofusins, PINK, and Parkin (Logan, 2006, 2010; Elgass et al., 2013; Labbé et al., 2014) are lacking. Interestingly, plants possess a highly organized ER with different subdomains including potential contact sites to mitochondria, with suggested functional links to the transfer of lipids and mitochondrial dynamics (Staehelin, 1997; Sparkes et al., 2009; Stefano et al., 2014a).

We recently identified a plant protein with LEA (Late Embryogenesis Abundant) and LysM domains and a conspicuous subcellular localization to ER and mitochondria (MELL1), which influences mitochondrial shape (Mueller et al., 2014). Here, we describe its influence on the association between mitochondria and ER and discuss future challenges in mitochondrial dynamics research.

RESULTS

In order to monitor mitochondria and ER simultaneously in a plant, we used fluorescently labeled organelles of the model moss *Physcomitrella patens*, which provides a uniquely high rate of homologous recombination in plants (Strepp et al., 1998) and is amenable to confocal microscopy studies (Abel et al., 1989; Furt et al., 2012; Vidali and Bezanilla, 2012; Müller et al., 2015). We generated a stable transgenic moss line constitutively expressing mitochondria-targeted mEOS (mtEOS; Mathur et al., 2010) and transiently transfected protoplasts of this line with an ER marker that comprises a signal peptide, mCerulean, and a C-terminal KDEL ER retention signal (spCerKDEL). We found that mitochondria in moss protoplasts were mostly small elongated tubular structures which move only at about a 10th of the speed of flowering plant mitochondria (max. speed in our hands was 75 nm/s), which supports previous findings (Pressel et al., 2008; Furt et al., 2012). ER tubules tightly wrapped most mitochondria of a cell (**Figure 1A**). When investigating high quality images of mitochondria and ER ($n = 51$), a third of the mitochondria showed an elongated shape with clear constriction sites (**Figure 1B**). In our dataset, 88% of these constriction sites showed a clear co-localization with ER tubules. Mitochondrial constriction sites did not always lead to fission events in the time frame of several minutes. But when fission events occurred (**Figure 1C**), ER was closely associated and ER tubules remained attached on both newly generated ends of daughter mitochondria. Thus, ER and mitochondrial dynamics are linked in moss, although the causality of this correlation is as yet unclear.

As mitochondria and ER dynamics correlate, we further investigated whether ER-mitochondria association is altered by overexpression of the ER-mitochondria localized protein we recently identified (MELL1; Mueller et al., 2014). **Figure 2A** depicts 3D reconstructions of a typical protoplast expressing spCerKDEL in the mtmEOS background line (bg) or spCerKDEL and MELL1:GFP in the mtmEOS background line (ox). Mitochondrial shape is severely altered toward large mitochondria. Mitochondrial number is significantly reduced and sphericity of mitochondria significantly increased (**Figure 2B**), whereas the total volume of mitochondria was not significantly altered (**Figure 2B**).The increase in sphericity induces a trend toward decreased surface area of the mitochondria, which was not statistically significant in our dataset (**Figure 2B**).

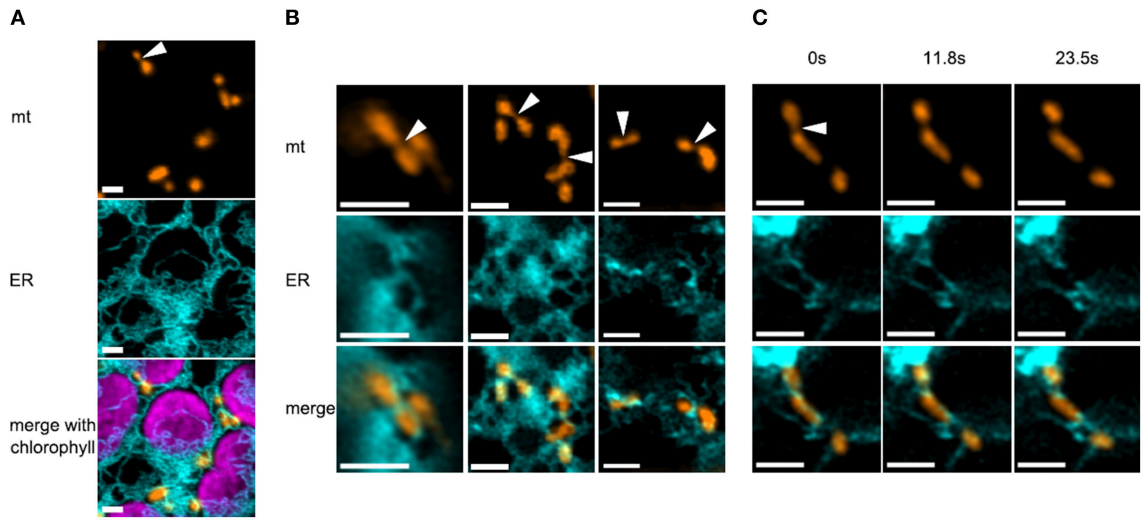

FIGURE 1 | Correlation between constriction sites in mitochondria and ER tubules in moss. (A) Overview of several mitochondria, the ER and chloroplasts in a protoplast of *Physcomitrella patens*. ER tubules are often closely associated to both organelles. The arrowhead points to a mitochondrion with a prominent constriction site, co-localizing with ER. Scale bars are 2 μm. **(B)** Several mitochondria show co-localization with the ER at constriction sites (arrowheads) and at their ends. Scale bars are 2 μm. **(C)** Time series (~2 min) of a mitochondrial fission event in moss, showing the close association of ER, which subsequently remains attached on both newly generated ends of daughter mitochondria. Scale bars are 2 μm.

Large mitochondria possess a common matrix space, as photobleaching of mtEOS lead to a concomitant decrease of fluorescence intensity in neighboring areas of the same mitochondrion (**Figure 2C**). We tracked the association of mitochondria and ER by Mander's co-localization coefficient (M1 **Figure 2D**) between different transfected cells (left), and over several time series (right, ~duration 2 min). The co-localization of mitochondria with the ER was significantly increased for cells over-expressing MELL1, compared to cells of the background line. Moreover, the co-localization coefficient remained elevated during the time courses in MELL1 over-expressing cells, in contrast to a higher variance of mitochondria-ER co-localization in the background line. Thus, the association of mitochondria and ER is increased in MELL1 over-expressing cells and shows a high temporal persistency. **Figure 2E** depicts details of the association between mitochondria and ER under MELL1 overexpression. Mitochondria are embedded in a dense network of ER tubules and occasionally (**Figure 2E** arrowhead) show tubular extensions [matrixules (Logan, 2006)].

DISCUSSION

Connectivity Between Organelles in Plant Cells

In plants, the existence of specialized contact domains between the ER and other organelles such as chloroplasts was evidenced by several experimental approaches, either exerting mechanical forces by optical tweezers (Andersson et al., 2007), or using transorganellar complementation to demonstrate biochemical continuity (Mehrshahi et al., 2013). Mitochondria and ER cooperate in several biosynthetic pathways and exchange phospholipids in plants (for review see Millar et al., 2008). However, the molecular identity of proteins mediating contact sites and connectivity between mitochondria and ER in plants is so far unknown.

Studies investigating organelle movement in plants point to the presence of tethers or hemifused membranes between the ER and other organelles, as organelle dynamics correlate, without evidence for luminal connectivity (Stefano et al., 2014a,b). Two factors modulating these interactions may be membrane curvature and shape, as well as movement on cytoskeletal elements (Stefano et al., 2014a). Thus, inhibition of both actin filaments and microtubules was found to promote mitochondrial fusion in plants (Sheahan et al., 2005), probably indicating that movement on cytoskeletal elements counteracts complete fusion, similar to the situation in the mammalian system (Liu et al., 2009). Further, when the actin and the microtubule cytoskeleton were perturbed simultaneously, mitochondria tended to cluster around chloroplasts and ER patches (Van Gestel et al., 2002), implying mechanisms for specific association that lead to typical plant subcellular positioning (Welchen et al., 2014). As we have shown here, mitochondria co-localize with ER in moss protoplasts (Mander's coefficient 0.69 ± 0.32), often with one or several ER tubules crossing parts of the mitochondrial surface and the ends of elongated mitochondria. This co-localization showed a high variance in the mtEOS-labeled line, indicating frequent changes in the amount of ER in the ultimate proximity of mitochondria. Similar to animal and fungal model systems, we found that ER labeled mitochondria constriction sites, suggesting an evolutionary conservation of mitochondria-ER interactions at constriction sites. In order to investigate the molecular basis and effect of this correlation, contact sites

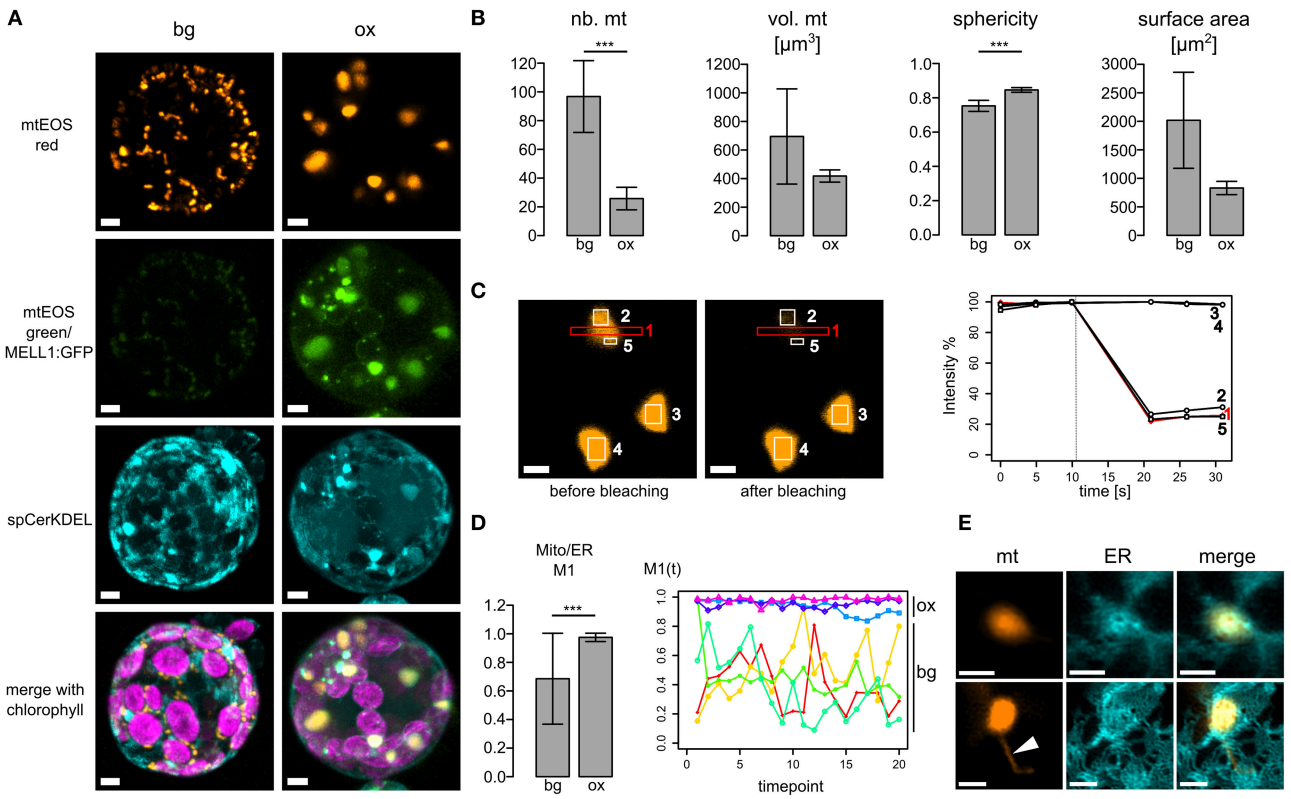

FIGURE 2 | Overexpression of MELL1 affects mitochondrial shape and association with the ER. (A) 3D Reconstructions of z-stacks of confocal planes from transfected moss protoplasts. Left panel: protoplast of a stably transformed mtEOS line (background bg) showing normal size and distribution of mitochondria and ER (spCeruleanKDEL). mtEOS is almost completely photoconverted to its red form. Right panel: MELL1:GFP overexpression (ox) leads to a profound change in mitochondrial shape and number, as well as to the accumulation of ER around mitochondria. Scale bars are 4 µm. **(B)** MELL1-overexpressing cells (ox) have significantly less mitochondria ($p < 0.01$, $n = 4$), whereas total mitochondrial volume was not significantly reduced ($p = 0.23$, $n = 3$). Mitochondria show a more spherical shape ($p < 0.01$, $n = 3$) and a concomitant trend to decreased surface area ($p = 0.07$, $n = 3$). **(C)** Large spherical mitochondria in MELL1-overexpressing cells are fused, as shown by photobleaching of mtEOSred (region 1) and a parallel drop in fluorescence intensity in neighboring regions of the same mitochondrion (regions 2 + 5), but not in other neighboring mitochondria (regions 3 + 4). **(D)** Mander's coefficient for co-localization of mitochondrial signal (mtEOSred) with ER signal (spCeruleanKDEL). Mitochondria co-localize significantly more with ER in different cells (***$p < 0.01$, $n = 13$ ox, $n = 6$ bg), as well as during time series of the same cell [M1(t), $p < 0.01$, $n = 3$ ox, $n = 4$ bg]. **(E)** Detail of large spherical mitochondria in MELL1-overexpressing protoplast, showing close association with a network of many ER tubules. Arrowhead points to matrixule. Scale bars are 2 µm.

between ER and mitochondria in plants await identification, as no homologs to ERMES or mitofusins are present in plant mitochondria.

MELL1 Level Influences the Association of Mitochondria to the ER

In differentiated plant cells, mitochondria undergo frequent fusion and fission (Arimura et al., 2004) without global changes in number or shape, whereas differentiating protoplasts show massive mitochondrial fusions (Sheahan et al., 2005), putatively to redistribute mtDNA. Overexpression of MELL1 led to large fused mitochondria, which were closely associated to a constitutively high amount of ER. In theory, this phenotype could either relate to increased fusion of mitochondria, or decreased fission. Interestingly, though major changes in mitochondrial shape and distribution occurred, mitochondria were not dysfunctional, as indicated by correct targeting of the mtEOS probe. Additionally, the ability to form tubular extensions (matrixules) was retained under MELL1 overexpression.

Using forward and reverse genetics, conserved molecular mechanisms behind mitochondrial fission as well as plant-specific modulators were characterized, such as *NETWORK/ELM* (ELongated Mitochondria) which is required for the localization of DRP3A to plant mitochondria (Arimura et al., 2008). In the model flowering plant *Arabidopsis thaliana*, the evolutionary conserved dynamin-related GTPases DRP3A and DRP3B mediate mitochondrial (and peroxisomal) fission (Fujimoto et al., 2009). Mutations of components of the fission machinery (DRP3A, DRP3B, FIS1A, FIS1B) lead to defects in mitochondrial shape and distribution, resulting in a reduced number of mitochondria with a more spherical shape (Scott et al., 2006; Zhang and Hu, 2008; Fujimoto et al., 2009), similar to our results. Other plant mutants exhibiting an aggregation of mitochondria include FRIENDLY, a homolog to mammalian CLUH (clueless homolog; Gao et al., 2014), which causes

clustering of mitochondria and an increase in matrix exchange, but no hyperfusion (El Zawily et al., 2014). CLUH was recently shown to bind mRNA of mitochondrially targeted proteins and may thus influence mitochondrial distribution indirectly via mitochondrial biogenesis (Gao et al., 2014). In MELL1 overexpressing protoplasts, mitochondria underwent complete fusion to large spherical mitochondria, with a common matrix space (**Figure 2C**) indicating a disturbed balance between fusion and fission. As this effect is accompanied by an increase in the association of ER to mitochondria, MELL1 might either directly or indirectly influence proteins at mitochondria-ER contact sites in plants. Whether MELL1 overexpression causes increased mitochondrial fusion or decreased mitochondrial fission is unclear so far. It is tempting to speculate that the increased mitochondrial association to the ER would disturb the fission machinery, as ER-mediated positional clues for fission, either provided by constriction via ER-tubules, or recruitment of the fission machinery to contact sites (Friedman et al., 2011), might be lacking. Alternatively, MELL1 might be a first link to the unknown mitochondrial fusion machinery in plants, although the protein does not contain a GTPase domain itself. An intriguing possibility is that MELL1 influences membrane curvature, as LEA domains may form alpha-helical structure which insert laterally into membranes (Tolleter et al., 2010; Candat et al., 2014). Future studies of knock-out mutants and mitochondrial dynamics in plants, as well as interacting proteins will address these open questions.

In conclusion, surprisingly little is known about the molecular identity of organelle contact sites in plants, but the evidence presented in this work points to an evolutionary conserved importance of mitochondrial dynamics and contacts to the ER between fungi, animals, and plants, while evolution may have shaped analogous molecular effectors. It will further be interesting to investigate whether there is any common mechanism in mitochondrial fusion shared by all eukaryotes. Future challenges include the identification of candidate proteins for organellar contact sites in plants, to further link changes in organellar form and function to the context of organelle connectivity, and to unravel the mechanisms behind balanced fusion/fission processes and quality control in mitochondria.

MATERIAL AND METHODS

Cloning

Mitochondria-targeted mEOS (Wiedenmann et al., 2004), containing the first 261 bp of the *Nicotiana plumbaginifolia* mitochondrial ATP2-1 coding sequence (X02868) as N-terminal targeting signal (Logan and Leaver, 2000; Mathur et al., 2010), was amplified via PCR (F ATAAGTCGACATGGCTTCTCGG AGGCTTCT, R ATCCGAGCTCTTATCGTCTGGCATTG) and ligated via the introduced SalI and SacI restriction sites into a newly assembled vector backbone containing the moss *Actin5* promoter (Weise et al., 2006) and a *NOS* terminator, as well as homologous regions for gene targeting to the "*P. patens* targeting site 2" (PTA2; Kubo et al., 2013) locus (*pAct5_PTA2*). To assemble this vector, PTA2 5′ homologous region (F GCT CTTCTCCTGGGGATTAATTATTGGAGG, R GAAAGAACG AATTCGATCGGATCCGCGACTAGTGAGAGAATGTT) and PTA2 3′ homologous region (F CTAGTCGCGGATCCGAT CGAATTCGTTCTTTCTGTCATTAACTGG, R GCTCTTCAT TGTTCAGGATAATGGTTC) were amplified from genomic DNA, joined with two template PCR (Tian et al., 2004) and ligated into a pJET1.2 vector (Thermo Fisher Scientific). The expression cassette of *Actin5* promoter, multiple cloning site, fluorescent protein, and *NOS* terminator (Mueller et al., 2014) was subsequently introduced between the PTA2 homologous regions with the restriction enzymes BamHI and EcoRI. To create an ER marker construct, the mCerulean coding sequence was amplified from *pGEMHE-X-Cerulean* (BIOSS toolbox Freiburg), and codons for the ER retention signal KDEL added to the C-terminus (F TACTGTCGACGTGAGCAAGGGCGAGGAG, R TTACAGCTCATCCTTCTTGTACAGCTCGTCCATGC). This construct was introduced in the *pAct5_PTA2* via restriction and ligation using SalI and Ecl136II. Subsequently, the signal peptide from moss aspartic protease (Schaaf et al., 2004) was PCR amplified from genomic DNA (F ATCAGTCGACATGGGGGC ATCGAGGAGTGTT; R ATTAGTCGACGCGAGGGCTTGCC TCAGCTA) and introduced in front of the *mCerulean::KDEL* with SalI restriction and ligation.

Moss Protoplast Transfection

Moss protoplasts of the *P. patens* (Hedw.) Bruch & Schimp. Gransden strain (International Moss Stock Center IMSC #40001) were prepared and transfected as described previously (Strepp et al., 1998; Hohe et al., 2004; Mueller et al., 2014). For stable transformation, an uncut plasmid containing the *nptII* neomycin resistance cassette (*pBSNNNEV*) was co-transfected in a ratio of 3:1 with the construct for homologous recombination. For transient transfection, *pAct5_PTA2* vectors containing organelle marker constructs and MELL1 overexpression vector (Mueller et al., 2014) were used uncut (10 μg per construct), whereas the construct was released from the vector creating homologous ends via BspQI restriction sites for stable transformation (30 μg used per transfection). Moss protoplasts were kept in the dark and imaged between 48 and 72 h after transfection. A stable mtEOS line (mtmEOS#44) is available from the International Moss Stock Center (IMSC #40776).

Confocal Microcopy and Image Analysis

All confocal images were taken with a Zeiss LSM 510 META with upright microscope Axio Imager Z1, using a C-Apochromat 63x/1.2 W Korr objective with water immersion. Fluorophores were excited with either an Argon laser (3% 488 nm for GFP/mtEOSgreen/chlorophyll), or diode lasers (3% 561 nm for mtEOSred; 3% 405 nm for Cerulean) using three separate tracks. Fluorescence was detected for chlorophyll from 670–756 nm (false colored magenta), for GFP from 505–550 nm (false colored green), for mtEOSred from 575–615 nm (false colored orange). Pinhole was set to 1 AU for Cerulean channel and section thickness adjusted accordingly in all other channels. Pixel dwell was 1.61 μs. Images were taken using 4 averages and 256 × 256 pixel for time series (∼7 s per time point) and using 16 averages and 512 × 512 pixel for snaps. The zoom factor was adjusted

to guarantee 1.5-2x overimaging of pixels, as recommended for deconvolution (see Huygens software manual). Bleaching (**Figure 2C**) settings for mtEOSred were used as follows: start after three scans, 300 iterations of bleaching, 100% 561 nm laser.

Confocal images were all deconvolved prior to subsequent analyses using Huygens Remote Manager (v3.2.2, Scientific Volume Imaging; SNR = 8 for time series, SNR = 10–15 for snaps). Co-localization analysis was performed in Huygens using Mander's coefficient (Manders et al., 1993). Three-dimensional reconstructions of z-stacks were performed after deconvolution using the Imaris software (Bitplane). Mitochondrial number was analyzed using the icy (http://icy.bioimageanalysis.org; de Chaumont et al., 2012) spot detector tool. Volume, shape, and surface area was analyzed by creating a surface from the mtEOSred channel in Imaris (Bitplane) and the surface statistics tool. Statistical analyses were conducted using the GraphPad Software Quickcalcs tools (http://www.graphpad.com/quickcalcs/), using two-tailed *T*-test. Bar graphs show mean and standard deviation.

AUTHOR CONTRIBUTIONS

SM and RR planned and designed the research. SM performed experiments and analyzed data. SM and RR wrote the manuscript. All authors discussed data and approved the final version of the manuscript.

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Microtubules Are Essential for Mitochondrial Dynamics–Fission, Fusion, and Motility–in *Dictyostelium discoideum*

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Mitochondrial function is dependent upon mitochondrial structure which is in turn dependent upon mitochondrial dynamics, including fission, fusion, and motility. Here we examined the relationship between mitochondrial dynamics and the cytoskeleton in *Dictyostelium discoideum*. Using time-lapse analysis, we quantified mitochondrial fission, fusion, and motility in the presence of cytoskeleton disrupting pharmaceuticals and the absence of the potential mitochondria-cytoskeleton linker protein, CluA. Our results indicate that microtubules are essential for mitochondrial movement, as well as fission and fusion; actin plays a less significant role, perhaps selecting the mitochondria for transport. We also suggest that CluA is not a linker protein but plays an unidentified role in mitochondrial fission and fusion. The significance of our work is to gain further insight into the role the cytoskeleton plays in mitochondrial dynamics and function. By better understanding these processes we can better appreciate the underlying mitochondrial contributions to many neurological disorders characterized by altered mitochondrial dynamics, structure, and/or function.

Keywords: mitochondria, fission, fusion, cytoskeleton, latrunculin, nocodazole, *Dictyostelium discoideum*, CluA

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INTRODUCTION

Mitochondria are highly specialized eukaryotic organelles responsible for producing the majority of a cell's adenosine triphosphate (ATP). They also play a vital role in many other cellular processes, such as the synthesis of heme groups and the regulation of membrane potential, calcium homeostasis, apoptosis, and cell differentiation (Mitchell, 1961; Frezza et al., 2006; Lill and Mühlenhoff, 2008; Baughman et al., 2011; De Stefani et al., 2011; Martinou and Youle, 2011; Maeda and Chida, 2013). To carry out these cellular processes the mitochondria must function properly, which is largely controlled by the organelle's morphology and distribution throughout the cell (Nunnari and Suomalainen, 2012).

For instance, in several organisms like animals, flies, and yeast, as well as specialized neuronal cells, the mitochondria exist in a reticular dynamic network, while in organisms like *Dictyostelium* and *Arabidopsis* the mitochondria exist as individual organelles (Chen, 1988; Bereiter-Hahn, 1990; Nunnari et al., 1997; Rizzuto et al., 1998; Schimmel et al., 2012; El Zawily et al., 2014). In either case, the appropriate morphologies are maintained by fission and fusion events (Nunnari et al., 1997; Bleazard et al., 1999; Gilson et al., 2003; Karbowski and Youle, 2003; Twig and Shirihai, 2011; El Zawily et al., 2014).

The cytoskeleton, in addition to affecting mitochondrial morphology plays a crucial role in maintaining mitochondrial distribution throughout the cell by facilitating organelle transport to areas with high metabolic demands (Van Gestel et al., 2002; Bereiter-Hahn et al., 2008; Kostal and Arriaga, 2011; Nekrasova et al., 2011; Wu et al., 2013). In order to rapidly respond to cellular demands, the cytoskeleton must have a communication system allowing it to influence organelle transport and position. This system usually encompasses various linker and motor proteins. The interaction between the cytoskeleton and the mitochondria is poorly understood; however several studies suggest that cytoskeletal network proteins interact with components of the fission and fusion machinery (Liesa et al., 2009), as well with the calcium sensing GTPase- Miro (Fransson et al., 2006; Frederick and Shaw, 2007).

Mitochondrial dynamics (fission, fusion, motility) also regulate the organelle's morphology and distribution. Disruption of these dynamics has been linked to a loss of metabolic function, an increase in ROS concentration, impairment of ATP synthesis, and a decrease in overall membrane potential (Margolin, 2000; Karbowski and Youle, 2003; Baloh et al., 2007; Chen and Chan, 2009; Nunnari and Suomalainen, 2012; Youle and van der Bliek, 2012; Picard et al., 2013). A variety of diseases, such as Alzheimer's, Parkinson's, Charcot-Marie-Tooth 2A, and Huntington's have also been linked to disruption in mitochondrial dynamics and morphology, though the mechanism driving these afflictions are unknown (Nunnari and Suomalainen, 2012).

Thus, to better understand mitochondrial dynamics and their role in disease we utilized *D. discoideum,* a lower eukaryotic model organism. In addition to lower eukaryotes sometimes being easier to tease out molecular mechanisms, *D. discoideum* is also a mitochondrial disease system (Barth et al., 2007; Annesley and Fisher, 2009; Annesley et al., 2014). Mitochondrial diseases caused by a specific mutation often manifest with a variety of clinical symptoms in humans. It has become apparent that unlike humans, *D. discoideum* cells do not exhibit this variation in symptoms, thus simplifying the study on mitochondrial diseases (Francione et al., 2011).

In *D. discoideum* mitochondrial distribution is maintained by the protein CluA (Zhu et al., 1997), which has homologs across a variety of organisms, including *Arabidopsis thaliana, Saccharomyces cerevisiae,* and *Drosophila melanogaster.* In all organisms studied to date, the absence of this protein results in clustered mitochondria (Zhu et al., 1997; Fields et al., 1998; Logan et al., 2003; Cox and Spradling, 2009). In plants, the organelles are found in small distinct clusters distributed throughout the cell (El Zawily et al., 2014), while larger, nuclearly centered clusters have been identified in *D. discoideum*. Further work has shown that these *D. discoideum* mitochondria are interconnected by thin membranous strands with limited movement (Fields et al., 2002). It has been hypothesized that CluA may represent a novel family of proteins which link the cytoskeleton to mitochondria. In addition to clustered mitochondria, the absence of CluA also decreases the rates of fission and fusion (Schimmel et al., 2012). Further, the nuclear localization of the mitochondrial clusters suggest they only move in an anterograde fashion (Zhu et al., 1997). Interestingly the *D. discoideum* Miro homolog, GemA, is not involved in mitochondrial transport along the cytoskeleton (Vlahou et al., 2011); further supporting the notion that CluA links the cytoskeleton and mitochondria. Therefore, it is conceivable that CluA has direct influence on mitochondrial dynamics by association with the cytoskeleton; however, it is presently unclear if CluA interacts with actin or microtubule motor proteins. Thus, to determine CluA's function the relationship between mitochondria and the cytoskeleton in *D. discoideum* must be elucidated.

It has been demonstrated that in animal cells mitochondria move along microtubules to travel long distances and use actin filaments for short distances (Wu et al., 2013). *Drosophila* mitochondria primarily use microtubules (Cox and Spradling, 2009), as do fission yeast (Yaffe et al., 2003), whereas actin is predominantly used by both plants and budding yeast (Van Gestel et al., 2002). There is even evidence that mitochondria use intermediate filaments in 3T3 fibroblast cells for proper motility regulation (Nekrasova et al., 2011). Thus, we analyzed *D. discoideum* mitochondria to establish whether microtubules or actin filaments were utilized for proper motility. Additionally, we examined whether or not *cluA*$^-$ mitochondrial morphology is dependent upon these cytoskeletal components and how these components affect motility of the *cluA*$^-$ mitochondria. Finally, due to mounting evidence linking impaired fission and/or fusion to aberrant mitochondrial motility and cellular health (Cagalinec et al., 2013), we assessed the influence of the cytoskeleton on mitochondrial fission and fusion.

To carry out these experiments, we disrupted the microtubules with nocodazole and the actin filaments with latrunculin-B. Through immunofluorescence and time-lapse imaging, we quantified mitochondrial morphology, motility, and fission and fusion rates. Taken together our results show that the *cluA*$^-$ clustered mitochondrial phenotype is partially dependent upon the actin and microtubule cytoskeletons, and that mitochondrial motility is not affected by loss of CluA. Therefore, we conclude that CluA does not play a significant role in connecting the mitochondria to the cytoskeleton. Further, we show that in *D. discoideum,* microtubules, but not actin, are important for both mitochondrial velocity as well as fission and fusion. Thus, we can infer that, as is the case with mammalian cells, microtubules play a much larger role in mitochondrial morphology and motility than actin. Finally, despite previous research, we did not find an interaction linking the mitochondria to the microtubule cytoskeleton through CluA, and conclude it may serve an unidentified function affecting mitochondrial morphology and distribution.

METHODS

Strain Culture and Growth Conditions

All *Dictyostelium discoideum* strains described were obtained from the Dicty-Stock Center (Fey et al., 2013). Wild-type (AX4) was deposited by Bill Loomis and *cluA*$^-$ by Margaret Clarke. The strains were cultured axenically in liquid HL5 medium supplemented with streptomycin (final concentration of 300

ug/ml) and ampicillin (final concentration 150 ug/ml) at 22°C shaking at 125 rpm.

Preparation of *D. discoideum* for Experiments

AX4 and *cluA*$^-$ cells were diluted to 3 × 10^4 cells/ml in HL-5 liquid media until cells reached log phase. Log phase cells (5.0 ml) were washed by centrifuging at 500 × g for 4 min and resuspended in 5 ml of room temperature Lo-Flo (Formedium). Cells were stained with 0.1 uM MitoTracker CMXRos (Invitrogen) and incubated for 4 h at room temperature while shaking. Excess MitoTracker was removed by washing the cells twice with Lo-Flo.

Cytoskeleton Disruption

During the 4 h Lo-Flo incubation period, the drugs or their appropriate vehicle control were added to the cells to disrupt the cytoskeleton. To inhibit the actin portion of the cytoskeleton, 10 uM latrunculin-B (Sigma) or equivalent volume of vehicle control (EtOH) was added to the cells for the final 30 min of incubation in Lo-Flo media. Nocodazole (10 ug/ml) (Sigma) or a vehicle control of dimethyl sulfoxide (DMSO) (Sigma) was used to inhibit the microtubule component of the cytoskeleton and was added in the final hour of the 4-h incubation period. To inhibit both actin and microtubules, latrunculin-B (10 uM) and nocodazole (10 ug/ml) were used with the equal volume of ethanol and DMSO for a control. For washout experiments, after drug treatment cells were washed and then incubated at room temperature with shaking for 1 h prior to processing for immunofluorescence.

Following incubation, the cells were washed twice at 500 × g for 4 min to remove excess MitoTracker and resuspended in 5 ml Lo-Flo plus the appropriate drug or vehicle in preparation for live cell imaging or immunofluorescence. Drug effectiveness was confirmed with immunofluorescence (see below), in all drug treatments the cytoskeleton was significantly different from vehicle controls.

Immunofluorescence of *Dictyostelium* Strains

AX4 (wild-type) and *cluA*$^-$ strains of *D. discoideum* were grown to log phase (about 2–4 × 10^6) then pelleted at 500 × g for 4 min and resuspended in Lo-Flo liquid medium (Formedium). Cells were treated with MitoTracker, nocodazole, latrunculin-B, or both or treated with DMSO, ethanol, or both, as previously described, to disrupt the cytoskeleton and stain the mitochondria. Stained and treated cells were washed by pelleting at 500 × g for 4 min two times, and resuspended in room temperature Lo-Flo liquid media to the original volume. Drugs or control were added back to the washed and stained cells. A 22 × 22 mm coverslip was placed into a 6-well plate. About 500 ul of washed and stained cells were added to the coverslips and allowed to adhere for 30 min. The coverslips with adhered cells were then washed twice with 10 mM MES-NaOH by gently adding and removing the solution to remove any cells that did not adhere to the coverslips. The adhered cells were fixed with 1 ml of 3% paraformaldehyde diluted in 10 mM Pipes (pH 6.0) for 30 min and then quenched with 100 mM glycine (1 ml) diluted in 1xPBS for 5 min. The membranes of the adhered and fixed cells were then permeabilized by using 0.02% Triton X-100 for 5 min. The permeabilized cells were washed three times by gently adding and removing 1xPBS and then blocked with 0.045% fish gelatin, 0.5% BSA in 1xPBS (PBG) for 1 h at room temperature. These cells were prepared for either actin or tubulin visualization.

To visualize actin, the blocked cells were stained for 1 h at room temperature in the dark using 0.5 ul of 6.6 uM phalloidin (Life Tech) in 500 ul PBG per coverslip. Excess phalloidin was removed by washing with 1xPBS three times for 5 min each before mounting the coverslips with SlowFade Gold (Invitrogen) onto glass slides.

To visualize tubulin, tubulin primary antibodies (mouse anti-tubulin, DSHB 12G10) were diluted 1:150 in PBG and added to the coverslips. The primary antibodies were allowed to sit overnight at 4°C. The following day, the cells were washed with 1xPBS three times for 5 min each. The secondary antibody (AlexaFluor 488 goat α mouse IgG) (Life Tech A11001) diluted 1:250 in PBG was added and incubated in the dark for 1 h at room temperature. The coverslips with treated cells were then washed with 1xPBS three times for 5 min each and mounted to glass slides with SlowFade Gold.

Quantification of Morphology

Cells were imaged using a Zeiss laser scanning LSM 510 Pascal confocal microscope to obtain z-stack images. The images were observed and classified according to the appearance of their microtubule and actin cytoskeleton and mitochondrial distribution. The microtubule cytoskeleton is present throughout the cell, while the actin localized around the cell at the membrane. The microtubule cytoskeleton morphology was classified as either little to none, patchy, or complete. Morphological classification was assessed by whether the microtubules appeared as disjointed and not extending throughout the cell (little to none), were in the astral configuration characteristic of the microtubule origin center (patchy), or branched throughout the cell and extending to the cell membrane (complete). The actin cytoskeleton was assessed by whether actin around the periphery of the cell was either absent or mostly absent (none), present but disjointed (patchy) or present and complete at the edge of the entire cell (complete). Mitochondrial morphology was determined to be distributed, loose cluster, or tight cluster (**Figure 1**). Distributed mitochondria were evenly dispersed throughout the cytoplasm, while clustered mitochondria were tightly aggregated toward the center or periphery of the cell. Loose clusters were considered loose mitochondrial aggregates.

Statistical Analysis-Immunofluorescence

Each experiment was repeated a minimum of three times per condition with a minimum of forty cells being quantified. Statistical analysis was performed using Prism Graph Pad 6.07. A Chi square (or Fisher when appropriate) analysis was conducted to determine statistical significance among treatments

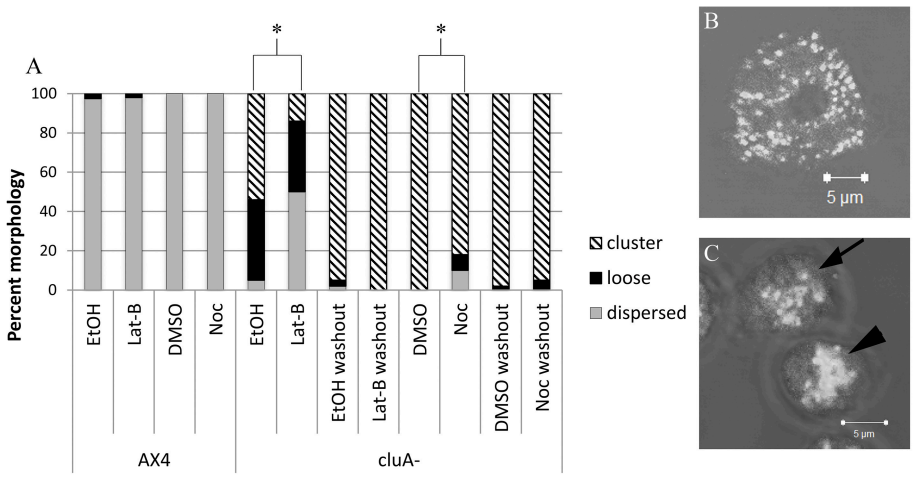

FIGURE 1 | Analysis of mitochondria distribution in AX4 and *cluA*$^-$ cytoskeleton disrupted cells. (A) AX4 mitochondrial morphology remained dispersed and was unaffected by all treatments. The *cluA*$^-$ clustered mitochondria phenotype was significantly decreased with more loose clusters and dispersed mitochondrial when treated with latrunculin-B ($p < 0.0001$) or nocodazole ($p < 0.0001$). **(B,C)** Examples of mitochondrial distribution. **(B)** AX4 cell with dispersed mitochondria, **(C)** *cluA*$^-$ cells where top cell (with arrow) shows loosely clustered mitochondria, bottom cell (with arrowhead) shows a tight cluster. *Indicates significant differences.

and strains. A p-value of less than 0.05 was considered statistically significant.

Quantification of Mitochondrial Fission and Fusion in *Dictyostelium* Strains

Washed and MitoTracker stained cells (0.5 ml) were placed in Nunc Lab-TekII 4-well chambered coverglass for imaging. A Zeiss laser scanning LSM Pascal confocal microscope with a pinhole setting of 144 um (1.36 airy units), resulting in an optical slice of 1.1 um was used to image washed, stained, and treated cells. A single plane was imaged every 677.38 ms for 100 time points, or until bleaching occurred.

To quantify fission events, mitochondria must be visible prior to the single organelle splitting into two. Fusion was quantified when two mitochondria approached and moved together for a couple of frames and then fused into a single organelle. If two organelles came together or split, then returned to their original state by the next frame, they were classified as "drive-bys" and were not quantified.

Statistical Analysis-Fission and Fusion

Statistical analysis was performed using JMP 11.0.0 (SAS Institute, Inc.) software. The rates of fission and fusion were calculated by averaging the number of events/min/cells for each strain and treatment. A minimum of 30 cells for each strain was used for quantification. Kruskal-Wallis analysis with a Steel-Dwass *post-hoc* was performed for statistical analysis. A p-value of less than 0.05 was considered statistically significant.

Kymograph Generation and Motility Analysis

Kymographs were generated using ImageJ from single plane confocal time lapse images (Schneider et al., 2012). A region of interest (ROI) was selected within a cell in the first image of each series. ROIs were drawn through the left, middle, and right portions of the cell in every instance where cells were visible throughout the entire series of images. The ROIs were stacked and converted to generate kymographs that depict mitochondrial movement within the region of interest over time. Kymographs were generated for a minimum of 20 cells in each treatment for AX4 and *cluA*$^-$ strains. To quantify motility, the speed in pixels/0.677 s was calculated and converted to micrometers/second for comparisons. Mitochondrial motility from the left, middle, and right portions were calculated and averaged for each cell. Kruskal-Wallis with Steel Dwass *post-hoc* analysis was conducted using JMP software for statistical comparisons of single drug vs. single control. A p-value of less than 0.05 was considered statistically significant.

The percent of mitochondria moving were counted from the kymographs created for motility analysis. Mitochondria in each kymograph were counted and classified as either moving or not moving, with stationary mitochondria being considered a straight vertical line from the top of the kymograph to the bottom. An average percent of mitochondria moving was calculated for each strain and treatment and compared; a minimum of 20 cells were analyzed for each treatment. For analysis, nonparametric Kruskal-Wallis and *post-hoc* Steel-Dwass statistical tests were used. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The Relationship between the Cytoskeleton and Mitochondrial Morphology in *D. discoideum*

We quantified mitochondrial morphology after disrupting actin with latrunculin-B (Lat-B) or microtubules with nocodazole (Noc) in wild-type (AX4) and *cluA*$^-$ strains. The morphology

and motility rates were compared to respective controls, as well as comparisons within treatment and across strains. These comparisons allowed us to determine the effect of each component of the cytoskeleton on mitochondrial morphology in *cluA*$^-$ cells.

As expected, alteration of the cytoskeleton with these pharmaceuticals does not affect mitochondrial distribution in AX4 cells when compared to vehicle control cells (Noc: $p = 1.0$; Lat-B: $p = 0.9999$; **Figure 1**). However, disruption of the cytoskeleton did alter mitochondrial distribution in *cluA*$^-$ cells. Treatment with either nocodazole or latrunculin-B changed *cluA*$^-$ mitochondria from their characteristic clustered morphology to a higher prevalence of loose clusters (Noc: $p < 0.0001$; Lat-B: $p < 0.0001$; **Figure 1**). As a further control, we treated the cells with vehicle or drugs then washed the cells and quantified mitochondrial morphology. Results from washout experiments indicate that indeed all changes to mitochondrial morphology are specific to the treatments, though interestingly the vehicles themselves have some effect (**Figure 1**). Especially EtOH, which increases the number of loose mitochondria compared to the washout controls. To determine if there was a synergistic effect between actin and microtubules, mitochondrial morphology of both strains was analyzed when the cells were exposed to nocodazole and latrunculin-B simultaneously. Our results indicate that there is no significant synergistic effect (data not shown), thus we can conclude that both cytoskeletal filaments are involved in maintaining the tightly clustered *cluA*$^-$ phenotype but do not affect the wild-type dispersed mitochondrial phenotype.

The Relationship between the Cytoskeleton and Mitochondrial Motility in *D. discoideum*

To determine the role of actin and microtubules in mitochondrial motility, cells were treated with cytoskeletal disrupting drugs and mitochondrial velocity and the percentage of organelles moving was calculated from kymographs. Again we analyzed both AX4 wild-type cells and *cluA*$^-$ cells, to determine not only the role of the cytoskeleton but also the role CluA may play in mitochondrial motility.

When actin is inhibited with latrunculin-B, AX4 had an average mitochondrial speed of 0.164 ± 0.007 um/s while the ethanol control averaged a speed of 0.165 ± 0.007 um/s, with no statistical difference between treatments ($p = 1.0$; **Figure 2A**). Similarly, the *cluA*$^-$ latrunculin-B and control treated cells averaged mitochondrial speeds of 0.161 ± 0.007 um/s and 0.158 ± 0.007 um/s, respectively, with no statistical significance ($p = 1.0$; **Figure 2A**). A comparison of treatments across strains also proved to not be statistically significant for latrunculin-B ($p = 1.0$) and ethanol ($p = 1.0$; **Figure 2A**). Thus, neither actin nor CluA has a significant role in mitochondrial velocity.

When measuring motility for microtubule inhibited cells, there was a significant difference between the drug treated cells and their control for both strains. In AX4, the DMSO control

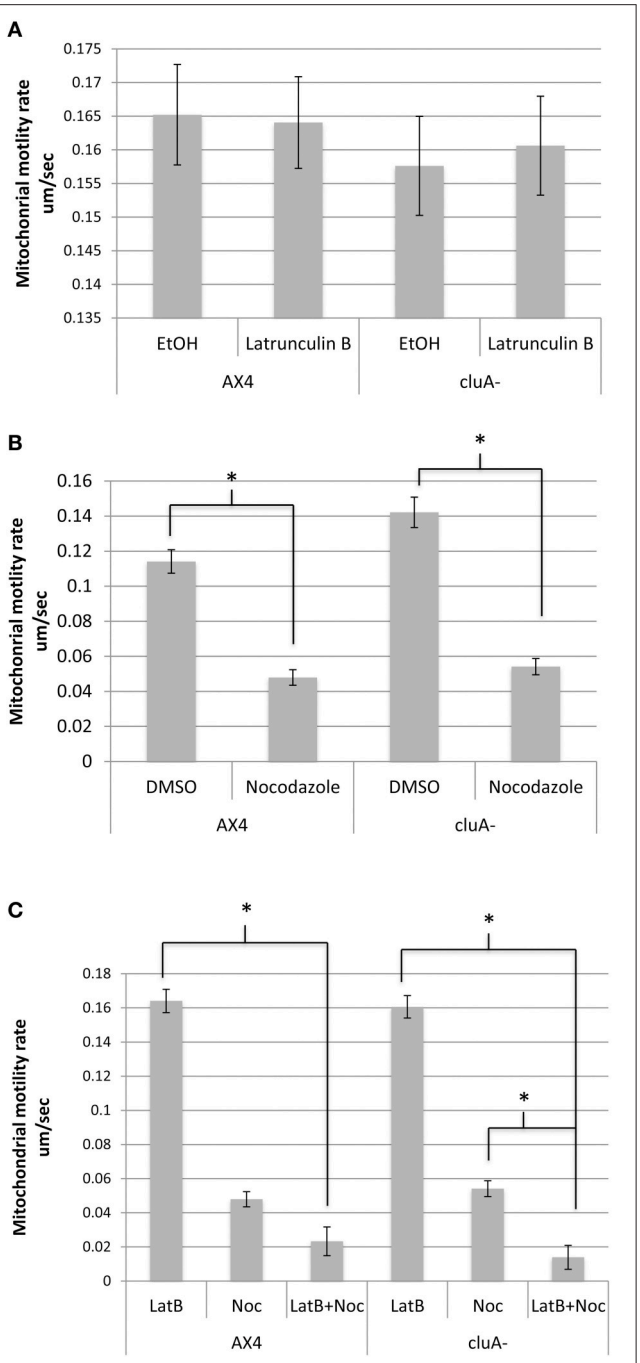

FIGURE 2 | Average mitochondrial speed compared across strains and treatments. (A) There was no significant difference between treatments in AX4 ($p = 1.0$) or *cluA*$^-$ ($p = 1.0$) when treated with latrunculin-B. Comparing the treatments across strains also failed to prove statistical significance in ethanol ($p = 1.0$) and latrunculin-B ($p = 1.0$) treatments. **(B)** AX4 and *cluA*$^-$ mitochondria treated with nocodazole moved significantly slower than in vehicle treated cells (AX4, $p < 0.0001$; *cluA*$^-$, $p < 0.0001$). AX4 DMSO mitochondrial speed was not significantly slower than the *cluA*$^-$ DMSO treatment ($p = 0.57$) or nocodazole treatment ($p = 0.99$). **(C)** Cells were treated with nocodazole and latrunculin-B simultaneously to determine if there was a synergistic effect between actin and microtubules. In AX4 only latrunculin-B ($p < 0.0001$) but not nocodazole ($p = 0.29$) single treatments had *(Continued)*

FIGURE 2 | Continued
significantly higher mitochondrial rates compared to the double treatment. In *cluA*$^-$ both single treatments (Lat-B: $p < 0.0001$; Noc: $p = 0.0031$) had significantly higher motility rates compared to the double drug treated *cluA*$^-$ cells. CluA plays no direct role in mitochondrial motility, while microtubules determine the speed of mitochondrial movement; though in the absence of CluA, actin does play a significant role also. *Indicates significant differences.

had an average rate of 0.1141 ± 0.007 um/s while the nocodazole treated mitochondria moved 55% slower at 0.0479 ± 0.004 um/s ($p < 0.0001$). Similarly, in *cluA*$^-$, mitochondrial speed in the control was 0.1422 ± 0.009 um/s and 0.0541 ± 0.005 um/s in the nocodazole treated cells, a 64% reduction in mitochondrial speed than the control cells ($p < 0.0001$; **Figure 2B**). Comparing treatments across strains proved that there was no significant difference in nocodazole or DMSO treated AX4 and *cluA*$^-$ (Noc: $p = 0.99$; DMSO: $p = 0.57$), though the DMSO vehicle AX4 cells had a 21% slower mitochondrial speed than DMSO *cluA*$^-$ (**Figure 2B**).

Again we determined if there was a synergistic effect between microtubules and actin in terms of mitochondrial velocity. In AX4, there is no statistical difference between nocodazole treatment and double drug treatment ($p = 0.29$; **Figure 2C**). Interestingly, in *cluA*$^-$ cells, nocodazole treated cells had a statistically higher motility rate, by 72%, than the double drug treated cells ($p = 0.0031$; **Figure 2C**). These results indicate that microtubules have the largest role in velocity, but when actin, microtubules, and CluA are disrupted, it is apparent that actin also has a contributory effect.

In addition to measuring the speed of mitochondrial movement we also quantified the percent of mitochondria moving from the kymographs. Approximately 72 and 87% of mitochondria are moving in the vehicle control EtOH and DMSO treated AX4 cells respectively (**Figure 3**). When treated with latrunculin-B, the percent of mitochondria moving was reduced by about 75% ($p < 0.0001$), while nocodazole treatment reduced the number of moving mitochondria by 39% ($p < 0.0005$). Further analysis showed that there is no synergism between microtubules and actin for determining how many mitochondria are moving. Thus, both actin and microtubules are necessary for determining how many mitochondria move, but actin is likely the predominant cytoskeletal element.

For *cluA*$^-$ single vehicle controls, 92% of mitochondria were moving in DMSO treated cells, with 68% moving in ethanol. When microtubules were inhibited, the percent of mitochondria moving was reduced by 28% ($p < 0.0013$), while inhibiting actin reduced the number of moving mitochondria by 81% ($p < 0.0001$; **Figure 3**). Again there is no synergistic effect, thus these results suggest in *cluA*$^-$, as in AX4, that actin plays the predominant role in the percent of moving mitochondria but both cytoskeletal elements are involved.

Overall, both actin and microtubules are necessary for mitochondrial motility in *D. discoideum,* while CluA seems to have no significant role. Our results indicate that microtubules play the largest role in determining velocity of movement, while actin seems to be more important for how many mitochondria are moving.

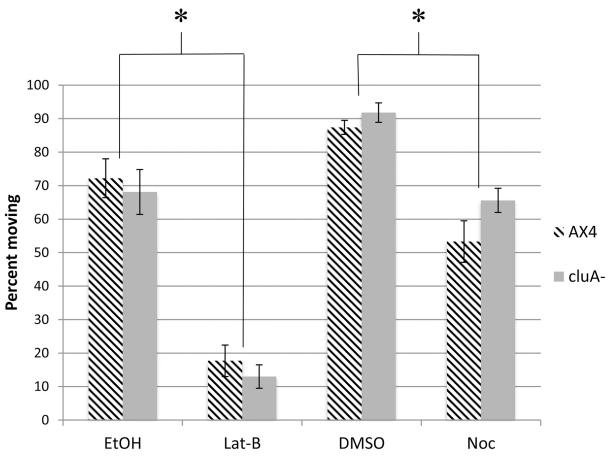

FIGURE 3 | Percent of mitochondria moving in cytoskeleton disrupted AX4 and *cluA*$^-$ strains. In AX4 cells, all drug treatments significantly lowered the percent of mitochondria moving in comparison to their appropriate controls (Lat-B: $p < 0.0001$; Noc: $p = 0.0005$). This also occurred in the *cluA*$^-$ strain (Lat-B: $p < 0.0001$; Noc: $p < 0.0013$). Inhibiting the cytoskeleton significantly decreased the percent of mitochondria moving in both wild-type and *cluA*$^-$ strains. *Indicates significant differences.

Assessing the Relationship between the Cytoskeleton and Mitochondrial Fission and Fusion in *D. discoideum*

Little is known about the cytoskeletal influences on mitochondrial fission and fusion but it is expected that the cytoskeleton would play a critical role. To give more insight to this potential interaction, we quantified both fission and fusion rates in the wild-type and *cluA*$^-$ strains when the microtubules or actin filaments were disrupted. The fission and fusion rates of drug treated cells were compared to an appropriate vehicle control, as well as across strains. Comparing these fission and fusion rates allowed us to determine the role of the cytoskeleton in *D. discoideum* fission and fusion.

Using laser scanning confocal microscopy, a series of single plane images of *D. discoideum* were captured that showed the real time mitochondrial movement in the cells. Fission and fusion events were quantified in each cell and a rate was calculated. When the actin cytoskeleton was inhibited in AX4 cells, fission ($p = 1.0$) and fusion ($p = 1.0$) rates were not significantly different compared to the control, additionally fission and fusion remained balanced within each treatment for both latrunculin-B ($p = 1.0$) and ethanol ($p = 1.0$) treated cells (**Table 1**).

The AX4 microtubule cytoskeleton was inhibited using nocodazole. In these cells the rates of fission and fusion remained balanced (Noc: $p = 1.0$; DMSO: $p = 1.0$; **Table 1**) though, it was apparent that inhibiting microtubules significantly lowered fission by 85% ($p = 0.004$) and fusion by 81% ($p = 0.003$; **Table 1**). Further analysis demonstrated there was no synergistic effect between microtubules and actin in these processes (data not

TABLE 1 | Data table for the comparison of fission and fusion rates, with standard error, of all AX4 treatments.

Treatment	Fission rate (events/min/cell)	Fusion rate (events/min/cell)
AX4 DMSO	0.79 ± 0.15	0.79 ± 0.14
AX4 Nocodazole	0.12 ± 0.04	0.15 ± 0.05
AX4 EtOH	0.89 ± 0.1	0.86 ± 0.1
AX4 Latrunculin-B	0.75 ± 0.1	0.74 ± 0.09

TABLE 2 | Data table for the comparison of fission and fusion rates, with standard error, of all *cluA*$^-$ treatments.

Treatment	Fission rate (events/min/cell)	Fusion rate (events/min/cell)
cluA$^-$ DMSO	0.97 ± 0.1	0.87 ± 0.1
cluA$^-$ Nocodazole	0.18 ± 0.08	0.19 ± 0.08
cluA$^-$ EtOH	0.81 ± 0.09	0.71 ± 0.08
cluA$^-$ Latrunculin-B	0.67 ± 0.09	0.57 ± 0.09

shown), thus microtubules are required for mitochondrial fission and fusion while actin plays little to no role in these processes.

As the cytoskeleton is required for fission and fusion, we wondered if disrupting the cytoskeleton would also decrease the rates of fission and fusion in cells lacking CluA. Our results show that DMSO treated *cluA*$^-$ mitochondria fission and fusion rates were balanced ($p = 1.0$) and similar to the ethanol control rates, which were also balanced ($p = 1.0$; **Table 2**). *cluA*$^-$ strains treated with nocodazole showed significantly lower fission and fusion compared to the DMSO control, 81 and 78% respectively (fission, $p < 0.0001$; fusion, $p < 0.0001$; **Table 2**). Within treatment, the rates of fission and fusion remained balanced (Noc: $p = 1.0$; **Table 2**). When treated with the actin inhibiting drug latrunculin-B, there was no difference in fission ($p = 1.0$) or fusion ($p = 1.0$) when compared to the ethanol control (**Table 2**). Again, as in wild-type cells, further analysis demonstrated there was no synergistic effect between microtubules and actin in fission and fusion in *cluA*$^-$ cells (data not shown). Thus, the microtubules exert a greater influence on regulating mitochondrial fission and fusion. Moreover, actin filaments were found to have no significant effect, even in the absence of CluA.

DISCUSSION

The Role of Microtubules in Mitochondrial Dynamics

Here we present data suggesting that microtubules are the predominant cytoskeletal element for moving and distributing *D. discoideum* mitochondria. We show an almost complete loss of motility in cells treated with nocodazole as well as the relaxed and more distributed clusters found in *cluA*$^-$ cells with disrupted microtubules. Additionally we and others have observed a small population of mitochondria that associate with the microtubules (Vlahou et al., 2011). Finally work by Vlahou et al demonstrates that mitochondrial distribution is dependent upon intact microtubules (Vlahou et al., 2011).

We also demonstrate that microtubules are essential for mitochondrial fission and fusion. It has not yet been teased out whether disruption of fission and fusion in these cells prevents motility or if motility must be functional for fission and fusion to take place. It has been suggested that blocks of fission and fusion will inhibit motility and distribution. Incomplete fission can result in a tangle of interconnected mitochondria and incomplete fusion can result in mitochondrial aggregates, thus motility's effect on the processes seems clear (Chen and Chan, 2009). On the other hand, it has been shown that loss of Miro, which inhibits motility, subsequently inhibits fusion (Cagalinec et al., 2013). It is logical to assume that motility facilitates fission and fusion as at least one mitochondrion must move toward another for fusion to take place and once divided the organelles must move apart to remain separate. Either way it is apparent that mitochondrial dynamics are intimately linked to motility and in *D. discoideum*, as suggested by our data, regulated by microtubules.

The Role of Actin in Mitochondrial Dynamics

Our results suggest that disruption of actin decreases the number of mitochondria moving, but the ones that are moving, move at the same speed and go to the same locations as in untreated cells. This suggests to us that while actin may not be a major highway for mitochondrial movement it may function as an entrance ramp, helping mitochondria get to the highway as needed. If this is the case, it is apparent that by actively targeting mitochondria, the cell can select the organelles that need to be transported to the sites of high energy needs or perhaps undergo fission and fusion to repair mitochondrial DNA preventing a buildup of damaged or older mitochondria. Microtubules can then move the selected mitochondria and regulate fission and fusion events. This model is similar to mitochondrial behavior in neurons. Neurons utilize the actin cytoskeleton to move mitochondria shorter distances and microtubules for long distance transport (Morris and Hollenbeck, 1995). The shorter distance movement is due to the neuron's need to retain mitochondria at sites of high ATP utilization (Boldogh and Pon, 2006).

The Role of CluA in Mitochondrial Dynamics

CluA is required for distribution and plays a role in fission and fusion. Our results indicate that tight cluster formation is dependent upon CluA, microtubules, and actin, but CluA is not a significant player in mitochondrial motility. Therefore, CluA is most likely not an adaptor protein linking mitochondria to the cytoskeleton. Instead we suggest that *D. discoideum* must have a novel adaptor protein not yet identified; perhaps an intermediate filament as indicated in 3T3 fibroblast cells (Nekrasova et al., 2011).

Interestingly while we suggest CluA is not a linker protein, the cytoskeleton does appear to play a larger role in mitochondrial distribution and motility when CluA is absent. The clustered phenotype of *cluA*$^-$ cells is relaxed by the disruption of the actin

and microtubule cytoskeletons, and there is a synergistic decrease in motility when CluA, microtubules, and actin are all disrupted. Perhaps this is simply a result of mis-regulation of fission and fusion in *cluA*$^-$ cells.

In conclusion, *D. discoideum* mitochondria move along the microtubule cytoskeleton, similar to what is reported in animal cells, and without this movement mitochondrial fission and fusion cannot take place. Finally, we propose that the link between mitochondria and the microtubules is not CluA and that this protein plays an as yet unidentified role in mitochondrial fission and fusion.

AUTHOR CONTRIBUTIONS

LW contributed to the acquisition, analysis and interpretation of the data, she drafted, revised, and approves the final version of the manuscript. GB contributed to the design and acquisition of the work. He revised and approves the final version of the manuscript. KN contributed to the design, acquisition, and interpretation of the work. She drafted, revised, and approves the final version of the manuscript. LW, GB, KN agree to be accountable for all aspects of the work.

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High Light Intensity Leads to Increased Peroxule-Mitochondria Interactions in Plants

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Peroxules are thin protrusions from spherical peroxisomes produced under low levels of reactive oxygen species (ROS) stress. Whereas, stress mitigation favors peroxule retraction, prolongation of the ROS stress leads to the elongation of the peroxisome into a tubular form. Subsequently, the elongated form becomes constricted through the binding of proteins such as dynamin related proteins 3A and 3B and eventually undergoes fission to increase the peroxisomal population within a cell. The events that occur in the short time window between peroxule initiation and the tubulation of the entire peroxisome have not been observed in living plant cells. Here, using fluorescent protein aided live-imaging, we show that peroxules are formed after only 4 min of high light (HL) irradiation during which there is a perceptible increase in the cytosolic levels of hydrogen peroxide. Using a stable, double transgenic line of *Arabidopsis thaliana* expressing a peroxisome targeted YFP and a mitochondrial targeted GFP probe, we observed sustained interactions between peroxules and small, spherical mitochondria. Further, it was observed that the frequency of HL-induced interactions between peroxules and mitochondria increased in the Arabidopsis *anisotropy1* mutant that has reduced cell wall crystallinity and where we show accumulation of higher H_2O_2 levels than wild type plants. Our observations suggest a testable model whereby peroxules act as interaction platforms for ROS-distressed mitochondria that may release membrane proteins and fission factors. These proteins might thus become easily available to peroxisomes and facilitate their proliferation for enhancing the ROS-combating capability of a plant cell.

Keywords: peroxisomes, peroxules, mitochondria, organelle interactions, *any1* mutant, ER

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INTRODUCTION

Peroxisomes are directly implicated in the scavenging of reactive oxygen and reactive nitrogen species (ROS and RNS, respectively) in plant cells (Corpas et al., 2013; Corpas, 2015). Peroxisome morphology in plants varies from ca. 0.5 to 2 μm diameter spheres to ca. 3–8 μm long tubules. Whereas, the spherical shape is typical for peroxisomes in a cell that is not overtly stressed, the elongated form is usually observed in a stressed cell and indicates that the peroxisome will soon undergo fission to produce more spherical peroxisomes (**Figure 1**; Schrader, 2006; Sinclair et al., 2009; Delille et al., 2010; Barton et al., 2014; Schrader et al., 2014). In addition, a transient form is encountered in response to mild ROS stress during which the peroxisome is neither completely

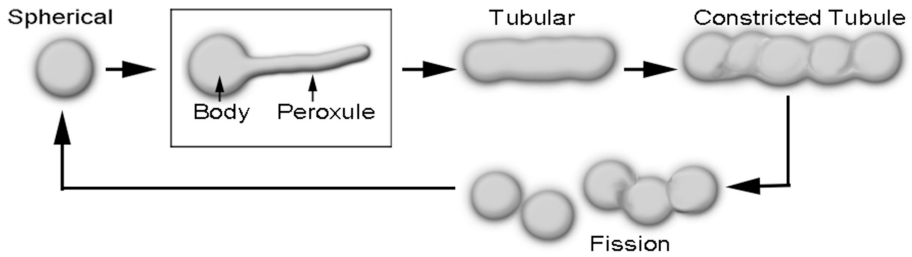

FIGURE 1 | Diagrammatic depiction of sequential changes in peroxisome morphology leading to their fission and proliferation. Peroxules extend and retract from a spherical peroxisome body and thus represent a transient, intermediate state between the spherical and the completely tubular, pre-constriction form of peroxisomes.

spherical nor tubular (Sinclair et al., 2009; Barton et al., 2013). During this phase thin, dynamic protrusions that have been named peroxules are observed extending and retracting from the spherical peroxisome body (**Figure 1**; Scott et al., 2007; Sinclair et al., 2009). Whereas, mitigation of a transient ROS stress results in peroxule retraction and reversion to the spherical peroxisome stage, prolonged stress, or higher stress intensity leads to the completely tubular peroxisomal form (Sinclair et al., 2009).

The formation of tubular peroxisomes is considered to be a multistep process involving the insertion of peroxisomal membrane proteins (PMPs) into the existing peroxisomal membrane (Li and Gould, 2003; Koch et al., 2004; Thoms and Erdmann, 2005; Schrader, 2006). The role of Peroxin11 (PEX11) isoforms appears to be especially important during the early tubulation phase and the remodeling of the peroxisome membrane (Lingard and Trelease, 2006; Kobayashi et al., 2007; Nito et al., 2007; Orth et al., 2007; Lingard et al., 2008; Delille et al., 2010; Koch et al., 2010). Interestingly, the ectopic expression of PEX11 family proteins from yeast, plants and mammalian systems results in the formation of juxtaposed elongated peroxisomes (JEPs; Koch et al., 2010), and tubular peroxisomal accumulations (TPAs; Delille et al., 2010). Diagrammatic depictions of single peroxisomes in these clusters suggest a strong morphological resemblance to peroxules. Although JEPs and TPAs inform about the role of PEX11 proteins in peroxisome proliferation they have been usually observed in response to overexpression of specific PEX11 proteins over several hours (Delille et al., 2010; Koch et al., 2010). By contrast, peroxules are produced within seconds in plant cells as a normal peroxisomal response to ROS (Sinclair et al., 2009). Nevertheless, the incorporation of additional PMPs (Delille et al., 2010) might be considered as a general mechanism leading to peroxule formation. The idea appears feasible since peroxule formation occurs as an intermediate stage that leads into the formation of tubular peroxisomes (**Figure 1**). The crucial question that remains unanswered is how the various PMPs and subsequently required fission factors become available so quickly in response to ROS.

Interestingly some of the major components of the peroxisomal division machinery are shared with mitochondria. Both organelles, once they have reached a certain degree of tubulation become constricted through the mechano-chemical activity of the GTPases Dynamin Related Protein (DRP3A and B/ADL2a and b, respectively, in plants: Arimura and Tsutsumi, 2002; Arimura et al., 2004; Logan et al., 2004; Mano et al., 2004; Zhang and Hu, 2009; Dlp1 in mammals: Pitts et al., 1999; Dnm1 in yeast: Bleazard et al., 1999). The GTPases are recruited from the cytosol and anchored to the membrane by FISSION1, a tail-anchored protein localized to both the peroxisome and outer mitochondrial membrane (Fis1/BIGYIN in plants: Scott et al., 2006; Zhang and Hu, 2008; 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). In both organelles membrane constriction produces a beaded appearance that ultimately leads to their fission and consequent increase in the mitochondrial and peroxisomal population within the cell (Barton et al., 2014; Jaipargas et al., 2015). While many details pertaining to the division machinery common to peroxisomes and mitochondria have been worked out it is still unclear whether the relevant proteins are recruited simultaneously to both organelles or becomes available in a hierarchical manner.

In a similar context while strong biochemical links exist between peroxisomes and mitochondria and both organelles respond to very similar stimuli, including ROS (Hoefnagel et al., 1998; Foyer and Noctor, 2003; Apel and Hirt, 2004; Brookes et al., 2004; Schrader, 2006; Schrader and Yoon, 2007; Schumann and Subramani, 2008; Gechev et al., 2010; Bhattacharjee, 2011), their actual behavior and cooperation in a living plant cell has not been visualized. Scenarios that evoke inter-organelle cooperation during ROS stress suggest that metabolites and proteins may be transferred between the two organelles through mitochondrial membrane extensions (Schumann and Subramani, 2008). We postulated that an opposite scenario might also operate whereby peroxisome extensions in the form of peroxules might be involved in interactions with mitochondria.

Here, we have investigated this idea to uncover the role of peroxules during a possible peroxisome-mitochondria interaction. We have used simultaneous imaging of mitochondria and peroxisomes in stable double transgenic lines expressing different fluorescent proteins targeted to the two organelles. Peroxules were induced by exposing wild type Arabidopsis plants to short periods of high light (HL). Further, an Arabidopsis mutant *anisotropy1* (*any1*; Fujita et al., 2013) was found to exhibit increased frequency of peroxules. Our observations clearly show that small, but not elongated, mitochondria cluster around peroxules in sustained interactions. Our work suggests that the

plant cell's ability to combat increased subcellular ROS levels relies upon a hierarchical relationship between mitochondria and peroxisomes where peroxules act as extended platforms for mitochondrial interactions.

MATERIALS AND METHODS

Double Transgenic Plants for Simultaneous Visualization of Peroxisomes and Mitochondria

Peroxisomes and peroxules were visualized in a transgenic Arabidopsis line expressing a yellow fluorescent protein (YFP) with a peroxisome targeting tri-peptide (SKL) appended to the carboxy terminus (YFP-PTS1; Mathur et al., 2002). Mitochondria were observed using a transgenic line expressing the N-terminal pre-sequence of the mitochondrial β-ATPase subunit fused to GFP (mitoGFP; Logan and Leaver, 2000). The two lines were crossed to obtain a double transgenic line where peroxisomes and mitochondria could be visualized simultaneously. YFP-PTS1 and mitoGFP were introduced into the *any1* mutant background by crossing with the respective wild type lines. The *any1* phenotype of isotropic expanded hypocotyl cells and trichomes as well as the GFP and YFP fluorescence were confirmed through light and epi-fluorescent microscopy. Double transgenic lines of *any1*-mitoGFP X YFP-PTS1 were created by crossing and stabilized over three generations.

An ER lumen-retained red fluorescent protein probe (RFP-ER; Sinclair et al., 2009) was introduced into stable mitoGFP YFP-PTS1 plants using the *Agrobacterium* floral dip method (Clough and Bent, 1998) (YFP-PTS1 mitoGFP RFP-ER). These triple transgenics were used to further investigate the rearrangements of the ER in response to HL and its role in the clustering of peroxisomes, mitochondria, and chloroplasts.

Seeds were germinated on Murashige and Skoog's medium (Murashige and Skoog, 1962) containing Gamborg B5 vitamins (M404; PhytoTechnology labs) and 3 g/L of Phytagel (Sigma-Aldrich), supplemented with 3% sucrose and with a pre-autoclaving pH adjusted to 5.8. All seeds were stratified for 2 days at 4°C.

Microscopy

Simultaneous imaging of GFP, YFP, RFP, and chlorophyll was carried out on a three channel Leica TCS-SP5 confocal laser-scanning unit equipped with 488 nm Ar and 543 nm He-Ne lasers. Emission spectra acquired were: GFP—503 to 515 nm (green); RFP- 540 to 630 nm (red); chlorophyll—650 to 710 nm (false colored blue). In double and triple transgenic plants the YFP fluorescence was picked up in both GFP and RFP channels and appeared yellow in the merged images.

Arabidopsis transgenics expressing cytosolic HyPer, a H_2O_2 responsive probe (http://www.evrogen.com/products/HyPer/HyPer.shtml, Evrogen, Russia; Belousov et al., 2006; Costa et al., 2010) were used to observe H_2O_2 formation in response to a short HL stimulus. Seedlings were given 1–5 min of HL and the change in fluorescence (ex. 488 nm; Em. Band collection 530–560 nm) was analyzed immediately after. The ImageJ RGB Profile Plot plugin was used to determine the changes in fluorescence intensity before and after HL treatment.

All images were captured at a color depth of 24 bit RGB. Tissue and 7–10 day old seedlings were mounted in tap water on a glass depression slide and placed under a coverslip. All images and movies were cropped and processed for brightness/contrast as complete montages or image stacks using either Adobe Photoshop CS3 (http://www.adobe.com) or the ImageJ/Fiji platform (http://fiji.sc/Fiji). Adobe Photoshop was used for annotation of movies.

Assessing Light-Induced Stress

Induction of HL Stress

Plants were all grown at low to intermediate light (50–164 μmol m^{-2} s^{-1}). Respective controls of the effects of light involved growing plants in complete darkness from stratification up to and including observations, unless stated otherwise. To evaluate the responses and the rapidity of the responses of the organelles to HL, plants were given short exposures (1–5 min) of HL (850 ± 50 μmol m^{-2} s^{-1}). Observations were taken immediately after the light treatments to give a better sense of how quickly responses may be.

3,3′-Diaminobenzidine Staining

To look at the effects of light-induced production of H_2O_2, Columbia and *any1* plants were grown in the light (164 μmol m^{-2} s^{-1}) for 8 days, transferred to the dark for 24 h and then exposed to light (164 μmol m^{-2} s^{-1}) for 30 min, 1 h, or 2 h. Cotyledon and hypocotyl tissue were submerged in a solution of 3,3′-diaminbenzidine (DAB) with a metal enhancer (SIGMA*FAST*™ DAB with Metal Enhancer, Sigma-Aldrich) or distilled water (control) and left under vacuum (−50 KPa) for 4 h. The tissue was cleared with ethanol by washing the samples with 100% ethanol, incubating them in 85% ethanol + 15% methanol overnight, and rinsing them in 70% ethanol and then distilled water. The samples were mounted in 50% glycerol and sealed. All images were acquired at the same light intensity and microscope settings to permit direct comparisons between treatments. The DAB stain intensity was measured as the average inverse gray value using ImageJ (http://imagej.nih.gov/ij/), which was subtracted from the background and considered as the average inverse gray values of distilled water treated seedlings. The staining intensity was representative of the amount of H_2O_2 produced during the relative light intensity treatments.

Characterizing *anisotropy1* Mutant in Relation to Wild Type

Scanning electron microscopy (SEM) and toluidine blue O (TBO) staining were used to assess the consequences of alteration of the cell wall in the *anisotropy1* (*any1*) mutant and the extent of its anisotropy. Scanning electron microscopy (SEM) of Arabidopsis wild type (ecotype Columbia) and *any1* plants grown in soil under 125 ± 10 μmol m^{-2} s^{-1} light intensity was carried out using uncoated tissue in a Tabletop Hitachi TM-1000 microscope with an electron beam accelerated at 15 kV. Leaf cross sections of 12 day old seedlings grown in low light (70 μmol

m^{-2} s^{-1}) were stained with TBO to analyse the alterations in cell isotropy and cell-to-cell connectivity.

Statistical Analysis

All experiments were carried out at least five times. Observations of mitochondrial length comparisons and their interactions with peroxisomes and peroxules were made with a minimum sample size of $n = 50$. Two-tailed *t*-tests were made to determine the significance of results. Significance was predetermined as having a $p < 0.01$ (99% confidence interval).

RESULTS

HL Irradiation Increases Cytosolic H_2O_2

It has been reported that the number of peroxisomes producing peroxules increases following 30–45 s of UV irradiation or exposure to H_2O_2 (Sinclair et al., 2009). HL is known to result in increased cytosolic ROS (Foyer and Noctor, 2003; Apel and Hirt, 2004). We used two different methods to assess this. First, transgenic Arabidopsis plants expressing the hydrogen peroxide (H_2O_2) responsive HyPer-cytosolic probe (Costa et al., 2010) showed a clear spike in fluorescence intensity relative to the basal pre-exposure levels following short exposures of 1–5 min to HL of 850 ± 50 μmol m^{-2} s^{-1}. The green fluorescence that had spiked to almost 2.5 times decayed back to basal levels within 8 s of illumination suggesting that the increase in the sub-cellular levels of H_2O_2 was transient and fell below fluorescence detection levels quickly (**Figures 2A,B**). Unless stated otherwise the 4 min exposure time was maintained as the minimum in subsequent experiments.

An alternative approach used the DAB staining method used to assess increased H_2O_2 production in tissues over a relatively longer period (**Figure 2C**). Based on the idea that the plant cell wall acts as micro lens and has a regulatory role in determining the intensity and property of light that reaches the cell interior (Haberlandt, 1914; Vogelmann, 1993; Vogelmann et al., 1996) we used *anisotropy1,* an Arabidopsis mutant for comparison with the wild type (WT) plants. The *any1* mutant in an Arabidopsis cellulose synthase (CesA) gene has reduced cell wall crystallinity (Fujita et al., 2013). As a result, epidermal cells in *any1* are nacreous and in comparison to the WT (**Figures 2D,G,I**) display increased curvature (**Figures 2E,F,H,J**) and larger inter-cellular spaces (**Figure 2K** vs. **Figure 2L**). Exposing 8 day old seedlings of WT and *any1* that had been kept in the dark for 24 h to achieve basal ROS level to 164 μmol m^{-2} s^{-1} light for 30, 60, and 120 min was followed by DAB staining for the presence of H_2O_2. The intensity of staining was higher in *any1* for every treatment (**Figure 2C**) suggesting that as compared to the WT the mutant plants had higher H_2O_2 levels.

Thus, the HL treatment on HyPer-cytosolic plants (**Figures 2A,B**) provided us with a short time window that could be used for assessing the effects of ROS on peroxisomes and mitochondria while the longer light treatment and DAB staining (**Figure 2C**) suggested *any1* as potential experimental material that might provide differences in organelle behavior during HL-induced ROS stress. WT and *any1* plants expressing YFP-PTS1 and mitoGFP that highlights peroxisomes (Mathur et al., 2002) and mitochondria, respectively (Logan and Leaver, 2000) were assessed next.

High Light Induces Elongated Peroxules but Results in Smaller Mitochondria

Peroxisomes in both WT and *any1* plants moved as part of the cytoplasmic stream at variable velocities ranging from 1.5 to 7 μm s^{-1} which is consistent with peroxisome motility rates observed earlier (Jedd and Chua, 2002; Mathur et al., 2002). No undue clustering of peroxisomes was observed in *any1* cells prior to HL exposure and attested to their healthy state. However, there was large variability between WT and *any1* cells in terms of peroxisomal response to HL. Only 6 ± 2% peroxisomes in cotyledon epidermal cells ($n = 50$ cells) from 11 to 13 day old WT plants exhibited peroxules after 8–10 min of HL exposure. Comparable cells in *any1*-YFP-PTS1 plants showed 30 ± 7% ($n = 80$ cells) peroxules upon exposure for less than 8 min (**Figure 3A**,**a**; **Movie S4**). Longer exposure time of up to 15 min was sometimes needed for hypocotyl tissue in both plant types, especially if the plants had already been in light for several hours. However, we did not observe chlorophyll bleaching or peroxisome clustering under HL light irradiation conditions in these plants suggesting that the cells, although stressed, were still functional after the prolonged exposure. While the dynamic behavior of peroxules (**Movie S4**), made it difficult to measure their precise dimensions, cotyledon cells of *any1* often had longer peroxules than similar cells in the WT plants. In addition, as judged by 488 nm laser induced photo-bleaching, the peroxules in *any1* appeared more robust and retained their fluorescence for a longer time as compared to peroxules in wild type plants (data not shown).

Similar HL treatments were carried out on WT and *any1* plants expressing mitoGFP and a comparison of mitochondrial size between the two kinds of plants under dark and light conditions showed significantly smaller mitochondria in the mutant (**Figure 3B**; **Figure S1**). Upon exposure to HL, mitochondria in *any1* appeared relatively fuzzy compared to mitochondria in WT cells. Further, the smallest mitochondrial size distribution of 0.5 ± 0.2 μm was observed in large, swollen epidermal cells in *any1* cotyledons whereas WT cotyledon cells maintained a predominant mitochondrial population of ca. 0.8 μm length. Whereas, we have reported earlier that mitochondria in WT plants grown in the dark appear long and tubular and undergo rapid fission upon exposure to light (Jaipargas et al., 2015), we were unable to ascertain a clear size difference between mitochondria in cotyledons of dark grown and light exposed *any1* plants.

Having made our baseline observations for both WT and *any1* plants under HL stress we investigated the behavior of peroxisomes and mitochondria simultaneously in double transgenic plants.

Small Mitochondria Cluster around Peroxules

Exposing 7–10 day old double transgenic, non-mutant plants expressing YFP-PTS1 and mitoGFP to HL for 4 min resulted in

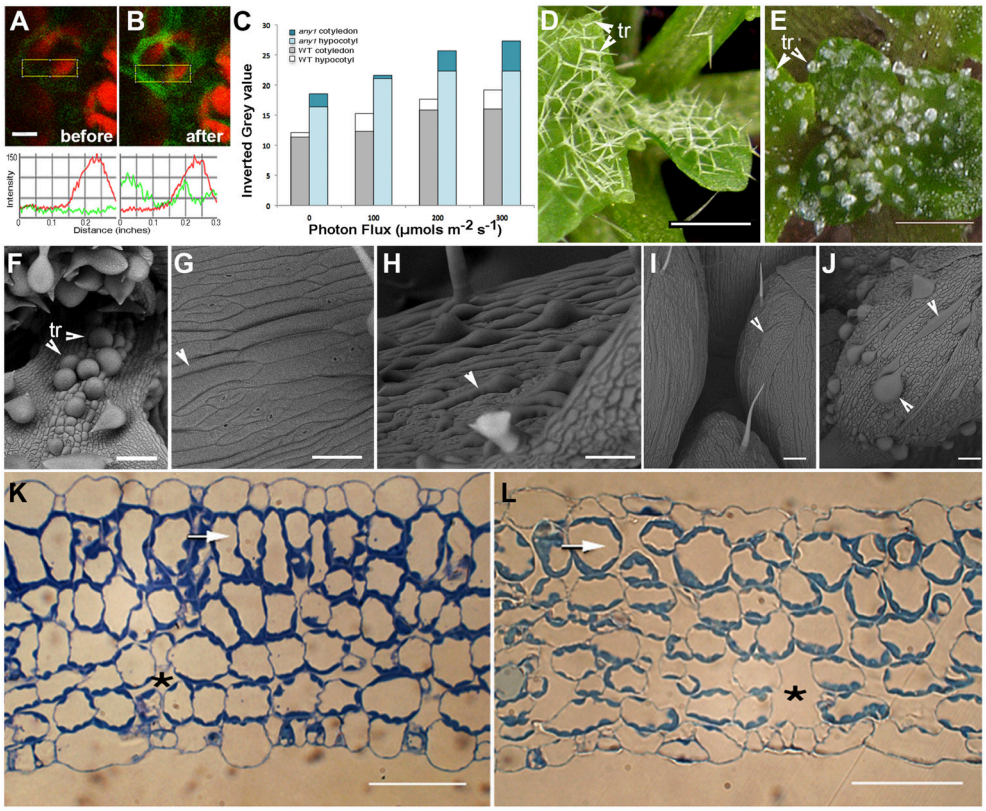

FIGURE 2 | Observations suggesting changes in subcellular H_2O_2 levels in response to HL and characteristic features of the *any1* mutant in comparison with wild type Arabidopsis. (A,B) A leaf epidermal cell in a transgenic *Arabidopsis* plant expressing HyPer-cytosolic probe **(A)** before and **(B)** after exposure to HL. Line traces based on the boxed areas are provided below the fluorescent images and show a nearly 2.5-fold general increase in green fluorescence intensity suggesting an increase in cellular H_2O_2 levels. Red chlorophyll auto-fluorescence is from chloroplasts in the mesophyll layer. **(C)** DAB staining of WT and *any1* mutant leaves exposed to increasing light intensity suggesting relatively higher H_2O_2 production and catalase activity in *any1* cells. **(D,E)** Leaf epidermal trichomes (tr) in WT **(D)** and the *any1* mutant **(E)** exhibit spectacular differences in growth anisotropy. **(F)** A scanning electron (SEM) image shows that the glassy-appearing trichomes as well as some non-trichome cells near the leaf base appear swollen due to isotropic growth in *any1*. **(G,H)** Arrowheads pointing to elongated cells in WT leaves suggest their relatively flat nature **(G)** as compared to the bulged cells in petioles of *any1* **(H)** mutant. **(I,J)** Giant cells in the sepal epidermis in WT (**I**; arrowhead) are relatively thin and unobtrusive compared to the large, swollen cells in the mutant (**J**; arrowheads). **(K,L)** Toluidine blue-O stained sections of WT **(K)** and *any1* **(L)** leaves at a similar stage of development show differences in the relative size of cells and intercellular spaces (*). The sub-epidermal layer in the mutant (arrows) is relatively disorganized and cells show approximately 35% decrease in elongation (19.3 µm vs. 29.7 µm long in wild type). Size bars in **(A)** = 5; **(D,E)** = 250; **(F–J)** = 100; **(K,L)** = 50 µm

altered interactions between organelles. Whereas, the majority of peroxisomes and mitochondria moved independently before HL irradiation there were clear clusters of organelles after the treatment (**Figure 3C**). However, the degree of organelle clustering varied widely between different cells and therefore cannot be provided as an average number. Mitochondria and peroxisomes of nearly similar sizes appeared together more often after HL irradiation while major clustering took place around chloroplasts. Notably, the peroxisome-mitochondria clusters around chloroplasts did not contain an equal number of the two organelles. In 72% of the clusters observed ($n = 100$) the ratio of mitochondria to peroxisomes was nearly double while extreme ratios approaching 5:1 were also observed (**Figures 3C,D**). The clustering appeared to take place through changes in the speed of cytoplasmic streaming and a perceptible slowdown occurred over 20 min of imaging. As reported earlier (Jaipargas et al., 2015), imaging of the same cell for more than 15 min using the 488 nm laser led to significant chlorophyll photo-bleaching and a hypoxia induced swelling of mitochondria. While mitochondria-peroxisome clusters observed in the non-mutant background exhibited peroxules only sporadically, cotyledon cells in *any1* readily exhibited a high frequency of these extensions. In both cases the dynamic, extending-retracting peroxules had clusters of small mitochondria with diameters of 0.7 ± 0.2 µm around them (**Figures 3E–I**). Whereas, mitochondria exhibit a wide morphological range from tubular to flattened disc-shaped giant mitochondria (Cavers, 1914; Lewis and Lewis, 1914; Van Gestel and Verbelen, 2002; Schrader, 2006; Logan, 2010; Jaipargas et al., 2015), we observed only the smallest forms clustering around peroxules. A cluster was scored as sustained if it was maintained for a minimum of five frames or 20 s (e.g., **Figure 3I**). Although such clustering around peroxules was

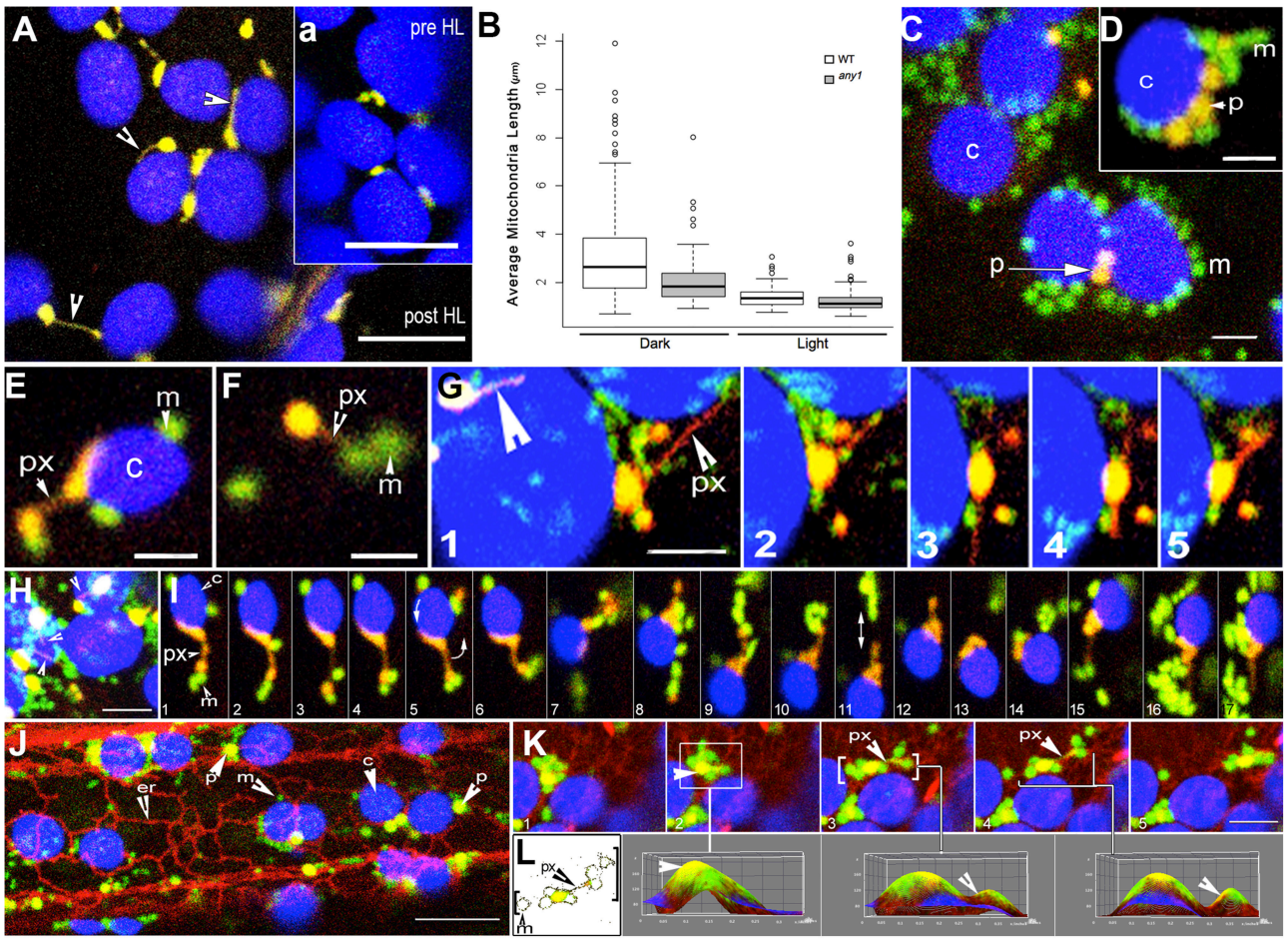

FIGURE 3 | Peroxisome and mitochondria in wild type and *any1* mutant *Arabidopsis* plants. (A) A single cotyledon mesophyll cell exhibits peroxules in the YFP-PTS1 line in the *any1* mutant. Time lapse sequence shown as **Movie S4**. The inset "**a**" shows a portion of the same cell before HL exposure. **(B)** Mitochondrial length, taken as an indicator of increased cellular ROS is significantly smaller in the *any1* mutant in comparison to WT under both light and dark growth conditions on medium without sugar ($p < 0.05$). **(C)** Mitochondria and peroxisomes often cluster around chloroplasts in cotyledon and hypocotyl cells of WT seedlings following HL exposure (**Movie S1** vs. **Movie S2**). **(D)** Similar clusters to "**c**" are observed in swollen cells of *any1* even under 125 ± 10 μmol m^2 s^1 light intensity. Peroxisome numbers are often lower than mitochondria in these clusters. **(E)** A peroxule (px) extended by a chloroplast-associated peroxisome shows mitochondrial (m) association. **(F)** An independent peroxisome with extended peroxule associates with two mitochondria following a 4-min exposure to HL. **(G)** Dynamic extension (frames 1–3), retraction (frame 4) and change of direction of extension (frame 4,5) of a peroxule (px) from a chloroplast associated peroxisome. Multidirectional peroxule extension while the parent peroxisome remains in one location allows interactions with several mitochondria on different sides. A second peroxisome with an extended peroxule is indicated (large arrowhead). **(H)** Single cotyledon cell in the *any1* mutant stably expressing YFP-PTS1 and mitoGFP shows several chloroplast-associated yellow-orange peroxisomes and peroxules (arrowheads) with clusters of mitochondria (green) (**Movie S5**). **(I)** A series of 17 sequential time-lapse snapshots of a hypocotyl cell of the double transgenic *any1* mutant show a chloroplast-associated peroxisome (orange-yellow) extending a peroxule. Note that the peroxisome-chloroplast association remains strong despite the rotation of the chloroplast (arrow in panel 5) while the mitochondrial population (green) that comes in contact with the extended peroxule is maintained even though individual mitochondria may join and leave the cluster (**Movie S6**). **(J)** Hypocotyl cell in a triple transgenic Arabidopsis plant shows the typical associations formed between chloroplasts (c-blue) mitochondria (m-green) peroxisomes (p-yellow) and the ER (er-red) following a 4-min HL exposure (**Movie S7**). **(K,L)** Analysis of sequential snapshots of a mitochondria-peroxisome cluster (yellow-green) moving past chloroplasts (blue) on an ER defined path. 3D surface plots corresponding to panels K2, K3, K4 show clear yellow peaks indicating the presence of peroxisomes within the cluster whereas color thresh-holding and skeletonizing in "L" reveals the presence of a thin peroxule amongst mitochondria in panels K3 and K4. Size bars = **(A,a)** = 5 μm; **(C,D)** = 2.5 μm **(E–I,K)** = 5 μm; **(J)** = 10 μm.

observed in 65 independent time-lapse imaging sequences the fact that some mitochondria joined the cluster while others left it (**Figures 3G,I**; **Movie S3**, **5**, **6**) did not allow us to ascertain the time that a single mitochondrion might spend in possible interaction with the peroxule. In addition several clusters were maintained even though the peroxule extended and retracted or the peroxisome swiveled around while moving as part of the cytoplasmic stream (**Figure 3I**; **Movie S6**). The independent streaming of mitochondria past different peroxules without joining a cluster suggested that only a particular subset of the mitochondrial population becomes involved in the sustained interaction.

A possible mechanism for organelle clustering in response to HL was investigated next.

The Endoplasmic Reticulum (ER) Enmeshes Peroxisomes, Mitochondria, and Chloroplasts

Along with specific and highly conserved mitochondrial fission factors (Schrader, 2006) the ER has been shown to mediate mitochondrial fission in yeasts, animal cells, and plants (Friedman et al., 2011; Jaipargas et al., 2015). The resultant mitochondria fall into the size range represented in the mitochondrial cluster around peroxules. A loose ER cage has been described around chloroplasts (Schattat et al., 2011) and it has been shown that under HL the ER accumulates around chloroplasts to form an ER-chloroplast nexus (Griffing, 2011). Moreover, earlier studies have implicated the ER in peroxisome behavior and peroxule extension (Sinclair et al., 2009; Barton et al., 2014). As the ER appears to be a major contributor to organelle motility, pleomorphy, and fission, we investigated its rearrangement as a possible mechanism for the observed clustering of peroxisomes, mitochondria and chloroplasts.

Triple transgenic lines expressing YFP-PTS1, mitoGFP, and RFP-ER were created. Using chlorophyll auto-fluorescence to distinguish chloroplasts allowed simultaneous visualization of all four organelles (**Figure 3J**). In more than 60% of observations (180 frames from seven time-lapse sequences) mitochondria and peroxisomes in the vicinity of chloroplasts were drawn into an ER-chloroplast nexus following HL exposure. Use of ImageJ color thresholding and 3D surface plot functions showed that peroxisomes and peroxules existed within the clusters (**Figures 3K,L**). In each case the movement, extension and retraction of peroxules and associated mitochondria occurred in tandem with the dynamic reorganization of neighboring ER tubules (**Figure 3K**). However, the ER-chloroplast nexus reorganized into dynamic tubules a few minutes after the HL exposure, while the mitochondria-peroxisome-chloroplast association was maintained for much longer periods. This suggested that while reorganization of the ER upon exposure to HL might facilitate increased proximity between organelles, their subsequent aggregation after the ER has reverted to its normal organization may involve other membrane factor(s) that are common to and shared between the organelles.

DISCUSSION

Live imaging has revealed numerous instances where transient changes in organelle morphology are observed in response to cellular stress (Mathur et al., 2012). For instance, it has been suggested that chloroplasts extend stroma filled tubules called stromules in response to internal ROS accumulation following inhibition of the electron transport chain (pETC; Brunkard et al., 2015) and other plastids respond to increased sugar levels in a cell (Schattat and Klösgen, 2011). Similarly, the rapid fission of tubular mitochondria increases their population in a cell in response to high cytosolic sugar content, high ROS levels and light stimuli (Yoshinaga et al., 2005; Yu et al., 2006, 2008; Jhun et al., 2013; Jaipargas et al., 2015). Here we have shown that HL also induces peroxule formation from peroxisomes. In an earlier study peroxule formation was observed as a direct morphological consequence of increased H_2O_2 levels in a plant cell (Sinclair et al., 2009).

The ROS-stress based mechanism for peroxule extension (Sinclair et al., 2009) is reinforced further by the observations presented here on the Arabidopsis *any1* mutant. In comparison to the WT Arabidopsis epidermal cells, the mutant cells have increased curvature due to reduced cellulose crystallinity (Fujita et al., 2013). In general, epidermal cells in plants are known to act as lenses that, depending upon characteristics such as the cell surface area, cell wall composition and the degree and uniformity of curvature, filter, and refract the sunlight that reaches the mesophyll and other internal layers in a plant (Haberlandt, 1914; Vogelmann, 1993; Vogelmann et al., 1996). If the radius of curvature (r), is small, the light is focused with minimal scattering to the top of the mesophyll layer. Conversely, if the radius is large (i.e., the cell is relatively flat), the light gets focussed farther below the epidermis. However, the deeper the focal point of light, the greater is the scattering and hindrance of absorption (Vogelmann et al., 1996). It would therefore be expected that the chloroplasts would be exposed to a higher light intensity if the cells in the outermost epidermal layer are more spherical. One of the consequences of increased light absorbance by the chloroplasts is an increase in the levels of photorespiration associated subcellular ROS. Indeed, as observed by us the typically round and bulbous epidermal cells in *any1* exhibit higher H_2O_2 levels as compared to the WT. Expectedly this leads to the increased peroxule formation in *any1* after HL exposure.

Observations on peroxules immediately prompt questions about the source of membranes that must become available for such extensions to be formed. A general mechanism that would involve different peroxisomal membrane proteins (PMPs) including PEX11 isoforms can be suggested. Indeed, the overexpression of different PEX11 isoforms over several hours has been shown to increase peroxisome tubulation and clustering (Delille et al., 2010; Koch et al., 2010). However, as shown here peroxules become visible within less than 5 min of HL-induced ROS stress. Their quick appearance seems to preclude the longer process requiring recruitment and incorporation of different PMPs and fission factors for peroxisome proliferation. Their rapid formation suggests the availability of a source of compatible membranes from the surroundings.

Recent research involving mitochondria derived vesicles (MDVs) suggests interesting possibilities involving mitochondria and peroxisomes (Neuspiel et al., 2008; Andrade-Navarro et al., 2009; Braschi et al., 2010; Mohanty and McBride, 2013). Schematic depictions suggest that transfer of mitochondrial matrix and inter-membrane metabolites or proteins might involve mitochondrial extensions that can increase both surface area and/or physical contact with peroxisomes (Figure 1 in Schumann and Subramani, 2008). Whereas, our observations have not shown any mitochondria extensions that might validate the diagrammatic depictions of Schumann and Subramani

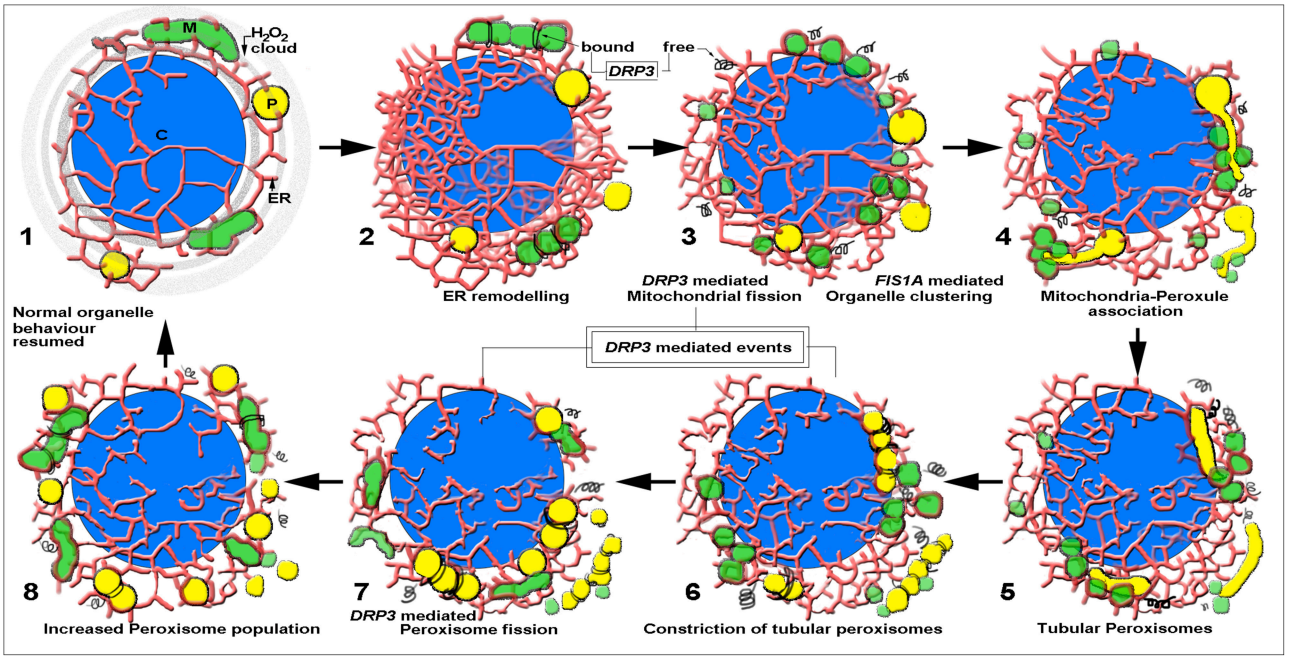

FIGURE 4 | A speculative diagrammatic depiction of the sequence of responses and interactions involving chloroplasts (C), endoplasmic reticulum (ER), mitochondria (M) and peroxisomes (P) following transient high-light (HL) induced ROS stress. 1. A chloroplast acts as a focal point for H_2O_2 release into the cell. Neighboring membranes and organelles are affected before the H_2O_2-cloud dissipates and gets neutralized. 2. ER polygons and cisternae around a chloroplast are affected by the transient increase in H_2O_2 and organize into an ER-chloroplast nexus that brings peroxisomes and mitochondria closer to the chloroplast. Mitochondria undergo fission rapidly in a DRP3 (shown as spirals) aided manner in response to increased H_2O_2/ROS. 3. The ER-chloroplast nexus disassembles but FIS1A present on chloroplast, mitochondrial and peroxisomal membranes tethers the organelles together and promotes their interactions. Note that the cytosolic concentration of free-DRP3 is speculated to increase after mitochondrial fission. 4. Small mitochondria cluster around thin dynamic peroxules extended by spherical peroxisomes. The sustained proximity might aid both non-vesicular and vesicular interactions between the organelles as well as promote the redistribution of DRP3 proteins. 5. Spherical peroxisomes are molded into tubules of nearly uniform diameter similar to that of tubular mitochondria. 6. Tubular peroxisomes become beaded as DRP3 binds and constricts at regular intervals. 7. Beaded peroxisomes undergo fission at DRP3-constricted sites through ER-aided contortions. 8. The peroxisomal population in the cell increases and aids rapid scavenging of H_2O_2/ROS to basal levels. Mitochondria resume their normal ER-mediated fusion-fission behavior. Normalcy is restored within the cell and the increased peroxisomal population fortifies the cell temporarily against harsher ROS stress.

(2008) our double transgenics do show clear peroxules that are surrounded by mitochondrial clusters.

Our observations thus strongly suggest that peroxules might act as platforms where mitochondrial outer membrane proteins might become incorporated into an existing peroxisome membrane. Whereas, we have not demonstrated the actual transfer of MDVs and their contents to peroxisomes in living cells, we have often observed the rapid thickening and elongation of peroxules that suggests such a possibility. Notably in all our experiments both mitochondria and peroxisomes are motile before HL exposure and do not appear to interact with each other beyond an occasional coincidental interaction that lasts for 1 or 2 time-lapse frames (about 6 s). Following HL exposure, however, there is a perceptible increase in mitochondria-peroxisome clusters both near and away from chloroplasts. In plants, both mitochondria and peroxisomes are linked metabolically with chloroplasts (Douce et al., 2001; Foyer and Noctor, 2003; Hayashi and Nishimura, 2003; Apel and Hirt, 2004; Ježek and Plecitá-Hlavatá, 2009). The same is true for the ER membranes that surround the different organelles. Since a short random diffusion distance of 1 μm is attributed to H_2O_2 (Halliwell and Gutteridge, 1989) all of these organelles might be expected to respond to it. One of the responses to increased ROS is the swelling of ER tubules (Margittai et al., 2008). This could lead to a more crowded ER with reduced flow characteristics that might account for the increased proximity of organelles that become enmeshed in it following HL-exposure.

However, as observed by us the change in ER dynamics and organization around chloroplasts was transient and the ER resumed its dynamic behavior after a few minutes. However, mitochondria and peroxisomes remained clustered around chloroplasts for a much longer period. We speculated that the 3-organelle cluster is maintained due to the presence of one or more proteins that is/are shared between them. FISSION1 represents such a protein. This tail-anchored membrane protein is highly conserved between different eukaryotes, exists with the same membrane topology in both peroxisomal and mitochondrial membranes, and has been shown to help recruit the DRPs to the peroxisomal and mitochondrial membrane for their eventual fission (Schumann and Subramani, 2008). Most important, in plants FIS1A has been shown to localize to peroxisomes, mitochondria and chloroplasts (Ruberti et al., 2014).

Our observations suggest a simple, presently quite speculative but testable model (**Figure 4**). As shown by us HL-induces a general increase in cellular ROS through the combined emanations from chloroplasts and other organelles. Increased mitochondrial ROS triggers their extensive fission and results in small, distressed mitochondria whose further breakdown would lead the cell into a cell death pathway. The increased subcellular ROS also has an effect on the ER and increases organelle proximity and interactivity. The close proximity possibly facilitates exchange of membranes proteins perhaps in a MDV mediated manner (Braschi et al., 2010; Mohanty and McBride, 2013). Increased availability of membranes and specific PMPs allows peroxules to attain a particular diameter that facilitates the recruitment of cytosol localized fission proteins such as DRP3A/B that might have been released following mitochondrial fission (Roux et al., 2010; Mears et al., 2011). DRP3 binding and constriction creates tubular-beaded peroxisomes and eventually results in peroxisome proliferation.

While details of this rather simplistic interpretation of HL induced events aimed at combatting ROS stress in a plant cell continue to be assessed critically, the work presented here clearly provides visual proof of sustained interactions between peroxisomes and mitochondria in living plant cells with peroxules acting as transient interaction platforms.

AUTHOR CONTRIBUTIONS

EJ and JM designed experiments and co-wrote the manuscript. NM and FD provided expert help with experiments and plant materials. GW provided seeds of *any1* mutant. All authors contributed critical comments and corrections and have approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00006

Figure S1 | A comparison of predominant mitochondrial size between dark grown plants of *Arabidopsis thaliana* ecotype Columbia "A" and the *any1* mutant "B" expressing GFP targeted to mitochondria. Size bar applies for **(A,B)** = 5 μm.

Movie S1 | Time-lapse sequence taken over 2 min of a portion of a cell from a low- light (60 μmol m^{-2} s^{-1}) grown double transgenic Arabidopsis seedling showing the general sub-cellular motility and lack of sustained association between chloroplasts (depicted in blue), mitochondria (green), and peroxisomes (yellow).

Movie S2 | The close association of mitochondria (green), peroxisomes (yellow) and a single chloroplast in a cell exposed to HL for 4-min and then tracked over approximately 7.5 min. Note that the association between the 3-peroxisomes on the plastid is maintained throughout while mitochondria appear to have relatively short but sustained contacts with the other two organelles.

Movie S3 | A time-lapse sequence taken over nearly 5.5 min shows mitochondria (green) and their strong but transient association with long peroxules (orange–yellow) extended from a chloroplast-attached peroxisome and an independent peroxisome (top left).

Movie S4 | A time-lapse sequence taken over 6 min of a cotyledon cell in the *any1* mutant expressing a peroxisomal targeted YFP shows numerous peroxules being extended and retracted in response to HL exposure for 4-min.

Movie S5 | Portion of a single cotyledon cell from a double transgenic *any1* seedling exposed to HL for 4-min exhibits numerous chloroplasts (blue) surrounded by small, punctate mitochondria (green), and peroxisomes (orange–yellow) with the latter extending and retracting 8–15 μm long peroxules.

Movie S6 | Portion of a cell in an *any1* plant double transgenic for YFP-PTS1-highlighted peroxisomes (yellow) and GFP-targeted mitochondria (green) observed over nearly 5 min shows organelle association with a chloroplast (auto-fluorescent chlorophyll depicted in blue). A peroxisome becomes closely associated with the chloroplast after a 4-min HL treatment and repeatedly extends and retracts thin, tubular peroxules that interact transiently with mitochondria in their vicinity.

Movie S7 | A portion of a cell from a triple transgenic Arabidopsis plant expressing YFP- PTS1 (peroxisomes-yellow), mitoGFP (green mitochondria) and RFP-ER (red) shows the phenomenon of aggregation around some chloroplasts (blue) after a 4-min HL illumination.

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REVIEW
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Redox interplay between mitochondria and peroxisomes

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Reduction-oxidation or "redox" reactions are an integral part of a broad range of cellular processes such as gene expression, energy metabolism, protein import and folding, and autophagy. As many of these processes are intimately linked with cell fate decisions, transient or chronic changes in cellular redox equilibrium are likely to contribute to the initiation and progression of a plethora of human diseases. Since a long time, it is known that mitochondria are major players in redox regulation and signaling. More recently, it has become clear that also peroxisomes have the capacity to impact redox-linked physiological processes. To serve this function, peroxisomes cooperate with other organelles, including mitochondria. This review provides a comprehensive picture of what is currently known about the redox interplay between mitochondria and peroxisomes in mammals. We first outline the pro- and antioxidant systems of both organelles and how they may function as redox signaling nodes. Next, we critically review and discuss emerging evidence that peroxisomes and mitochondria share an intricate redox-sensitive relationship and cooperate in cell fate decisions. Key issues include possible physiological roles, messengers, and mechanisms. We also provide examples of how data mining of publicly-available datasets from "omics" technologies can be a powerful means to gain additional insights into potential redox signaling pathways between peroxisomes and mitochondria. Finally, we highlight the need for more studies that seek to clarify the mechanisms of how mitochondria may act as dynamic receivers, integrators, and transmitters of peroxisome-derived mediators of oxidative stress. The outcome of such studies may open up exciting new avenues for the community of researchers working on cellular responses to organelle-derived oxidative stress, a research field in which the role of peroxisomes is currently highly underestimated and an issue of discussion.

Keywords: antioxidant systems, interorganellar cross-talk, mitochondria, oxidative stress, peroxisomes, pro-oxidant systems, redox signaling

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Abbreviations: ACOX, acyl-CoA oxidase; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPHX, epoxide hydrolase; ETC, electron transport chain; 4-HNE, 4-hydroxy-2-nonenal; GLRX, glutaredoxin; GPX, glutathione peroxidase; GSH, reduced glutathione; GSR, oxidized glutathione reductase; GSSG, oxidized glutathione; MAM, mitochondria-associated membrane; MDA, malondialdehyde; mPTP, mitochondrial permeability transition pore; NOS, nitric oxide synthase; NOX, NADPH oxidase; OXPHOS, oxidative phosphorylation; PUFA, polyunsaturated fatty acid; PRDX, peroxiredoxin; RCC, respiratory chain complex; RIRR, ROS-induced ROS release; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TRX, thioredoxin; TSC, tuberous sclerosis complex; TXNRD, thioredoxin reductase; VLCFA, very-long-chain fatty acid; X-ALD, X-linked adrenoleukodystrophy; XDH, xanthine dehydrogenase.

Introduction

All life on earth is powered by reduction-oxidation (redox) reactions, in which electrons are transferred from a donor to an acceptor molecule. To survive, cells had to evolve mechanisms to control redox potential intervals in which biological processes and signaling pathways can take place (Foyer and Noctor, 2011). To cope with this challenge, cells have developed spatially compartmentalized redox circuits that are regulated by various small molecule- and protein-based redox buffer systems (Forman et al., 2010; Mallikarjun et al., 2012). As organisms need to sense and respond to changing environmental conditions, these circuits have to be sufficiently flexible to (locally) respond to exogenous stimuli and endogenous metabolic alterations. Shifts in the intracellular redox equilibrium may favor either beneficial or detrimental outcomes (**Figure 1**). The outcomes are determined by a combination of factors, including the types of oxidants produced, their concentration and localization, and their kinetics of production and elimination (Trachootham et al., 2008; Forman et al., 2010). A shift of the redox equilibrium in favor of oxidized biomolecules gives rise to a phenomenon called "oxidative stress." High levels of oxidative stress are capable of causing damage to all major biomolecules and of initiating cell death (Jones and Go, 2010). However, low levels of oxidative stress may promote cell proliferation and survival pathways (Holmström and Finkel, 2014). As such, it is not surprising to see that changes in the cellular redox environment significantly contribute to the development of virtually all major chronic human disorders, including atherosclerosis, cancer, diabetes, and neurodegeneration (Groitl and Jakob, 2014; Holmström and Finkel, 2014).

Redox signaling refers to the concept that electron-transfer processes play a key messenger role in biological systems (Rigas and Sun, 2008; Burgoyne et al., 2012). Cells produce two different types of redox signaling molecules: the first type comprises reactive oxygen species (ROS), such as the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$); the second type covers reactive nitrogen species (RNS), such as the nitric oxide radical ($^{\bullet}NO$), the nitrogen dioxide radical ($^{\bullet}NO_2$), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$) (Nathan and Ding, 2010). Oxidative modifications of specific target molecules by various ROS/RNS are covalent but often reversible. The best studied reversible oxidation reactions include those of H_2O_2 with sulfhydryl groups (RSH) to yield disulfides (RSSR'), sulfenic acids (RSOH), or sulfinic acids (RSO_2H), and of $^{\bullet}NO$ with RSH to yield S-nitrosothiols (RSNO) (Nathan and Ding, 2010). Irreversible oxidation products most frequently include hydroxylations, carbonylations, nitrations, the formation of sulfonic acids (RSO_3H), and the destruction of iron-sulfur (FeS) clusters (Nathan and Ding, 2010). As both protein cysteine thiols and lipids are among the most prominent targets of ROS/RNS (Trachootham et al., 2008; Hekimi et al., 2011), many biologically-relevant redox signals are conveyed through cysteine oxidation and lipid peroxidation. Here, it is of particular importance to be aware that (i) many signaling components like kinases, phosphatases, transcription factors, caspases, and metalloproteases contain active site- or zinc finger-coordinating cysteines that can be reversibly modified in a redox-responsive manner (Forman et al., 2010; Corcoran and Cotter, 2013; Berridge, 2014), and (ii) multiple lipid peroxidation products [e.g., malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE)] can act as important messengers in signaling events that lead to cell proliferation, differentiation, senescence, or apoptosis (Fritz and Petersen, 2013; Ayala et al., 2014). Finally, to counteract oxidative stress, cells are also equipped with various antioxidant defense systems. These systems can be classified into two broad categories: the enzymatic antioxidants [e.g., superoxide dismutases (SODs), catalase, glutathione peroxidases and reductases, peroxiredoxins, etc.) and the low molecular weight antioxidants [e.g., reduced glutathione (GSH), ascorbate (vitamine C), α-tocopherol (vitamin E), etc.] (Nathan and Ding, 2010).

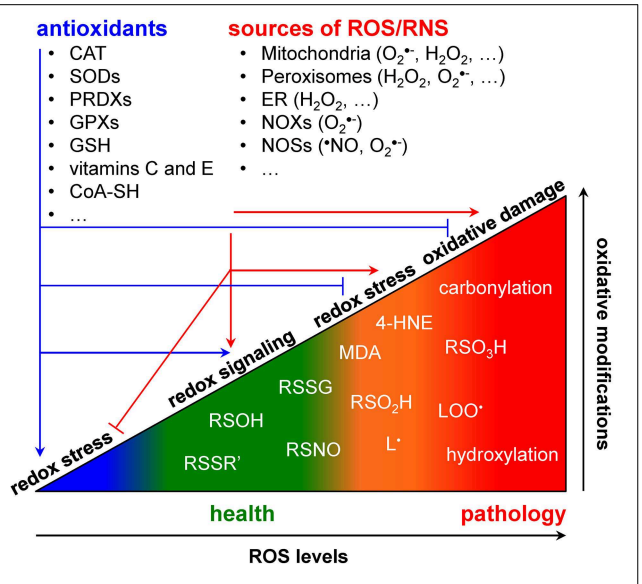

FIGURE 1 | ROS/RNS, antioxidants, and cellular redox balance in health and disease.

Major sites of cellular ROS/RNS production include mitochondria, peroxisomes, the endoplasmic reticulum (ER), and the NADPH oxidases (NOXs) and nitric oxide synthases (NOSs) that are located in distinct subcellular locations (Trachootham et al., 2008; Fransen et al., 2012). In the following two sections, we will review the pro- and antioxidant systems of mitochondria and peroxisomes, with a focus on the situation in mammals. For a detailed description of the other systems, the reader is referred to other reviews (Bedard and Krause, 2007; Appenzeller-Herzog, 2011; Förstermann and Sessa, 2012).

The Redox Metabolism of Mitochondria

Pro-oxidant Systems

Mitochondria are key players in cellular redox metabolism. Important sources of mitochondrial ROS include (i) complex I and complex III of the electron transport chain (ETC) in the inner mitochondrial membrane, (ii) dihydrolipoamide dehydrogenase (DLD), a common subunit of the α-ketoglutarate dehydrogenase

and pyruvate dehydrogenase multi-enzyme complexes, in the mitochondrial matrix (Andreyev et al., 2005; Circu and Aw, 2010), and (iii) monoamine oxidase, a mitochondrial outer membrane-resident flavoprotein (Andreyev et al., 2005; Orrenius et al., 2007). Monoamine oxidase is an important source of H_2O_2 (Orrenius et al., 2007). Complex I, complex III, and dihydrolipoamide dehydrogenase contain redox centers that are potentially capable of $O_2^{\bullet-}$ production (Andreyev et al., 2005). Importantly, while complex I can only produce $O_2^{\bullet-}$ in the mitochondrial matrix, complex III can release this radical on both sides of the mitochondrial inner membrane (Orrenius et al., 2007). Excessive production of $O_2^{\bullet-}$ may not only lead to the SOD-catalyzed formation of H_2O_2, but also cause the release of Fe^{2+} from FeS-containing proteins (e.g., complex I, aconitase) (Dixon and Stockwell, 2014). These events may in turn give rise to $^{\bullet}OH$ via the non-enzymatic Haber-Weiss and Fenton reactions (Winterbourn, 1995) and initiate a cascade of reactions resulting in the formation of carbon-centered lipid radicals ($L^{\bullet}$), lipid peroxide radicals ($LOO^{\bullet}$), and multiple lipid peroxidation products such as MDA and 4-HNE (Ayala et al., 2014). The role of mitochondria in cellular RNS production is less well documented. However, here it is worthwhile to mention that the mitochondrial inner membrane contains at least three $NO^{\bullet}$-producing enzymes: a posttranslationally modified splice variant of neuronal NOS (NOS1α), called mitochondrial NOS (mtNOS) (Ghafourifar and Richter, 1997; Aguirre et al., 2012); and two molybdopterin-containing amidoxime-reducing enzymes, called MARC1 and MARC2 (Sparacino-Watkins et al., 2014). Wheareas, in general, NOSs oxidize L-arginine with O_2 to form citrulline and $^{\bullet}NO$, molybdopterin enzymes have the capacity to reduce NO_2^- to $^{\bullet}NO$ (Sparacino-Watkins et al., 2014).

Antioxidant Systems

Mitochondria also contain a network of enzymatic and non-enzymatic antioxidants that protect the organelle from oxidative damage. The main antioxidant enzymes include thioredoxin 2 (TRX2), thioredoxin reductase (TXNRD2), the glutaredoxins 2 (GLRX2) and 5 (GLRX5), the peroxiredoxins 3 (PRDX3) and 5 (PRDX5), GSH peroxidase 1 (GPX1), oxidized glutathione (GSSG) reductase (GSR), and the copper/zinc (SOD1)- and manganese (SOD2)-containing SODs (Kalinina et al., 2008). The major non-enzymatic antioxidants are GSH, coenzyme A (CoA-SH; for more details, see The Redox Metabolism of Peroxisomes - Antioxidant Systems), ubiquinol (Maroz et al., 2009), vitamin C, and vitamin E (Sagun et al., 2005; Marí et al., 2009; Lauridsen and Jensen, 2012). Vitamin E, a fat-soluble nutrient, is present in relatively low concentrations in mitochondria, and its main function is to trap $LOO^{\bullet}$, thereby preventing the propagation of lipid peroxidation (Forkink et al., 2010). GSH and vitamin C, two hydrophilic antioxidants, can directly recycle vitamin E to its reduced active form. GSH is the most important low molecular weight thiol (∼1-6 μmol/g tissue), and its concentration in mitochondria is estimated at ∼10–14 mM, similar to the cytosolic levels. GSH can also directly neutralize $^{\bullet}OH$ and function as a cofactor of GPX1 to scavenge H_2O_2 and lipid peroxides (LOOH) (Forkink et al., 2010). The regeneration of GSH from GSSG is carried out by GSR, an NADPH-consuming enzyme. Note that, as GSH is only synthesized *de novo* in the cytosol, mitochondria have to import this molecule across their inner membrane (Marí et al., 2009). An alternative mechanism to convert mitochondrial H_2O_2 to H_2O involves the oxidation of PRDX3 or PRDX5. The oxidized forms of these peroxiredoxins are subsequently reduced by TRX2, which in turn is regenerated by TXNRD2, an NADPH-dependent FAD-containing enzyme (Forkink et al., 2010). Two comments should be added at this point. First, the GSH/GPX/GLRX and PRDX/TRX/TXNRD redox pathways are considered to be the most important redox regulating systems in mitochondria (Murphy, 2012). Second, as (i) GSR and TXNRD2 receive their reducing equivalents from the mitochondrial NADPH pool, and (ii) the pool of NADPH is kept reduced by the tricarboxylic acid (TCA) enzymes nicotinamide nucleotide transhydrogenase (NNT), malic enzyme 3 (ME3), and isocitrate dehydrogenase 2 (IDH2), it is clear that a functional TCA cycle is essential for the regeneration of the antioxidant capacity of the mitochondrial matrix (Kohlhaas and Maack, 2013).

Metabolic Factors Affecting ROS Production

The net release of ROS from mitochondria strongly depends on the (patho) physiological state of the cell. For example, according to the classical concept, the rate of ROS production from the ETC increases when substrates (e.g., glucose, fatty acids) are available, but energy consumption is low (Kohlhaas and Maack, 2013). Indeed, when TCA-derived high-energy electron carriers NADH and FADH2 donate more electrons to the ETC and mitochondria are not making ATP, the ETC is highly reduced and electrons are more likely to slip to O_2 to produce $O_2^{\bullet-}$ (Kohlhaas and Maack, 2013). In addition, also stress situations causing impairment and uncoupling of specific respiratory chain complexes (RCCs) may provoke the formation of free radicals (Schönfeld and Wojtczak, 2008; Marí et al., 2009).

The Redox Metabolism of Peroxisomes

Pro-oxidant Systems

As peroxisomes contain large sets of pro- and antioxidant enzymes, also these organelles have the potential to play a significant role in cellular redox metabolism and signaling. The most abundant class of ROS-producing enzymes inside peroxisomes are the flavin-containing oxidases, which reduce O_2 to H_2O_2 [for a detailed overview of these enzymes, the reader is referred to (Antonenkov et al., 2010)]. Depending on the organism, the tissue and cell type, and the cellular environment, peroxisomes also contain two potential sources of $O_2^{\bullet-}$ and $^{\bullet}NO$ production: xanthine dehydrogenase (XDH) and the inducible form of NOS (NOS2) (Angermüller et al., 1987; Stolz et al., 2002; Loughran et al., 2013). XDH is a key enzyme in the purine degradation pathway that catalyzes the conversion of hypoxanthine to xanthine and of xanthine to uric acid, a potent antioxidant and free radical scavenger (Nishino et al., 2008). However, a select set of posttranslational modifications (e.g., sulfhydryl oxidation, proteolytic processing) can rapidly convert the NAD^+-dependent dehydrogenase form of the enzyme to an oxidase form that catalyzes the reduction

of O_2 to $O_2^{\bullet-}$ (Nishino et al., 2008). As XDH, like MARC1 and MARC2 (see The Redox metabolism of Mitochondria - Pro-oxidant Systems), is a molybdopterin-containing enzyme, it can also reduce nitrates and NO_2^- to $NO^\bullet$ (Harrison, 2002). NOS2 is a homodimeric heme-containing enzyme that normally catalyzes the oxidation of L-arginine to $NO^\bullet$ and citrulline in a complex reaction requiring O_2, NADPH, tetrahydrobiopterin (BH4), FMN, and FAD (Del Río, 2011). However, in the absence of substrate or in its monomeric form, the enzyme can also produce significant amounts of $O_2^{\bullet-}$ (Stuehr et al., 2001). Interestingly, the peroxisomal pool of NOS2 appears to be monomeric (Loughran et al., 2005). Finally, as (i) $NO^\bullet$ may rapidly combine with $O_2^{\bullet-}$ to form $ONOO^-$ (Pacher et al., 2007), (ii) within the heme protein-rich environment of peroxisomes, H_2O_2 may give rise to $^\bullet OH$ through the Fenton reaction (Loughran et al., 2005), and (iii) $^\bullet OH$ is one of the prime catalysts for the initiation of lipid peroxidation (Ayala et al., 2014), it is very likely that peroxisomes also function as potential sources of $ONOO^-$, $^\bullet OH$, $L^\bullet$, $LOO^\bullet$, MDA, and 4-HNE (Ayala et al., 2014).

Antioxidant Systems

Like mitochondria, peroxisomes are also well equipped with multiple enzymatic and non-enzymatic antioxidant defense systems that scavenge harmful H_2O_2 and free radicals, thereby protecting the organelle from oxidative stress. The best characterized peroxisomal antioxidant enzyme is catalase, a heme-containing enzyme that can remove H_2O_2 in a catalatic ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) and peroxidatic ($H_2O_2 + AH_2 \rightarrow A + 2\ H_2O$) manner (Kirkman and Gaetani, 2007). Typical peroxidatic electron donors (AH_2) are low molecular weight alcohols, formate, nitrite, and formaldehyde (Kirkman and Gaetani, 2007). Other antioxidant enzymes include SOD1, PRDX5, glutathione transferase kappa (GSTK1), "microsomal" glutathione S-transferase 1 (MGST1), and epoxide hydrolase 2 (EPHX2). As already mentioned above, SOD1 can convert $O_2^{\bullet-}$ to O_2 and H_2O_2 (see The Redox metabolism of Mitochondria - Pro-oxidant Systems), and PRDX5 can reduce H_2O_2 to H_2O (see The Redox metabolism of Mitochondria - Antioxidant Systems). PRDX5 can, in addition, also reduce alkyl hydroperoxides (ROOH) to their respective alcohols, and $ONOO^-$ to NO_2^- (Knoops et al., 2011). GSTK1 and MGST1 are thought to play a role in LOOH detoxification processes (Antonenkov et al., 2010; Johansson et al., 2010; Wang et al., 2013b), EPHX2 can convert epoxides to the corresponding dihydrodiols (Decker et al., 2009), and peroxisomal PRDX5 has recently been shown to exert a cytoprotective function against H_2O_2- and LOOH-induced oxidative stress (Walbrecq et al., 2015). For a detailed description of these enzymes, the reader is referred to other reviews (Antonenkov et al., 2010; Fransen et al., 2012). Interestingly, there is some indirect evidence that also GSH and vitamin C may play a role in the regulation of the peroxisomal redox state. Indeed, as the peroxisomal membrane contains a nonselective pore-forming protein (PXMP2) with an upper molecular size limit of 300-600 Da (Rokka et al., 2009), these low molecular weight antioxidants can most likely freely diffuse through the peroxisomal membrane. The observations that (i) a peroxisomal variant of roGFP2, a genetically-encoded redox sensor that specifically equilibrates with the GSSG/GSH redox pair, quickly responds to redox changes in the peroxisomal matrix, and (ii) supplementation of vitamin C to the cell culture medium causes an increase in the intraperoxisomal redox state, are in line with this hypothesis (Ivashchenko et al., 2011). However, the precise mechanisms underlying the latter, rather unexpected observation remain to be unraveled. Nevertheless, in the context of this review, it is tempting to speculate that, in a heme-rich environment such as the peroxisomal matrix, vitamin C can reduce Fe^{3+} to Fe^{2+}, and that this further drives the generation of free radicals through the Fenton reaction. Note also that it is not yet clear how GSSG (molar mass, 612.63 g mol^{-1}) can be reduced in or exported out of the peroxisomal matrix. Here it is important to point out that, in contrast to mitochondria (see The Redox metabolism of Mitochondria - Antioxidant Systems), peroxisomal thioredoxins and glutaredoxins have not yet been identified in mammals. Finally, it should be noted that—although peroxisomes contain (i) enzymes that can produce and consume $NAD(P)^+$ and NAD(P)H (Visser et al., 2007), and (ii) shuttle systems that permit the transfer of reducing equivalents without physical exchange of these redox cofactors between the lumen of the organelle and the cytoplasm (Rottensteiner and Theodoulou, 2006; Antonenkov et al., 2010; Schueren et al., 2014)—it is also not yet clear how changes in peroxisomal $NAD(P)^+$ and NAD(P)H metabolism influence the intra- and extraperoxisomal redox state.

Metabolic Factors Affecting ROS Production

So far, no consensus has been reached about whether peroxisomes function as a net source or sink of ROS/RNS (Fransen et al., 2013). However, as for mitochondria, this most likely depends on the (patho) physiological state and growth environment of the cell. This idea is in line with the observation that the intraperoxisomal redox state is strongly influenced by various genetic and environmental factors (Ivashchenko et al., 2011). In the following subsections, we further discuss how peroxisomal metabolism of fatty acids, acyl-CoA esters, and plasmalogens may impact on cellular redox state alterations. Not unexpectedly, most of these lipids are linked to two of the major metabolic pathways in peroxisomes: β-oxidation and plasmalogen synthesis.

Fatty Acids and acyl-CoA esters

Mammalian genomes code for at least three functional peroxisomal acyl-CoA oxidases (ACOX1, ACOX2, ACOX3), and evidence exists for a fourth gene (*ACOXL*) (Van Veldhoven, 2010). Additionally, the *ACOX1* gene gives rise to two transcripts via alternative splicing, and this splicing is conserved in eukaryotes (Morais et al., 2007). Through these different ACOXs, combined with an uptake mechanism for CoA-esters that is not controlled and restricted by a carnitine-acylcarnitine translocase as in mitochondria (Rubio-Gozalbo et al., 2004), peroxisomes can β-oxidize a broad range of carboxylates, including medium-, long-, and very-long-chain fatty acids, mono- and polyunsaturated fatty acids, pristanic acid and other 2-methyl-branched fatty acids, as well as carboxylates with a bulky or rigid ω-end such as prostanoids, bile acid intermediates

and xenobiotics, either with or without an α-methyl group (Van Veldhoven, 2010). Moreover, these organelles can degrade carboxylates containing a 3-methyl or 2-hydroxy group via α-oxidation (Van Veldhoven, 2010), whereby the one-carbon shortened products can be passed onto the β-oxidation system. Exposure of cells to carboxylates (or their precursors) that are desaturated by these ACOXs will generate peroxisomal H_2O_2. This has been shown in different systems [e.g., cells, perfused organs (Foerster et al., 1981; Handler and Thurman, 1987), and intact animals (Van den Branden et al., 1984)] and with different types of carboxylates [e.g., medium-chain fatty acids (Skorin et al., 1992); long-chain saturated and mono- and polyunsaturated fatty acids (Mannaerts et al., 1979; Foerster et al., 1981; Chu et al., 1995; Okamoto et al., 1997)]; medium-chain dicarboxylic acids (Leighton et al., 1989); and xenobiotics such as *N*-(α-methylbenzyl)azelaamic acid (Yamada et al., 1986; Suzuki et al., 1990), ω-phenyl-substituted fatty acids (Yamada et al., 1987), and PCA16, a metabolite of the cytosine arabinoside antileukemic prodrug YNKO (containing a stearic acid side chain) (Yoshida et al., 1990).

The amount of H_2O_2 produced depends strongly on the chain length of the fatty acids. For example, when comparing fatty acids ranging from C8:0 to C18:0 in isolated rat hepatocytes, a maximal activity is observed around C10:0-C12:0 (Yamada et al., 1987; Suzuki et al., 1990; Skorin et al., 1992). Similarly, ω-phenyldodecanoic acid (Yamada et al., 1987) and dodecanedioic acid (Leighton et al., 1989) are optimal compared to their analogs. Finally, monounsaturated oleic acid is a better H_2O_2-source than palmitic acid in rat hepatocytes (Mannaerts et al., 1979) and perfused rat liver (Foerster et al., 1981). In mice, starvation increases the H_2O_2 production by liver, likely due to increased fatty acid plasma levels (Van den Branden et al., 1984). However, in cardiac tissue there is no change (Kerckaert and Roels, 1986). Treatment with fibrates increases the rate of H_2O_2 production in hepatocytes (Mannaerts et al., 1979; Foerster et al., 1981; Yamada et al., 1987; Leighton et al., 1989; Yoshida et al., 1990).

For various fatty acids linked to peroxisomal metabolism, an influence on ROS levels has been reported. Examples include (i) phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a dietary fatty acid that is degraded via peroxisomal α-oxidation, (ii) pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), the breakdown product of phytanic acid, that is degraded via β-oxidation, (iii) polyunsaturated fatty acids (PUFAs) that are produced via the retroconversion pathway, and (iv) VLCFAs that are shortened via β-oxidation (Van Veldhoven, 2010). Interestingly, long-chain fatty acids that are normally degraded by mitochondria have been shown to be toxic to insulin-producing cells (e.g., RINm5F, INS-1E, and primary rat islet cells), due to the combined effect of increased peroxisomal H_2O_2 generation and the intrinsically low activity of catalase in these cells (Gehrmann et al., 2010).

Plasmalogens

An important class of ROS-protecting lipids are plasmalogens. This class of phospholipids contains a vinyl ether bond at position one of the glycerol moiety. The initial steps of their biosynthesis are confined to peroxisomes: glyceronephosphate O-acyltransferase (GNPAT) acylates dihydroxyacetone-phosphate, while alkylglycerone-phosphate synthase (AGPS) replaces the acylgroup by a fatty alcohol (Braverman and Moser, 2012). Subsequently, the 2-oxogroup is reduced by 1-alkylglycerone-phosphate reductase (DHRS7B), an enzyme found in the membrane of both peroxisomes and the ER (Keller et al., 2009; Lodhi et al., 2012), thereby generating 1-alkylglycero-3-phosphate which will undergo further metabolic conversions in the ER, similar to those of 1-acylglycero-3-phosphate [the precursor of esterglycero(phospho)lipids]. Finally, the double bond adjacent to the ether bond is introduced in the ER (Nagan and Zoeller, 2001). At the sn-2 position, plasmalogens are generally enriched in PUFAs. In some tissues (e.g., brain, testis, and heart), a substantial portion (10–30%) of the phospholipids (especially the ethanolamine-phospholipids) are plasmalogens. The absence of plasmalogens causes a very specific phenotype in man, described as rhizomelic chondrodysplasia punctata (dwarfism, shortening of proximal limbs, etc.), but how these symptoms are linked to plasmalogens is not clear (Braverman and Moser, 2012).

The vinyl-ether bond makes plasmalogens sensitive to attack by different ROS-species, both *in vitro* and *in cellulo*. Hence, one can consider them as (lipophilic) antioxidants (Lessig and Fuchs, 2009), often comparable to tocopherol with regard to potency. *In vitro*, plasmalogens delay the oxidative degradation of PUFAs as good as vitamin E (Engelmann et al., 1994; Reiss et al., 1997; Hahnel et al., 1999a), most likely due to the fact that the vinyl-ether bond can scavenge peroxy radicals and oxidized PUFA products. As this bond can complex with Cu^{2+}, plasmalogens also attenuate Cu^{2+}-induced lipid oxidation (Hahnel et al., 1999b). Depending on the type of oxidative stress, different metabolites can be formed. For example, while UV light-induced oxidation of plasmalogens generates aldehydes via dioxetane intermediates, Fe^{2+}/ascorbate treatment results in the formation of α-hydroxy-aldehydes via plasmalogen epoxides (Stadelmann-Ingrand et al., 2001). Plasmalogens do protect cells against chemical hypoxia induced by antimycin A or cyanide (by scavenging produced ROS), as shown in the murine monocyte/macrophage cell line RAW 264.7 (Zoeller et al., 1999) and human pulmonary arterial endothelial cells (PAEC) (Zoeller et al., 2002). During UV-exposure of Chinese hamster ovary (CHO) cells photosensitized with ω-pyrene-substituted fatty acids or Merocyanine 540, plasmalogens disappear (Zoeller et al., 1988). This is most likely due to the fact that singlet oxygen converts the vinyl-ether bond into a dioxetane intermediate that subsequently decomposes into a 2-lysophospholipid, formic acid, and an n-1 aldehyde (Zoeller et al., 1988). Another ROS-molecule that is scavenged by plasmalogens is hypochlorous acid (HOCl). This reactive chlorinating species is produced by myeloperoxidase and promotes the selective cleavage of plasmalogens into 1-lysophosphatidylcholine and 2-chloro-fatty aldehydes. This has been shown *in vitro* (Albert et al., 2001; Messner et al., 2006; Skaff et al., 2008; Ullen et al., 2010), in activated neutrophils (Thukkani et al., 2002) and monocytes (Thukkani et al., 2003), and in mouse brain with lipopolysacharide-induced neuroinflammation (Ullen et al., 2010). Likewise, 2-bromo-fatty aldehydes are produced by stimulated neutrophils (Albert et al., 2002) or eosinophils (Albert et al., 2003). Note that 2-halo-fatty aldehydes, being

chemoattractants for phagocytes and stimulators of expression of phagocyte tethering proteins in endothelial cells, sustain the inflammatory response. Finally, in thyroid cells, plasmalogens are susceptible to iodine attack. This results in the formation of 2-iodo-fatty aldehydes (Panneels et al., 1996), a major thyroid iodolipid that—similar to iodide—regulates thyroid metabolism. Cells with higher plasmalogen levels are more resistant to H_2O_2, hyperoxia, and the $O_2^{\bullet-}$ generator plumbagin (Zoeller et al., 2002), whereas the protective actions are gone in cells lacking plasmalogens. Photosensitisized plasmalogen-deficient CHO cells and mouse embryonic fibroblasts are hypersensitive to light treatment (Zoeller et al., 1988; Wang et al., 2013b), and plasmalogen-deficient RAW 264.7 cells are more sensitive to electron transport inhibitors (Zoeller et al., 1999). However, plasmalogens are apparently not important to protect cells against lactic acid-induced oxidative stress, at least not in primary rat astrocytes (Fauconneau et al., 2001).

To conclude this subsection, it should be noted that plasmalogen-derived oxidation products, and especially 2-hydroxy-fatty aldehydes (Liu and Sayre, 2003; Stadelmann-Ingrand et al., 2004) and 2-halo-fatty aldehydes (Stadelmann-Ingrand et al., 2004; Wildsmith et al., 2006), are reactive molecules that can modify amino groups of lipids and proteins (via Schiff base formation) as well as sulfhydryl groups in proteins. This probably contributes to their short half-life in cerebral cortex homogenates (Stadelmann-Ingrand et al., 2001). In addition, oxidation of 2-chloro-fatty aldehydes to 2-chloro-fatty acids leads to increased ROS, ER-stress, and finally apoptosis in activated primary human monocytes, THP-1 human monocytes, and RAW 264.7 mouse macrophages (Wang et al., 2013a). Together with the observations that (i) α-hydroxy fatty aldehydes and plasmalogen epoxides accumulate in aged brain and chronic disorders (Lessig and Fuchs, 2009), and (ii) 2-chloroaldehyde levels are elevated in atherosclerotic plaques and infarcted rat myocardium (Ford, 2010), these findings question the scavenger role of plasmalogens. However, it might be that the pathways degrading these oxidative metabolites are less active under these conditions.

Mitochondria as Redox Signaling Nodes

Currently, it is widely accepted that redox signals to and from mitochondria are at the core of a wide variety of biological processes, including cell proliferation and differentiation, adaptation to hypoxia, autophagy, immune function, and hormone signaling (**Figure 2**) (Collins et al., 2012; Chandel, 2014). The most studied and best characterized mitochondrial redox signaling molecule is H_2O_2, which is relatively stable *in vivo* and can pass easily through mitochondrial membranes (Bienert et al., 2006). For example, it has been shown that, under physiological hypoxia, mitochondrial H_2O_2 can stabilize hypoxia-inducible factor 1α (HIF-1α), a transcription factor playing a key role in the cellular adaptation to oxygen availability (Chandel et al., 1998). In addition, also other transcription factors (e.g., FOXO3A, NF-kB, p53, and PGC-1α) and signaling components (e.g., c-Jun N-terminal kinase, protein tyrosine phosphatases, cysteine protease Atg4, the mitochondrial peroxiredoxins, the NLRP3 inflammasome, etc.) have been identified as targets of mitochondrial H_2O_2 (Chandel et al., 2000a,b; Nemoto et al., 2000; Valle et al., 2005; Scherz-Shouval et al., 2007; Chiribau et al., 2008; Cox et al., 2010; Zhou et al., 2011; Chae et al., 2013; Frijhoff et al., 2014; Long et al., 2014; Marinho et al., 2014). However, although it is well known that H_2O_2 can selectively modify proteins containing cysteine residues with a low pKa (Veal et al., 2007), the precise mechanisms by which mitochondria-derived H_2O_2 coordinates or relays (retrograde) signaling events thereby provoking adaptive or maladaptive responses are not yet entirely clear (Forkink et al., 2010). Two main models have been proposed: (i) in the redox relay model, H_2O_2 scavenging enzymes are first oxidized and subsequently transfer the oxidative equivalents to other target proteins (Toledano et al., 2004); and (ii) in the floodgate model, scavenging enzymes act as molecular floodgates, keeping H_2O_2 away from susceptible targets under basal conditions, but permitting signaling events to occur at H_2O_2 thresholds sufficient to inactivate the scavenging enzymes (Wood et al., 2003).

Another important class of redox signaling molecules are cardiolipins, which are virtually exclusively localized in the inner mitochondrial membrane (Ren et al., 2014). Most cardiolipin species have four unsaturated acyl chains that are oxidation-sensitive. Currently, it is well documented that—upon mitochondrial injury and depolarization—a significant portion of these lipids is externalized to the mitochondrial surface where they function as an "eat-me"-signal for the autophagic machinery (Chu et al., 2013). In addition, detailed studies have established that cardiolipin peroxidation is a critical first step in cytochrome c release and apoptosis (Kagan et al., 2014). The precise mechanisms through which peroxidized cardiolipin species fulfill their action remain to be established, but most likely they facilitate outer mitochondrial membrane permeabilization (Kagan et al., 2014; Raemy and Martinou, 2014).

Over the years, it has become clear that mitochondria are central integrators and transducers for ROS signals from other cellular sources (Sena and Chandel, 2012). In this context, it is interesting to briefly discuss the concept of ROS-induced ROS release (RIRR), a phenomenon wherein mitochondria respond to elevated ROS concentrations by increasing their own ROS production (Zorov et al., 2000). RIRR is a mechanism for ROS amplification and regional ROS generation. The process involves the opening of two different channels in the mitochondrial inner membrane: the mitochondrial permeability transition pore (mPTP) and the inner membrane anion channel (IMAC) (Aon et al., 2009; Zorov et al., 2014). Opening of the pores (e.g., upon elevated Ca^{2+} or ROS levels) may, among other responses [e.g., a dissipation of the mitochondrial inner membrane potential ($\Delta\Psi m$), a ceased production of ATP, etc.], elicit ROS bursts. Depending on the extent of pore opening and how fast ROS released from mitochondria are eliminated by intracellular antioxidant systems, RIRR may (i) constitute an adaptive housekeeping mechanism to release accumulated toxic levels of mitochondrial ROS, (ii) activate pools of redox-sensitive proteins in the vicinity of mitochondria, (iii) trigger RIRR in neighboring mitochondria, and (iv) lead to the destruction of mitochondria,

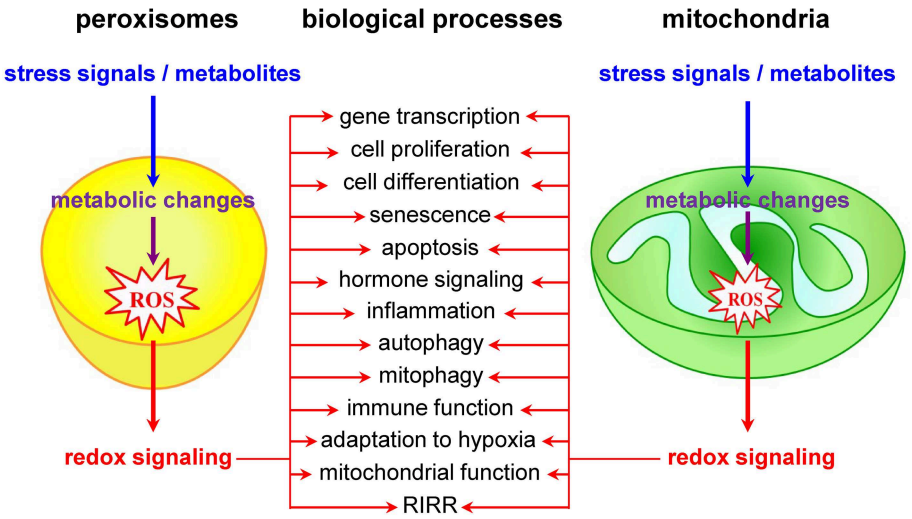

FIGURE 2 | Peroxisomes and mitochondria actively contribute to transcompartmental redox signaling.

and—if propagated from mitochondrion to mitochondrion—of the cell itself (Zinkevich and Gutterman, 2011; Zorov et al., 2014).

Peroxisomes as Redox Signaling Nodes

Although it is already known for years that peroxisomal metabolism and cellular redox equilibrium are closely intertwined (Reddy and Rao, 1987), peroxisomes have long been underestimated and largely ignored as potential redox signaling platforms. However, a limited but growing number of studies lend strong support to the idea that these organelles do actively contribute to transcompartmental ROS signaling in mammalian cells (**Figure 2**). For example, alterations in peroxisomal H_2O_2 metabolism have been shown to influence the cellular protein disulfide content (Yang et al., 2007; Ivashchenko et al., 2011), NF-kB activation (Li et al., 2000; Han et al., 2014), E-cadherin expression (Han et al., 2014), the secretion of matrix metalloproteinases (Koepke et al., 2008; Han et al., 2014), mTORC1 activity and autophagy (Zhang et al., 2013), neuronal activity (Diano et al., 2011), and cell fate decisions in response to different stressors (Carter et al., 2004; Chen et al., 2004; Elsner et al., 2011). Unfortunately, the precise molecular mechanisms underlying most of these observations remain poorly understood and sometimes even controversial. For example, although these and other observations strongly indicate that H_2O_2 can rapidly cross the peroxisomal membrane (Boveris et al., 1972; Fritz et al., 2007), the molecular identity of the channels involved remains to be determined. In addition, virtually nothing is known about how peroxisome-derived H_2O_2 can coordinate or relay signaling events. In this context, it is important to highlight and briefly discuss one of the potentially most significant recent breakthroughs in this research area. It concerns the discovery that the tuberous sclerosis complex (TSC) signaling node (TSC1, TSC2, TBC1D7, and Rheb) can localize to peroxisomes, and that this localization is essential to regulate mTORC1 activity in response to (peroxisomal) ROS (Zhang et al., 2013). However, as (i) the peroxisomal localization of TSC2 could not yet be confirmed by others (Menon et al., 2014), and (ii) peroxisome proliferator-activated receptor (PPAR) agonist-induced ROS production and exogenous H_2O_2 addition do not faithfully mimic the spatial and temporal signaling pattern of peroxisome-derived H_2O_2 [e.g., PPAR agonists increase ROS production by both peroxisomal and non-peroxisomal enzymes (Pyper et al., 2010)], these findings should be interpreted with care and warrant future research. In the context of this section, it is also worth mentioning that, albeit the intraperoxisomal redox status is strongly influenced by environmental growth conditions, peroxisomes resist—within limits—oxidative stress generated elsewhere in the cell (Ivashchenko et al., 2011). However, as an increase in the redox state of the cytosol reduces the import efficiency of peroxisomal matrix proteins (Legakis et al., 2002; Apanasets et al., 2014), it is very likely that conditions chronically disturbing the redox state of the cytosol will affect peroxisome function. Finally, as alterations in peroxisomal ROS production rapidly trigger changes in the mitochondrial balance (Ivashchenko et al., 2011), these organelles may act as upstream initiators of mitochondrial ROS signaling pathways (for more details, see next section).

To which extent peroxisomal β-oxidation influences the cellular or mitochondrial redox state under physiological conditions is not documented. What is interesting to note here is that African green monkey kidney cells (CV-1 cells) (Chu et al., 1995), mouse fibroblasts (LM-tk cells) (Dadras et al., 1998), and rat urothelial cells (MYP3 cells) (Okamoto et al., 1997) overexpressing rat ACOX1 are transformed upon long-term culturing in the presence of fatty acids such as linoleic acid, erucic acid, or nervonic acid. Given that CV-1 cells overexpressing urate oxidase undergo a similar transformation upon exposure to uric acid (Chu et al., 1996), H_2O_2 – and not an acyl-CoA – is thought to be the causative factor. However, in the intact animal, the supply of fatty acids to cells will be a limiting factor. For example, in control rats, hepatic H_2O_2 production increases when plasma

fatty acids levels are higher (Van den Branden et al., 1984). Nevertheless, under basal conditions, hepatic H_2O_2 production is comparable in rats and deer mice with or without peroxisome proliferation (and hence altered ACOX1 levels) (Handler et al., 1992).

Clearly, when discussing peroxisomal β-oxidation and ROS, only H_2O_2 production is emphasized. However, one should recall that this pathway (as well as any other pathway requiring an activated carboxylate) acts on acyl-CoAs, and not on free fatty acids. Most reviews on the role of the thiol/disulfide redox state in biological systems completely neglect CoA-SH as possible modulator of ROS-mediated signaling events (Hansen et al., 2009). Nevertheless, levels of free CoA-SH are substantial (∼10–150 nmol/g tissue), being highest in liver. The majority of this cofactor is confined to the mitochondrial matrix, reaching mM concentrations [e.g., depending on the diet and nutritional status, it was estimated at 3.5–8.5 mM in rat liver (Van Broekhoven et al., 1981; Horie et al., 1986)]. The cytosolic levels are lower, ranging from 0.02 to 0.20 mM (Van Broekhoven et al., 1981; Horie et al., 1986). This indicates that CoA-SH is second in range to glutathione as the most prominent non-protein thiol in mitochondria (for more details, see Antioxidant Systems). Moreover, cellular CoA-SH concentration drops upon treatment with t-butylhydroperoxide and, based on the presence of mixed CoA-glutathione disulfides and their increase during H_2O_2 metabolism in perfused rat liver, an interplay between these two thiol compounds exists (Crane et al., 1982). Finally, the level of this cofactor is regulated by complex metabolic pathways, involving more than 25 acyl-CoA synthetases (Watkins et al., 2007), 13 acyl-CoA thioesterases, and 30 acyltransferases, either N-acyltransferases (conjugating enzymes) or O-acyltransferases (acyl-carnitine transferases and other acyltransferases) (Hunt et al., 2005; searches in the HGNC database of human gene names). Hence, addition of β-oxidizable peroxisomal substrates does not only lead to H_2O_2 production, but also to a lowering of cellular (cytosolic) CoA-SH. When the CoA-ester is poorly or not degradable, CoA-SH levels can drop significantly. This phenomenon, also described as "CoA sequestration" (Brass, 1994), can occur in metabolic disorders (Mitchell et al., 2008) or upon exposure to xenobiotics and drugs (Brass, 1994). In addition, CoA-esters are chemically reactive species that are known to transacylate the cysteinyl-thiol of GSH, and glutathione depletion has been described upon treatment of cells with certain carboxylates (Grillo, 2011). In conclusion, the CoA-SH/CoA-ester ratio can influence the cellular (mitochondrial) GSH/GSSG balance. This occurs when dealing with xenobiotic carboxylates that can neither be degraded by peroxisomal α- or β-oxidation, nor by mitochondrial β-oxidation. As such, it is very likely that the GSH/GSSG balance is altered in patients with fatty acid oxidation defects.

Redox Signaling Between Peroxisomes and Mitochondria

Over the years, it has become increasingly clear that several cellular processes (e.g., fatty acid oxidation, antiviral signaling, and cell fate decisions) require the proper cooperation between mitochondria and peroxisomes (Dixit et al., 2010; Van Veldhoven, 2010; Fransen et al., 2013; Nordgren and Fransen, 2014; Odendall et al., 2014). This is further evidenced by the observations that both organelles share key proteins of their division machinery (Schrader et al., 2013), and that peroxisomal dysfunction can cause mitochondrial abnormalities (e.g., structural alterations of the inner mitochondrial membrane, reduction in the activities of several RCCs, depletion of mitochondrial DNA, increase in oxidative stress, increase in biogenesis, etc.) (Goldfischer et al., 1973; Baes et al., 1997; Baumgart et al., 2001; Maxwell et al., 2003; Dirkx et al., 2005; Ferrer et al., 2005; López-Erauskin et al., 2013; Peeters et al., 2015; Salpietro et al., 2015). Recently, it was demonstrated that these mitochondrial perturbations closely follow the loss of functional peroxisomes in time (Peeters et al., 2015). However, the molecular mechanisms underlying these changes remain poorly understood. It also remains virtually completely unstudied to what extent mitochondrial damage contributes to peroxisomal dysfunction. In the following subsections, we review and discuss emerging evidence that peroxisomes and mitochondria share an intricate redox-sensitive relationship and cooperate in cell fate decisions. Key issues include possible messengers, mechanisms, and physiological significance.

Peroxisomes and Mitochondria Share an Intricate Redox-sensitive Relationship

Mitochondria and peroxisomes are central organelles in setting cellular redox balance and homeostasis (Noctor et al., 2007; Nordgren and Fransen, 2014). Increasing evidence now also indicates that disturbances and/or deficiencies in peroxisomal lipid and ROS metabolism have, directly or indirectly, an impact on the mitochondrial redox balance (**Figure 3**). For example, *in cellulo* experiments have shown that inhibition of catalase activity (and hence a concomitant increase in H_2O_2 levels) rapidly increases mitochondrial ROS production (Koepke et al., 2008; Ivashchenko et al., 2011; Walton and Pizzitelli, 2012). In addition, it has been observed that catalase, a non-canonical PTS1-containing enzyme (Purdue and Lazarow, 1996), mislocalizes to the cytosol during cellular aging (Legakis et al., 2002) and that this phenomenon precedes the age-dependent decrease in mitochondrial inner membrane potential (Koepke et al., 2007). Importantly, as expression of catalase-SKL, a variant with enhanced peroxisome targeting efficiency, can repolarize mitochondria and reduce the number of senescent cells in late passage cell cultures of human fibroblasts (Koepke et al., 2007), it is reasonable to postulate that peroxisome-derived oxidative imbalance may rapidly impair mitochondrial function (Fransen et al., 2012; Walton and Pizzitelli, 2012). In support of this hypothesis are, among others, the findings that (i) inactivation of ABCD1, a peroxisomal VLCFA transporter causative for X-linked adrenoleukodystrophy (X-ALD), causes oxidative damage to mitochondrial proteins and impairs oxidative phosphorylation (OXPHOS) in the spinal cord of mice (López-Erauskin et al., 2013), (ii) acute and chronic loss of PEX5 function quickly impair the activities of the RCCs I, III, and V in hepatocytes from mice (Peeters et al., 2015), and (iii) the activities of the muscle

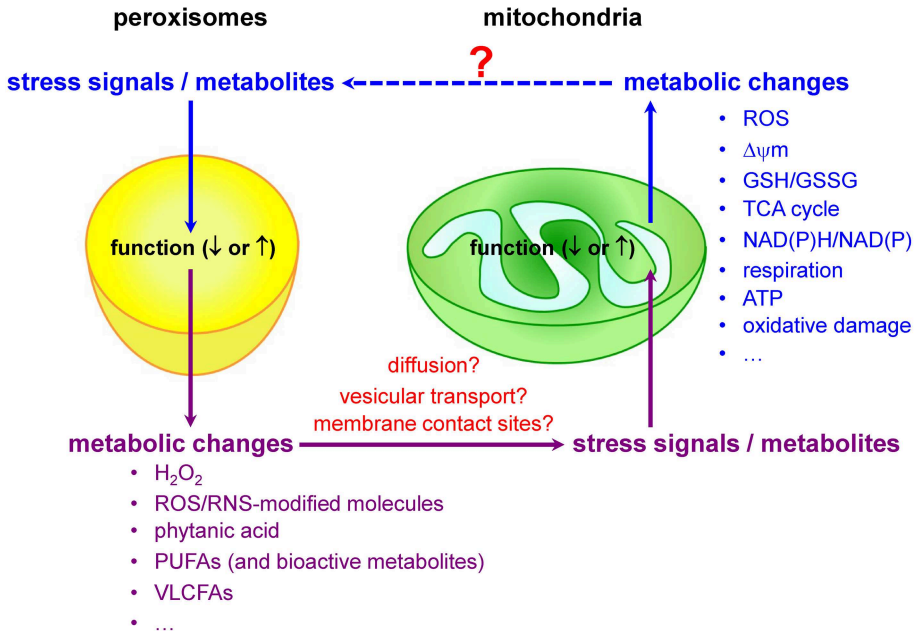

FIGURE 3 | Peroxisomes and mitochondria share an intricate redox-sensitive relationship.

mitochondrial RCCs II, III, and IV are decreased in a Zellweger syndrome (ZS) patient with homozygous pathogenic mutations in the *PEX16* gene (ZS is the most severe of the peroxisome biogenesis disorders) (Salpietro et al., 2015).

Redox Messengers and Modulators

Currently, little is known about the biological messengers that convey redox information between peroxisomes and mitochondria. Potential messengers may include primary ROS/RNS, ROS/RNS-modified molecules, and metabolites. Indeed, it is well known that peroxisomes and mitochondria are actively involved in the metabolism of H_2O_2, $^{\bullet}NO$, and certain lipids that act as signaling molecules (Wang et al., 2014), and—as such—it is very likely that alterations or disturbances in peroxisomal or mitochondrial metabolism may trigger communication events between these organelles. As—to our knowledge—virtually nothing is known about how alterations in mitochondrial activity affect peroxisome function, the next subsections will focus on peroxisomal substrates and metabolites that, upon changes in organelle function, may trigger changes in mitochondrial ROS production due to metabolic stress.

Hydrogen Peroxide

As (i) peroxisomes contain copious amounts of enzymes that can produce or degrade H_2O_2 (see Pro-oxidant Systems and Antioxidant Systems), (ii) peroxisomal H_2O_2 can leak into the cytosol (Mueller et al., 2002), and (iii) changes in catalase activity (and hence in peroxisomal H_2O_2 metabolism) have a profound impact on mitochondrial redox balance (see Peroxisomes and Mitochondria Share an Intricate Redox-sensitive Relationship) and respiration (Barbosa et al., 2013), it is plausible to suppose that peroxisome-derived H_2O_2 can act as a signaling molecule between peroxisomes and mitochondria (Camões et al., 2014). However, the underlying physiological mechanisms are still poorly understood, and it remains to be determined whether peroxisomal H_2O_2 exerts its action on mitochondria directly (e.g., via a RIRR response) or indirectly through the activation of non-mitochondrial stress response pathways. In this context, it is interesting to note that inhibition of peroxisomal catalase activity rapidly leads to a decrease in mitochondrial aconitase activity in early-passage human fibroblasts (Walton and Pizzitelli, 2012) and a reduced phosphorylation of CREB1 and PGC1α transcription in skeletal muscle cells (CREB1 is a cAMP response element binding protein that activates the transcription of PGC1α, a transcriptional co-activator critical for mitochondrial biogenesis and function) (Barbosa et al., 2013).

Phytanic Acid

Phytanic acid is best known for its accumulation in Refsum's disease (plasma levels: ~1.0 mM; normal: <30 μM), a disorder affecting adults and that is clinically characterized by retinitis pigmentosa, peripheral neuropathy, and cerebellar ataxia (Wanders et al., 2011). In addition, the levels of this branched-chain fatty acid are also elevated in patients with rhizomelic chondrodysplasia punctata (RCDP), a more severe disease with cerebellar atrophy caused by death of both Purkinje cells and granular neurons (Powers et al., 1999). Interestingly, loss of these Purkinje cells was postulated to be caused by the incorporation of phytanic acid into cellular membranes, thereby altering intracellular calcium levels and causing mitochondrial dysfunction (Powers et al., 1999). In the meantime, it is known that—when administered to rat hippocampal astrocytes—phytanic acid (50–100 μM) causes a transient rise in cytosolic Ca^{2+}, mitochondrial depolarization and ROS generation,

and cell death within a few hours of exposure (Reiser et al., 2006; Schönfeld et al., 2006). Phytanic acid (100–500 μM) also enhances the production of $O_2^{\bullet-}$ in mitochondria isolated from rat brain and heart tissue (Schönfeld et al., 2006; Grings et al., 2012). In these tissues, such treatment also resulted in lower mitochondrial GSH and NAD(P)H levels, a decreased membrane potential, the oxidative modification of both lipids and proteins, and cytochrome c release (Schönfeld et al., 2006; Grings et al., 2012). One concern related to the studies with brain-derived cells is that the phytanic acid concentration used might not be physiological, given that its levels in cerebrospinal fluid are many fold lower than in plasma (<12 nM in controls) (ten Brink et al., 1993). In addition, it has recently been shown that phytanic acid causes Neuro2a cell death via activation of histone deacetylase activity (Nagai, 2015).

Pristanic Acid

Pristanic acid, the breakdown product of phytanic acid, is further degraded in peroxisomes via β-oxidation. Hence, the plasma level of this 2-methyl-branched fatty acid is increased in patients lacking peroxisomes or with a deficiency in one of the involved β-oxidation enzymes (5-80 μM vs. <3 μM in controls). Based on the clinical phenotype of the latter patients, pristanic acid seems to be linked to adult-onset sensory motor neuropathy as well as visual (retinitis pigmentosa) and intellectual problems. As such, many studies on the toxicity of pristanic acid focus on neurons. Rat astrocytes, oligodendrocytes, and neurons of the hippocampus and cerebellar granule cell layer have been reported to generate more ROS upon exposure to pristanic acid (50–200 μM) (Rönicke et al., 2009; Busanello et al., 2014). Compared to phytanic acid, pristanic acid has a stronger cytotoxic effect on the hippocampal cells: it causes a more profound mitochondrial depolarisation and induces a stronger ROS production (Rönicke et al., 2009). However, whether or not the latter could be due to peroxisomal oxidation was not addressed or investigated in these studies. Note that pristanic acid apparently exerts its toxic effect mainly through its protonophoric action, at least in human skin fibroblasts (Komen et al., 2007). When given to post-nuclear supernatant fractions prepared from rat brain cortex, pristanic acid also causes ROS-generation, as evidenced by decreased GSH levels and increased levels of MDA and protein oxidation (Leipnitz et al., 2011; Busanello et al., 2014). In mitochondrial preparations of rat brain, pristanic acid decreases the $\Delta\Psi m$ and NAD(P)H levels and causes mitochondrial swelling. As the latter process can be prevented by N-acetylcysteine, swelling is most likely caused by ROS-induced damage of the mPTP (Busanello et al., 2012). Note that the *in vitro* effects of pristanic acid can only be observed at rather high concentrations (200 μM).

Polyunsaturated Fatty Acids and their Bioactive Metabolites

Due to the lack of Δ4-desaturase in mammals, PUFAs containing a double bond at position 4,5 are generated via retroconversion, a process consisting of elongation, Δ6-desaturation, and one β-oxidation cycle, the latter mainly by peroxisomes. This process ensures the formation of important PUFAs like arachidonic (C20:4; ARA) and docosahexaenoic (C22:6; DHA) acid. Once incorporated into membrane phospholipids, PUFAs are main players in the generation of ROS by a non-enzymatic process, called autooxidation. An initial oxidative event, the formation of a hydroperoxy-derivative, will trigger a series of reactions leading, via double bond migration, to the generation of 4-HNE and MDA, better known as thiobarbituric acid-reactive compounds (TBARS) (Gardner, 1989). These lipo-oxidative end-products oxidatively modify proteins. PUFAs are not only important phospholipid constituents, but also the precursors of a large class of bioactive fatty acid derivatives, called eicosanoids (derived from ARA) or docosanoids (derived from DHA). Among the best known are prostanoids (prostaglandins, prostacyclins, thromboxanes), leukotrienes, and ω/ω-1-hydroxy- and epoxy-derivatives, all being classified as signaling lipids. After inactivation, these carboxylates are degraded via peroxisomal β-oxidation (Van Veldhoven, 2010). PUFA-epoxides are oxidative products but, in contrast to TBARS, they are formed enzymatically (Spector and Kim, 2014). PUFA-epoxides are mainly inactivated by the cytosolic EPHX2 (Morisseau and Hammock, 2013). However, due to the presence of a weak PTS1, this enzyme is also targeted to peroxisomes in rat (Arand et al., 1991) and in man (Luo et al., 2008). Given that EPH2 is apparently only found in liver and kidney peroxisomes (Enayetallah et al., 2006), these organelles are unlikely to be important for the hydrolysis of PUFA-epoxides. Nevertheless, the impact of peroxisomes on PUFA levels and metabolism is not only substantial, but also quite complex. For example, in peroxisome biogenesis disorders and some peroxisomal β-oxidation enzyme deficiencies, brain PUFA levels (and especially DHA) are lower (Martinez, 1992). This decrease is seen in all cellular phospholipids, including the mitochondrial ones (Peeters et al., 2015). Additionally, the presence of abnormal very-long-chain PUFAs (generated via a runaway process) has been documented in Zellweger patients (Poulos et al., 1988) and some β-oxidation deficiencies (Infante et al., 2002; Huyghe et al., 2006). Whether or not these PUFA-related changes have particular consequences for ROS-signaling or mitochondrial functioning is not known. PUFA-dependent ROS formation in cultured cells is generally linked to stimulation of plasma membrane-bound NADPH-oxidase. However, in PC12 cells, PUFAs increase the fluorescence intensity of MitoSOXred, indicating mitochondrial ROS production, likely by impairing the electron flux in the respiratory chain (Schönfeld et al., 2011). When given to isolated bovine heart mitochondria, ARA—like other fatty acids—causes uncoupling via inhibition of complex I and III (Cocco et al., 1999). Note that, under these conditions, also more H_2O_2 is produced (Cocco et al., 1999).

Very-long-chain Fatty Acids

The accumulation of VLCFAs (C24:0-C30:0) is a biochemical hallmark of X-ALD, a disorder linked to mutations in the peroxisomal ABCD1 membrane transporter and characterized by demyelinisation of the central nervous system. How VLCFAs, mainly found in the cholesterylesters in white matter and adrenals, cause neurodegeneration is not entirely clear, but particular lipids with low abundancy are thought to be involved. Examples include C24:0-lysophosphatidylcholine

causing abnormal activation of microglia and apoptosis (Eichler et al., 2008) and VLCFA-gangliosides activating CD8 cytotoxic T-cells via aberrant binding to CD1 (Ito et al., 2001). More recently, oxidative stress has been proposed to contribute to the pathology. This is mainly based on the facts that (i) X-ALD plasma has increased levels of TBARS, carbonyls, and GSSG/GSH ratios (Vargas et al., 2004; Petrillo et al., 2013), (ii) X-ALD red blood cells display increased GPX activity (Vargas et al., 2004), and (iii) cultured X-ALD fibroblasts contain increased levels of modified lysine residues (and especially N1-carboxyethyl-lysine and N1-malondialdehyde-lysine) (Fourcade et al., 2008), elevated catalase and SOD activities (Vargas et al., 2004), and a higher sensitivity to L-buthionine-sulfoximine, an inhibitor of GSH synthesis (Fourcade et al., 2008). Similarly, two-fold more $O_2^{\bullet-}$ and H_2O_2 is produced in ABCD1 (or ACOX1)-silenced 158N murine oligodendrocytes (Baarine et al., 2012b).

Various findings in X-ALD suggest that VLCFAs (or related compounds) affect the mitochondrial compartment. For example, in the dorsal root ganglia of adult X-ALD patients, atrophic neurons were observed with lipidic inclusions in the mitochondria (Powers et al., 2001), and abnormal mitochondria (condensed cristae, myelinoid figures, mitochondrial dissolution) were found in the adrenal cortical cells of (presymptomatic) 12–13 month-old ABCD1-deficient mice (McGuinness et al., 2003). In addition, a defective OXPHOS could be observed in *ex vivo* spinal cord slices from such mice (López-Erauskin et al., 2013), and signs of ROS (e.g., increased MDE-lysine levels) in this tissue could already be demonstrated as early as 3.5 months of age (Fourcade et al., 2008). At 12 months, MDE-lysine levels tended to normalize while markers for carbonylation and glycoxidation increased (Fourcade et al., 2008). Very recently, Reiser and colleagues showed that VLCFAs also diminish mitochondrial Ca^{2+} retention capacity, and that brain mitochondria prepared from 6 month-old *Abcd1* null mice show slightly but significantly higher Ca^{2+} retention capacity than those from corresponding wild-type mice (Kruska et al., 2015). In the context of the OXPHOS observations, it is important to mention that the respiratory chain is apparently normal in mitochondria isolated from skeletal muscle tissue of 9 month-old ABCD1-deficient mice, despite the acculumation of VLCFAs (Oezen et al., 2005). Whether these differences in OXPHOS function represent age-related or sample-specific variations, remains to be investigated. However, here it is of interest to note that (i) the spinal cord is the main X-ALD target tissue (in man) (Berger et al., 2014), (ii) OXPHOS complexes are also severely impaired in hepatocytes isolated from liver-specific *Pex5* null mice (Peeters et al., 2015), and (iii) isolated mitochondria and intact cells may respond differently to an increase in VLCFAs because these lipids also exert detrimental influence on pyridine nucleotide regeneration in the cytosol (Kruska et al., 2015). By treatment of the *Abcd1*$^{-/-}$ mice with a mixture of antioxidants (N-acetyl-cysteine, α-lipoic acid, and α-tocopherol), oxidative stress, axonal degeneration, and locomotor impairment were reversed (López-Erauskin et al., 2011). Variable effects of VLCFAs (C24:0, C26:0) on cultured cells have been reported. Most likely, multiple factors (e.g., the concentration, the presence of albumin/serum, the number of cells, and the dissolution and delivery mode of VLCFAs) do contribute to this variation. Unfortunately, these experimental details are rarely documented in detail. Above 20 μM, C24:0, and C26:0 appear to be toxic, and a loss of mitochondrial potential was seen in 158N murine oligodendrocytes, rat C6 glioma cells, rat primary neuronal-glial cells, and rat primary oligodendrocytes (Baarine et al., 2012a). This toxicity was accompanied by an increased production of mitochondrial $O_2^{\bullet-}$, mitochondrial vacuolization, the destabilization of lysosomes, and a decrease in catalase activity (Baarine et al., 2012a). However, at physiological VLCFA concentrations (1–5 μM, levels found in X-ALD plasma), no effects were seen. Further studies on the 158N oligodendrocytes showed that 20 μM of C24:0 or C26:0 (complexed to cyclodextrin) triggered oxidative stress characterized by overproduction of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}NO$ associated with lipid peroxidation (e.g., increased levels of 4-HNE, total 7-hydroxycholesterols, and total hydroxyoctadecadienoic acids), protein carbonylation, increased SOD2 activity, and decreased catalase activity and GSH/GSSG ratios (Baarine et al., 2012a). Silencing of the expression of ABCD1 or ACOX1 enhanced the effects of VLCFAs (Baarine et al., 2012a). However, neutral lipid accumulation was only observed with ACOX1 silencing. Human skin fibroblasts appear to be less affected by VLCFAs. Exposure to 100 μM C26:0 did only have minor effects on the inner mitochondrial potential and intracellular ROS production, and only SOD2 was upregulated (Fourcade et al., 2008).

Compared to normal skin fibroblasts, X-ALD fibroblasts are more sensitive to C26:0 in that ROS production starts at lower concentrations (from 10 μM on vs. 50 μM) and GSH levels drop more (Fourcade et al., 2008). Exposure of mouse spinal cord slices to C26:0 (100 μM) resulted in higher expression levels of GPX1 but lower expression levels of SOD1 and SOD2. Related to the cellular studies with VLCFAs, it should be emphasized that C26:0 levels in plasma from healthy controls ($<$1.5 μM, being the sum of free and esterified C26:0) and X-ALD patients ($<$ 5μM) are extremely low (ten Brink et al., 1993). At physiological VLCFA concentrations (1–5 μM, levels found in X-ALD plasma), no effects were seen in 158N murine oligodendrocytes, rat C6 glioma cells, rat primary neuronal-glial cells, and rat primary oligodendrocytes (Baarine et al., 2012a).

Mechanisms

A hypothesis gaining prominence is that disturbances in peroxisomal metabolism can trigger redox-related signaling events that ultimately result in increased mitochondrial stress and the activation of mitochondrial stress pathways (Titorenko and Terlecky, 2011; Beach et al., 2012; Fransen et al., 2013). However, the communication pathways involved remain to be established. Potential mechanisms may include (i) the diffusion of signaling molecules from one compartment to the other via the cytosol, (ii) the exchange of molecules via direct membrane contact sites or vesicular transport mechanisms, and (iii) retrograde signaling. Naturally, the communication pathway may differ depending on the identity, reactivity, and selectivity of the messenger. For example, as H_2O_2 can diffuse out of peroxisomes (see Hydrogen Peroxide), it is very likely that this molecule can modulate the activity of extra-peroxisomal redox-sensitive proteins (e.g.,

transcription factors, kinases, and phosphatases) involved in the (transcriptional) control of mitochondrial biogenesis and function. One such example may be AKT1, a serine-threonine protein kinase that positively regulates the activity of CREB1 [see Hydrogen Peroxide and (Barbosa et al., 2013)] and is degraded by the ubiquitin-mediated proteasome pathway in conditions with elevated H_2O_2 (Kim et al., 2011). As the biological half-life of some ROS is extremely short (e.g., $O_2^{\bullet -}$, $\sim 10^{-6}$s; $^{\bullet}OH$, $\sim 10^{-9}$s), it is unlikely that these molecules will be directly transported from one compartment to the other by diffusion or vesicular transport mechanisms (Fransen et al., 2012; and references therein). In this context, it is interesting to note that we recently discovered that the production of excess $O_2^{\bullet -}$ inside peroxisomes causes cellular lipid peroxidation, and that this in turn triggers a complex network of signaling events eventually resulting in increased mitochondrial H_2O_2 production (Wang et al., 2013b). Note that, as (i) there is some evidence that the propagation of ROS signals from the ER to mitochondria is facilitated by membrane contact sites (Verfaillie et al., 2012), and (ii) such contact sites may also exist between peroxisomes and mitochondria (Horner et al., 2011; Schrader et al., 2015), it is possible that these sites are also involved in the redox communication between peroxisomes and mitochondria. Finally, as mitochondria have the ability to generate mitochondria-derived vesicles (MDVs) that selectively transport mitochondrial proteins to either peroxisomes or lysosomes (Neuspiel et al., 2008; Soubannier et al., 2012a), such vesicular transport pathways may also exist for peroxisomes. Here it is interesting to note that, albeit the MDVs destined for the lysosomes are selectively enriched for oxidized proteins, the functional importance of MDV-mediated protein delivery to peroxisomes has not yet been determined (Soubannier et al., 2012b).

Physiological Significance

Currently, it is widely accepted that mitochondrial ROS levels are crucial to regulate the fitness of eukaryotic organisms (Hamanaka and Chandel, 2010). In addition, it is becoming increasingly clear that mitochondria can act as dynamic receivers, integrators, and transmitters of oxidative stress derived from various sources (Nickel et al., 2014). We and others have shown that also disturbances in peroxisomal redox metabolism have an immediate impact on mitochondrial ROS production, both *in cellulo* (Walton and Pizzitelli, 2012; Wang et al., 2013b) and *in vivo* (López-Erauskin et al., 2013; Peeters et al., 2015). In addition, there is strong evidence that defects in peroxisome function as well as excessive ROS-production inside these organelles can trigger mitochondria-mediated cell death (López-Erauskin et al., 2012; Wang et al., 2013b). These and other findings clearly demonstrate that peroxisomal and mitochondrial fitness are closely intertwined, and—as such—it may not come as a surprise that both organelles play a cooperative role in the pathogenesis of at least some neurometabolic diseases. For example, defects in peroxisome biogenesis have been reported to lead to secondary dysfunction of mitochondria, and this may in turn determine—at least in part—the severe phenotype of Zellweger syndrome (Salpietro et al., 2015). The finding that peroxisomes and mitochondria cooperatively function in redox regulation may also offer therapeutic potential for at least some patients with peroxisomal deficiencies. Indeed, as has been shown in preclinical experiments with *Abcd1* null mice, boosting mitochondrial function with pioglitazone (Morató et al., 2013) or activators of SIRT1 (Morató et al., 2015) may normalize redox balance and prevent axonal demise.

Promises and Potential of "Omics" Approaches

Oxidative and nitrosative stress-induced modifications are central to a broad range of stress responses, and the accumulation of ROS/RNS can be expected to leave traces of biomarkers at the genome, transcriptome, proteome, and metabolome levels (Aebersold, 2003; Ma et al., 2013). As state-of-the-art "-omics" technologies allow detection of subtle biological variations, such platforms offer unique opportunities for researchers to study the molecular effects of oxidative stress at system level. The goal of this section is to provide the reader with a few examples of how data mining of publicly-available large-scale data may be used to gain additional insights into potential redox signaling pathways between peroxisomes and mitochondria (we do not intend to give an exhaustive overview).

A first example is RedoxDB, a curated database of experimentally-verified protein oxidative modifications (http://biocomputer.bio.cuhk.edu.hk/RedoxDB/) (Sun et al., 2012). Searching this database revealed that multiple peroxisomal proteins in mammalian cells [e.g., catalase, 3-ketoacyl-CoA thiolase (ACAA1), and hydroxyacid oxidase 2 (HAO2)] contain cysteine thiol groups that are susceptible to oxidation by ROS/RNS and can undergo S-nitrosylation (RSNO), S-sulfenylation (RSOH), and/or S-thiolation (RSSG) (Doulias et al., 2013). Note that ACAA1 catalyzes the final step in the oxidation of straight-chain acyl-CoAs, and that HAO2 is an FMN-dependent enzyme that oxidizes long-chain L-2-hydroxy acids to ketoacids at the expense of O_2 with concomitant production of H_2O_2. The physiological relevance of these observations remains to be determined. However, as (i) these modifications are likely to impact enzyme activity and stability (Stolz et al., 2002; Ortega-Galisteo et al., 2012; Gould et al., 2013), and (ii) alterations in peroxisomal fatty acid β-oxidation and H_2O_2 metabolism have been shown to affect mitochondrial function (see Peroxisomes and Mitochondria Share an Intricate Redox-sensitive Relationship and Redox Messengers and Modulators), such reversible cysteine modifications may participate in signaling processes between mitochondria and peroxisomes.

The second example is AGEMAP (Atlas of Gene Expression in Mouse Aging Project), a gene expression database that catalogs changes in gene expression in mice as a function of age (Zahn et al., 2007). The idea behind this database is that the identities of age-related genes provide important clues about mechanisms (e.g., stress response) that drive transcriptional changes in old age (e.g., oxidative stress). Upon profiling the effects of aging on gene expression in different tissues dissected from mice of ages 1, 6, 16, and 24 months and comparing these data with DNA microarray data on aging from human muscle, Becker and colleagues

discovered that 17 genesets are commonly age-regulated in multiple human and mouse tissues (Zahn et al., 2007). The most relevant ones in the context of this manuscript are the genesets associated with peroxisomes and the mitochondrial electron transport chain, both of which show an overall decrease in expression with age (for comparison, the genesets associated with lysosomes and the inflammatory response showed a common increasing trend in expression with age).

A third and last example concerns the proteomic analysis of a subdomain of the ER, called the mitochondrial-associated ER membrane (MAM). Previous confocal microscopy studies have shown that MAM also physically connects mitochondria to peroxisomes during antiviral response (Horner et al., 2011). In addition, activation of the antiviral innate immune response has been reported to alter peroxisomal and mitochondrial morphology (Dixit et al., 2010; Horner et al., 2011). Evidence that mitochondria and peroxisomes physically interact, can also be inferred from proteomic datasets. Indeed, proteomic analysis of MAM fractions isolated from mouse brain (Poston et al., 2013), cytomegalovirus-infected human fibroblasts (Zhang et al., 2011), and SenV- or hepatitis C virus-infected Huh7 human hepatoma cells (Horner et al., 2015) have demonstrated that these fractions also contain peroxisomal matrix (e.g., AGPS, ACOX1, CAT, etc.) and membrane (e.g., ABCD1, PEX11β, PEX14, etc.) proteins. Interestingly, some of these proteins (e.g., CAT and PEX14) are differentially enriched in MAM fractions from infected and non-infected cells. Although the biological significance of these observations remains to be established, it has been hypothesized that the changes in expression of individual proteins may reflect changes in organelle interactions during the antiviral signaling response and the generation of new signaling sites through these organelle interactions (Horner et al., 2015). Importantly, as the MAM compartment represents a hot spot for the intracellular signaling of important pathways, including phospholipid synthesis and ROS generation and activity (Giorgi et al., 2015), it is tempting to speculate that this compartment also provides an axis through which stress stimuli and metabolites can be transmitted from peroxisomes to mitochondria (see Mechanisms). However, experimental evidence to support this hypothesis is currently lacking.

Concluding Remarks

Peroxisomes and mitochondria are pivotal team players in cellular redox metabolism. In addition, growing evidence suggests that mitochondria can act as dynamic receivers, integrators, and transmitters of peroxisome-derived mediators of oxidative stress, and that alterations in the peroxisomal redox state are likely to impact mitochondrial redox activity. Therefore, in order to understand how a decline in peroxisome function can be associated with cellular aging and the initiation and progression of oxidative stress-related diseases, it is critical to gain more insight into the molecular mechanisms by which peroxisomes and mitochondria communicate. Future studies should focus on (i) the link between peroxisomal/mitochondrial (dys)function and cellular redox balance, (ii) the identification of the proximal targets of peroxisome-derived ROS/RNS, (iii) the molecular mechanisms underlying the redox communication between peroxisomes and mitochondria, and (iv) the validation of novel targets and mechanisms in primary cells and tissues from patients and mice suffering from peroxisomal or oxidative stress-related disorders. The outcome of such studies may open up exciting new avenues for the community of researchers working on cellular responses to organelle-derived oxidative stress, a research field in which the role of peroxisomes is currently highly underestimated and an issue of discussion.

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REVIEW
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Hypoxia signaling pathways: modulators of oxygen-related organelles

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Oxygen (O_2) is an essential substrate in cellular metabolism, bioenergetics, and signaling and as such linked to the survival and normal function of all metazoans. Low O_2 tension (hypoxia) is a fundamental feature of physiological processes as well as pathophysiological conditions such as cancer and ischemic diseases. Central to the molecular mechanisms underlying O_2 homeostasis are the hypoxia-inducible factors-1 and -2 alpha (HIF-1α and EPAS1/HIF-2α) that function as master regulators of the adaptive response to hypoxia. HIF-induced genes promote characteristic tumor behaviors, including angiogenesis and metabolic reprogramming. The aim of this review is to critically explore current knowledge of how HIF-α signaling regulates the abundance and function of major O_2-consuming organelles. Abundant evidence suggests key roles for HIF-1α in the regulation of mitochondrial homeostasis. An essential adaptation to sustained hypoxia is repression of mitochondrial respiration and induction of glycolysis. HIF-1α activates several genes that trigger mitophagy and represses regulators of mitochondrial biogenesis. Several lines of evidence point to a strong relationship between hypoxia, the accumulation of misfolded proteins in the endoplasmic reticulum, and activation of the unfolded protein response. Surprisingly, although peroxisomes depend highly on molecular O_2 for their function, there has been no evidence linking HIF signaling to peroxisomes. We discuss our recent findings that establish HIF-2α as a negative regulator of peroxisome abundance and suggest a mechanism by which cells attune peroxisomal function with O_2 availability. HIF-2α activation augments peroxisome turnover by pexophagy and thereby changes lipid composition reminiscent of peroxisomal disorders. We discuss potential mechanisms by which HIF-2α might trigger pexophagy and place special emphasis on the potential pathological implications of HIF-2α-mediated pexophagy for human health.

Keywords: endoplasmic reticulum, ER stress, hypoxia, HIF-α, mitochondria, mitophagy, peroxisomes, pexophagy

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Introduction

Life with oxygen (O_2) began around 2.4 billion years ago, when photosynthetic organisms prospered and multiplied, leading to a progressive increase of atmospheric O_2. O_2-related organelles, such as mitochondria, peroxisomes, and plastids, must have been acquired after that date (De Duve, 2007; Semenza, 2007). The appearance of O_2 was one of the defining moments in

evolution, as it offered organisms the advantage of generating energy more efficiently. Because of the high energy potential of O_2, aerobic organisms have become dependent on this gas for their performance and survival. O_2 is an essential substrate in cellular metabolism, bioenergetics, and signaling and as such inseparably linked to the survival and normal function of all metazoans. Hence, aerobic species developed mechanisms to sense O_2 levels and regulate O_2 consumption, in order to cope with conditions of insufficient O_2 supply. This Review focuses on the role of hypoxia-inducible factors (HIFs) as master regulators of O_2 homeostasis and, in particular, on recent advances in understanding their roles in regulating major O_2-consuming organelles, namely mitochondria, the endoplasmic reticulum (ER), and peroxisomes.

Regulation of HIFs

Central to the molecular mechanisms underlying O_2 homeostasis are HIF-1α and HIF-2α that function as master regulators of the adaptive response to hypoxia. HIFs form a heterodimer consisting of a constitutively expressed ARNT/HIF-1β subunit and O_2-regulated α subunits (HIF-1α or EPAS1/HIF-2α) (Majmundar et al., 2010; Keith et al., 2012). A third HIF-α subunit (HIF-3α) has also been described. *HIF3A* mRNA is differentially spliced to produce multiple HIF-3α isoforms that either promote or inhibit the activity of other HIF complexes (Keith et al., 2012). Under normoxia, HIF-α subunits are hydroxylated by prolyl hydroxylases (PHD1-3) and recognized and targeted for proteasomal degradation by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex (**Figure 1A**). PHD enzymes are 2-oxoglutarate- and iron-dependent dioxygenases, whose activity is absolutely dependent on O_2. Hence, the rate of HIF-α hydroxylation is suppressed under hypoxia. Hypoxia or loss of functional VHL stabilizes HIF-α subunits. HIF-α either dimerizes with HIF-1β and binds to hypoxia-responsive elements in promoters of target genes to promote a concerted transcriptional response (Keith et al., 2012) (**Figure 1A**) or it physically interacts with other non-HIF proteins (Uniacke et al., 2012; Hubbi et al., 2013), enabling convergence of HIF O_2 sensing with other signaling pathways.

HIF-α subunits can also be stabilized under non-hypoxic conditions, a phenomenon termed "pseudohypoxia." In addition to O_2, PHDs are sensitive to changes in certain Krebs cycle intermediates. Mutations in four genes involved in the metabolism of citrate have the potential to stabilize HIF-α by inhibiting HIF-α hydroxylation and are linked to various tumors (**Figure 1B**) (Raimundo et al., 2011; Losman and Kaelin, 2013). Succinate dehydrogenase and fumarate hydratase deficiencies lead to accumulation of succinate and fumarate, respectively, and these metabolites compete with 2-oxoglutarate to inhibit PHDs. Mutations in isocitrate dehydrogenases 1 and 2 could promote HIF-α stabilization as a result of low levels of 2-oxoglutarate, which is an essential co-substrate of PHDs (Thompson, 2009). Tumor-associated isocitrate dehydrogenase mutations cause a gain of function, leading to high-level production of (*R*)-2-hydroxyglutarate and depletion of 2-oxoglutarate (Losman and Kaelin, 2013). PHD enzymes were initially reported to be inhibited by (*R*)-2-hydroxyglutarate (Zhao et al., 2009). However, further studies have shown that (*R*)-2-hydroxyglutarate potentiates PHD activity and blunts the induction of HIF-α in response to hypoxia (Losman and Kaelin, 2013).

HIF-1α is expressed ubiquitously, whereas HIF-2α is selectively expressed in distinct cell populations of most organs (Majmundar et al., 2010). HIF-1α and HIF-2α have both overlapping and distinct target genes (Keith et al., 2012) and they are differentially regulated in various physiological settings (e.g., embryonic development) and function in pathophysiological conditions such as cancer and ischemic diseases (Semenza, 2012). They have also different roles in tumorigenesis dependent on specific tumor microenvironments (Majmundar et al., 2010; Keith et al., 2012). HIF-induced transcription promotes angiogenesis, erythropoiesis, metastasis and metabolic reprogramming, such as shifting cell metabolism from oxidative phosphorylation to glycolysis. HIF activation due to hypoxia or loss of VHL function also reprograms lipid metabolism leading to lipid accumulation (Huss et al., 2001; Boström et al., 2006; Rankin et al., 2009; Kucejova et al., 2011; Qu et al., 2011; Walter et al., 2014).

Mitochondria and HIF-α

Mitochondria, metabolism, and O_2 are inextricably intertwined. In the following sections we will discuss the numerous mechanisms by which HIF signaling can affect mitochondrial function.

HIF-dependent Regulation of Mitochondrial Metabolism

The hypoxia-dependent increase in the abundance and activity of HIF-1α and the HIF-1α-dependent transcriptional program have

Abbreviations: ALFY, autophagy-linked FYVE protein; ALOX15, 15-lipoxygenase-1; ATF, activating transcription factor; Atg, autophagy-related protein; BNIP3, Bcl-2 and adenovirus E1B 19-kDa-interacting protein 3; BNIP3L/NIX, BNIP3-like; ccRCC, clear cell renal cell carcinoma; CHO, Chinese hamster ovary; CHOP, C/EBP homologous protein; CK2, casein kinase 2; COX, cytochrome c oxidase; DAO, D-amino acid oxidase; DHA, docosahexaenoic acid; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERR, estrogen-related receptor; FA, fatty acid; FOXO3a, forkhead-box protein O3a; FUNDC1, FUN14 domain containing 1; GABARAP, γ-aminobutyric acid-receptor-associated protein; GADD34, growth arrest DNA-inducible gene 34; GLUT, glucose transporter; GRP, glucose-regulated protein; HIF, hypoxia-inducible factor; HTT, Huntingtin; IRE1, inositol-requiring protein 1; LC3, microtubule-associated protein-1 light chain 3; LDHA, lactate dehydrogenase A; LIR, LC3-interacting region; LONP, Lon protease; MAX, MYC-associated factor X; MCT4, monocarboxylate transporter 4; mtROS, mitochondrial reactive oxygen species; MXI1, MAX-interacting protein 1; NBR1, neighbor of BRCA1 gene; NRF, nuclear respiratory factor; OMM, outer mitochondrial membrane; OxPhos/ETC, oxidative phosphorylation and electron transport chain; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PERK, protein kinase RNA-like ER kinase; PEX, peroxin; PGAM5, phosphoglycerate mutase family member 5; PHD, prolyl hydroxylase; PGC-1, PPARγ coactivator 1; PINK1, PTEN-induced putative protein kinase 1; PMP, peroxisomal membrane protein; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin homolog; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RPC, receptor protein complex; SQSTM1/p62, sequestosome 1; TNBC, triple-negative breast cancer; ULK1, UNC51-like kinase 1; UOX, urate oxidase; UPR, unfolded protein response; VDAC, voltage-dependent anion channel; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau; VLC, very long-chain; XBP1, X-box-binding protein 1.

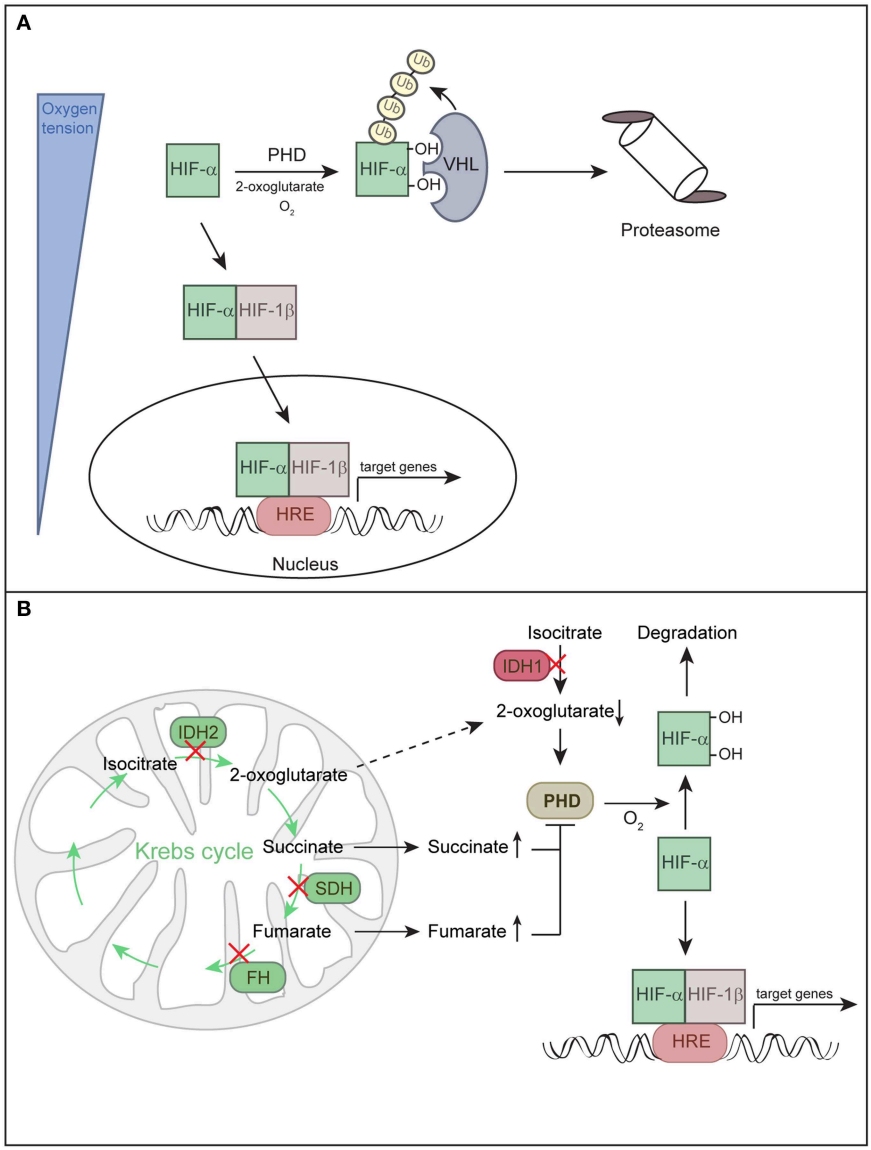

FIGURE 1 | Regulation of HIF-α subunits. (A) Hypoxia-inducible factors (HIFs) are transcription factors composed of O_2-regulated α subunits (HIF-1α or HIF-2α) and a constitutively expressed HIF-1β subunit. Together these subunits bind hypoxia response elements (HRE) to mediate adaptive responses to hypoxia. HIF-α activity is directly linked to oxygen partial pressure. Under normoxia, HIF-α is hydroxylated by prolyl hydroxylase domain protein (PHD) and targeted for proteasomal degradation by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex. Under hypoxia, hydroxylation is inhibited and HIF-α is stabilized, it dimerizes with HIF-1β and enters the nucleus to induce target gene transcription. **(B)** HIF-α can be stabilized irrespective of O_2 tension due to inhibition of PHDs, a state defined as pseudohypoxia. Mutations in the Krebs cycle enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) lead to accumulation of succinate and fumarate, respectively, whereas mutations in isocitrate dehydrogenases 1/2 (IDH1 and IDH2) lead to low levels of 2-oxoglutarate. Succinate and fumarate inhibit PHDs, while low levels of the co-substrate 2-oxoglutarate decrease the activity of PHDs. Decreased activity of PHDs leads to a low rate of HIF-α hydroxylation under normoxic conditions and stabilization of HIF-α.

three major effects on metabolism that serve to equilibrate O_2 consumption with O_2 supply. First, HIF-1α promotes glycolytic energy production by inducing genes that encode glucose transporters (e.g., *GLUT1*, *GLUT3*) and glycolytic enzymes (**Figure 2A**) (Semenza, 2010). HIF-1α also upregulates lactate dehydrogenase A (LDHA), which converts pyruvate to lactate and regenerates NAD^+ for continuous supply for glycolysis, and monocarboxylate transporter 4 (MCT4), which transports lactate out of the cell (**Figure 2A**).

Second, HIF-1α suppresses both the Krebs cycle and oxidative phosphorylation within mitochondria. HIF-1α induces pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates the mitochondrial enzyme pyruvate dehydrogenase that catalyzes the conversion of pyruvate to acetyl-CoA, thereby

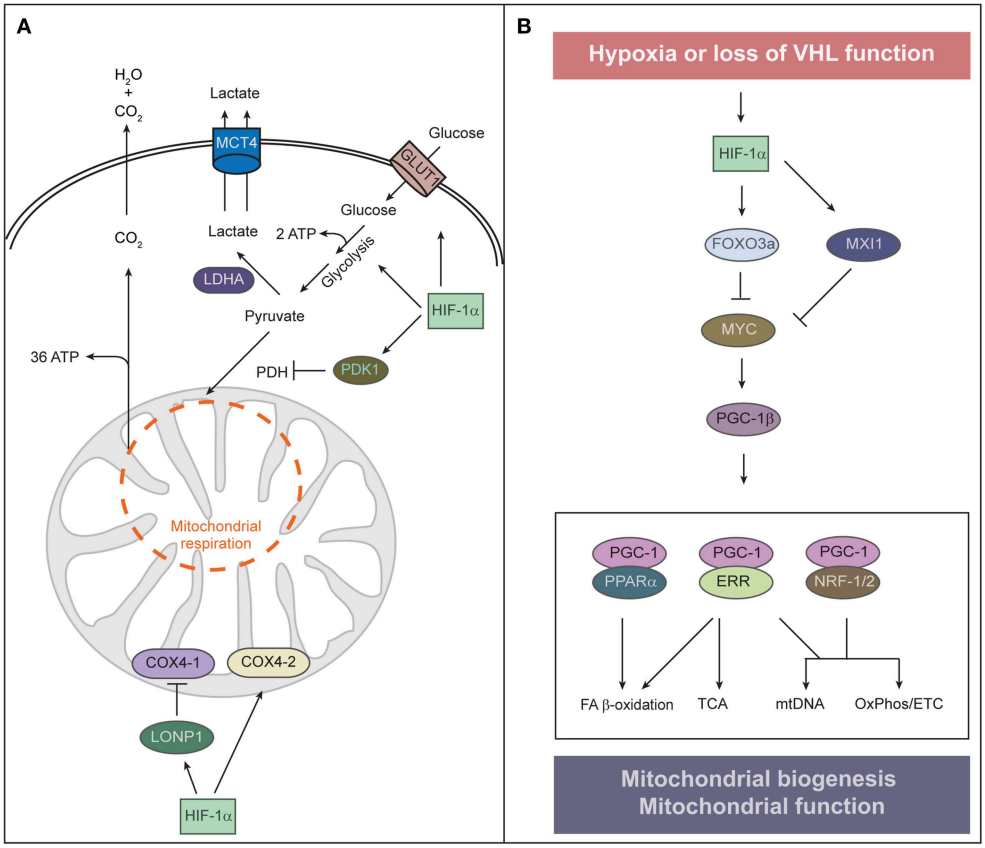

FIGURE 2 | Regulation of mitochondrial function and abundance by HIF-α. (A) To adapt to low oxygen tension, cells undergo two HIF-1α-mediated alterations of cellular metabolism: O_2-independent ATP production and reduction of mitochondrial O_2 consumption. HIF-1α signaling also contributes to the Warburg effect of aerobic glycolysis—that is, an uncoupling of glycolysis from O_2 levels—by stimulating the expression of the glucose transporter GLUT1 and glycolytic enzymes. Increased glycolysis generates increased levels of pyruvate, which is largely converted to lactate by HIF-inducible lactate dehydrogenase A (LDHA) and removed from the cell by the monocarboxylate transporter 4 (MCT4). HIF-1α induces pyruvate dehydrogenase kinase 1 (PDK1), which inhibits pyruvate dehydrogenase (PDH) and blocks conversion of pyruvate to acetyl-CoA, resulting in decreased flux through the tricarboxylic acid (TCA) cycle. Decreased TCA cycle activity attenuates oxidative phosphorylation and excessive mitochondrial ROS production. Under normoxia, COX4-1 is the predominant isoform of COX4 present in complex IV of the electron transport chain, which transfers electrons to O_2. Under hypoxia, HIF-1α upregulates the expression of *COX4-2* and the mitochondrial protease *LONP1*, which in turn degrades COX4-1. COX4-2 is more efficient at facilitating the electron transfer to O_2 and thereby protects the cell from oxidative damage during hypoxia. **(B)** Control of mitochondrial biogenesis by HIF-α. HIF-1α induces the expression of MAX-interacting protein 1 (MXI1), a repressor of MYC activity, and thereby represses a subset of MYC target genes such as *PGC-1β*. HIF-1α-dependent activation of FOXO3a inhibits MYC activity by reducing MYC protein stability. Interaction between PGC-1 and transcription factors such as PPARα, ERR, and NRF-1/2 orchestrates the major functions of mitochondria. HIF-1α-mediated inhibition of MYC and PGC-1 results in reduced mitochondrial biogenesis.

shunting pyruvate away from mitochondria and diminishing hypoxic mitochondrial respiration (Kim et al., 2006; Papandreou et al., 2006). A benefit of attenuating mitochondrial respiration under hypoxia is reducing the amount of toxic mitochondrial reactive oxygen species (mtROS) generated by inefficient respiration. Mouse embryonic fibroblasts lacking *Hif1α* undergo cell death as a result of excess mtROS production due to a failure of PDK1 induction (Kim et al., 2006). HIF-1α also reduces mtROS production under hypoxic conditions by optimizing respiration efficiency through inducing cytochrome c oxidase (COX) subunit IV isoform 2 (COX4-2) and the mitochondrial protease LONP1, which degrades the less efficient COX4-1 (Fukuda et al., 2007). Mitochondrial ROS also modulate cell signaling through stabilization of HIF-1α, due to PHD inhibition (Sena and Chandel, 2012). Cells utilize an acute increase in mtROS to stabilize HIF under hypoxia and subsequently restrain ROS production under chronic hypoxia to avoid cellular damage. ROS have a relatively short diffusion distance and thus, mtROS signaling may rely on proximity of ROS-producing mitochondria to their sites of action. Intriguingly, perinuclear clustering of mitochondria triggered by hypoxia is accompanied by the accumulation of nuclear ROS and required for maximal HIF-1α binding to the *VEGF* promoter and *VEGF* expression (Al-Mehdi et al., 2012).

Third, hypoxia shifts mitochondrial glutamine metabolism from oxidation to reductive carboxylation (Sun and Denko,

2014). The activity of the α-ketoglutarate dehydrogenase complex is decreased under hypoxia, since HIF-1α induces the E3 ubiquitin ligase SIAH2 that mediates the proteasomal degradation of the E1 subunit of α-ketoglutarate dehydrogenase. Increased α-ketoglutarate levels drive the reverse reaction at isocitrate dehydrogenase, and the glutamine-derived citrate can be transported to the cytoplasm in order to generate acetyl-CoA for anabolic processes under hypoxia.

Cancer cells exhibit a high rate of glycolysis even in the presence of O_2, a phenomenon known as aerobic glycolysis or the "Warburg effect" (Vander Heiden et al., 2009). HIF-1α activation as a result of *VHL* loss or pseudohypoxia contributes also to the development of the Warburg effect. Furthermore, although the shift from oxidative phosphorylation to glycolysis is attributed to the activity of HIF-1α, at least in the liver HIF-2α induces the same genes involved in this metabolic adaptation (Rankin et al., 2009; Walter et al., 2014).

Mitochondrial Biogenesis and HIF-α

A second key remodeling of mitochondria in hypoxia is suppression of mitochondrial biogenesis to decrease mitochondrial mass and O_2 consumption. Mitochondrial biogenesis involves replication of the mitochondrial genome and coordinated expression of nuclear- and mitochondrial-encoded gene products. It depends upon the activity of a hierarchy of nuclear transcription factors that includes the peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 family of transcriptional coactivators [PGC-1α, PGC-1β, and the PGC-related coactivator (PRC)], the nuclear respiratory factors (NRF1 and NRF2), and estrogen-related receptors (ERRα, ERRβ, and ERRγ) (**Figure 2B**) (Scarpulla et al., 2012). PGC-1α is the master regulator of all aspects of mitochondrial biogenesis, including activation of respiratory chain and fatty acid oxidation genes, increased mitochondrial number, mtDNA replication, and augmentation of mitochondrial respiratory capacity. It exerts these effects through direct interaction with and coactivation of PPARs, NRFs, and ERRs (Scarpulla et al., 2012). Nutrient supply and cellular energy balance regulate the activity of PGC-1α at both the transcriptional and posttranslational level (Dominy et al., 2010; Scarpulla et al., 2012). PGC-1β and PRC interact with and coactivate many of the same transcription factors as PGC-1α to promote mitochondrial biogenesis (Scarpulla et al., 2012).

The oncogenic transcription factor MYC promotes mitochondrial biogenesis through activation of PGC-1β (Li et al., 2005; Zhang et al., 2007). MYC dimerizes with MYC-associated factor X (MAX) and binds specific E-box sequences in target gene promoters to activate transcription. Heterodimers of MAX with MAX-interacting protein 1 (MXI1) antagonize MYC function and repress transcription by binding to the same promoter regions of MYC target genes. Cross-talk between HIF and MYC has been defined at a number of levels, however, HIF-1α and HIF-2α exert opposing roles on MYC interaction with its transcription cofactors (Dang et al., 2008; Keith et al., 2012). HIF-1α activation inhibits mitochondrial biogenesis by promoting MYC degradation and by inducing MXI1 expression (Zhang et al., 2007). This inhibition is mediated by the transcription factor FOXO3a (Forkhead-box protein O3a) which is activated in hypoxia downstream of HIF-1α (Peck et al., 2013). FOXO3a can also inhibit MYC activity by reducing MYC protein stability and by increasing the expression of miRNAs that perturb the translation of *MYC* mRNA (Peck et al., 2013). Huang et al. (2014) reported that HIF-1α, but not HIF-2α, represses MYC in human hepatoma cells and thereby decreases *PGC-1*β expression, leading to decreased expression of medium- and long-chain acyl-CoA dehydrogenases and subsequent inhibition of mitochondrial fatty acid β-oxidation. It has not been addressed if HIF-1α-mediated downregulation of PGC-1β also affects mitochondrial biogenesis and mass in hepatoma cells. HIF-2α actually enhances MYC transcriptional activity by binding to and stabilizing the MAX-MYC heterodimer (Gordan et al., 2007, 2008). The cooperation of HIF-2α with MYC increases MYC effects on various cell cycle regulators and drives tumorigenesis. Given the opposite effects of HIF-1α and HIF-2α on MYC, it remains to be established how MYC is modulated in cells that express both HIF isoforms.

However, with the exception of HIF-1α-mediated inhibition of PGC-1β, data about HIF-α mediated regulation of PGC-1α, the master regulator of mitochondrial biogenesis and function, are relatively scarce. Several studies demonstrated an induction of PGC-1α by hypoxia independently of HIF-1α activity (Shoag and Arany, 2010). Overexpression of PGC-1α under normoxia induces mitochondrial biogenesis which increases O_2 consumption and decreases intracellular O_2 levels, leading to HIF-1α protein stabilization and activation of HIF-1α target genes (O'Hagan et al., 2009). ROS accumulation in hypoxic cancer cells induces expression of *PGC-1α/β* to promote detoxification through induction of antioxidative enzymes (Austin and St-Pierre, 2012). The precise relationship between the PGC-1α and HIF-α pathways with respect to mitochondrial biogenesis obviously needs further clarification.

HIF-dependent Regulation of Mitophagy

The third mechanism by which HIF-α controls mitochondrial function is selective mitochondrial autophagy (mitophagy). Autophagy is an evolutionary conserved catabolic process for degradation of macromolecules and organelles. Both non-selective "bulk" autophagy and selective autophagy of specific proteins or organelles have been described (Mizushima et al., 2011; Schreiber and Peter, 2014). Selective and non-selective autophagy share a set of autophagy-related (Atg) proteins referred to as the core autophagic machinery (Stolz et al., 2014). Yeast Atg8 and its mammalian homologs of the microtubule-associated protein-1 light chain 3 family (LC3A, LC3B, LC3C) and γ-aminobutyric acid-receptor-associated (GABARAP, GABARAPL1, GABARAPL2) proteins are covalently conjugated to the lipid phosphatidylethanolamine upon induction of autophagy. Besides playing a pivotal role in different steps of autophagosome biogenesis, the LC3 family members are important for target recognition during selective autophagy. Selective autophagy requires specific receptors, which recognize cargo tagged with degradation signals, connect it to the autophagosomal membrane through their LC3-interacting regions (LIR), and are degraded together with their cargo within autolysosomes (Stolz et al., 2014).

Mitophagy can be initiated in several ways to arbitrate mitochondrial quality control via the selective removal of superfluous or damaged mitochondria. The best-studied mechanism for mitophagy in mammalian cells is the PINK1-Parkin-mediated pathway, which is elegantly reviewed elsewhere (Scarffe et al., 2014). Briefly, phosphatase and tensin homolog (PTEN)-induced putative protein kinase (PINK1), which is rapidly degraded in healthy mitochondria, accumulates upon mitochondrial membrane depolarization at the outer membrane, leading to the recruitment of the E3 ubiquitin ligase Parkin to mitochondria and ubiquitination of several outer mitochondrial membrane (OMM) proteins (Winklhofer, 2014). The recruitment of ubiquitinated mitochondria to autophagic structures is mediated by LC3 family members and ubiquitin-binding adaptor proteins such as sequestosome 1 (SQSTM1/p62), NBR1 (neighbor of BRCA1 gene 1), and optineurin (Rogov et al., 2014).

Although mitophagy has been extensively studied in mammals, mitophagy-specific factors still remain controversial. In yeast Atg32 has been identified as receptor protein for mitophagy (Kanki et al., 2009, 2015; Okamoto et al., 2009). Atg32 localizes to the OMM, harbors a classical LIR consensus sequence and interacts with Atg8 and the scaffold protein Atg11 to enable the assembly of the core autophagy machinery around the cargo (**Figure 3A**). Casein kinase 2 (CK2) regulates mitophagy by phosphorylating Atg32, which stabilizes the Atg32-Atg11 interaction and promotes mitophagy. So far no mammalian homolog has been identified for Atg32.

Similar to Atg32 in yeast, the mammalian mitophagy receptors BNIP3 (Bcl-2 and adenovirus E1B 19-kDa-interacting protein 3), BNIP3-like (BNIP3L/NIX), and FUNDC1 (FUN14 domain containing 1) are OMM proteins which can directly bind to LC3 via their LIR motifs (**Figures 3B,C**). BNIP3 and NIX were originally thought to promote apoptosis or programmed necrosis (Zhang and Ney, 2009). They can activate autophagy by binding to Bcl-2 and thereby disrupting the interaction between Beclin-1 and Blc-2/Bcl-X_L (Bellot et al., 2009; Boland et al., 2013). BNIP3 and NIX are hypoxia-inducible HIF-α target genes and it has been suggested that they act either in hypoxia-induced macroautophagy or mitophagy (Sowter et al., 2001; Tracy et al., 2007; Zhang et al., 2008; Bellot et al., 2009). While BH3 domains of BNIP3 and NIX are sufficient to induce the general autophagy response (Bellot et al., 2009), induction of mitophagy requires the LIR domain of NIX (Novak et al., 2010) and BNIP3 (Hanna et al., 2012; Zhu et al., 2013). Phosphorylation of BNIP3 at serines flanking its LIR domain promotes binding to LC3 family members and thereby increases mitophagy (Zhu et al., 2013), however, involved kinases are unknown (**Figure 3B**). It is not clear how phosphorylation of BNIP3 is regulated under hypoxia and if phosphorylation regulates NIX.

Recently, FUNDC1 has been implicated in mediating hypoxia-induced mitophagy (Liu et al., 2012). FUNDC1-mediated mitophagy is regulated at the posttranslational level by reversible phosphorylation. Under normal physiological conditions FUNDC1 is phosphorylated by Src kinase at Tyr18, which is located in the LIR motif, and at Ser13 by CK2 (**Figure 3C**) (Chen et al., 2014a). In response to hypoxia or loss of mitochondrial membrane potential the mitochondrially localized phosphoglycerate mutase family member 5 (PGAM5), a Ser/Thr phosphatase, dephosphorylates FUNDC1 at Ser13, whereas Tyr18 phosphorylation seems to be prevented before mitophagy-induction due to inactivation of Src kinase (**Figure 3C**) (Chen et al., 2014a). Dephosphorylated FUNDC1 displays a higher binding affinity to LC3, resulting in selective autophagosome incorporation and autophagic degradation of mitochondria (Liu et al., 2012; Chen et al., 2014a). Moreover, a study showed that hypoxia or mitochondrial uncouplers elevate protein levels of the autophagy-initiating kinase ULK1 (UNC51-like kinase 1) and target ULK1 to damaged mitochondria where it phosphorylates FUNDC1 at Ser17 and thereby enhances FUNDC1 binding to LC3 (**Figure 3C**) (Wu et al., 2014). However, it remains to be determined how BNIP3, NIX and FUNDC1 are interconnected or even needed during hypoxia-induced mitophagy.

Hepatic HIF-α Signaling and Mitochondrial Abundance

We examined the effect of HIF-α signaling on hepatic mitochondrial abundance in control and liver-specific $Vhl^{-/-}$, $Vhl^{-/-}/Hif1\alpha^{-/-}$, and $Vhl^{-/-}/Hif2\alpha^{-/-}$ mice. Mitochondrial protein levels are similar in control and knockout livers (Walter et al., 2014), suggesting that constitutive HIF-α signaling does not affect hepatic mitochondrial mass. However, subcellular fractionation of livers from control and $Vhl^{-/-}$ mice shows that mitochondrial protein levels are decreased in the heavy mitochondrial fraction of $Vhl^{-/-}$ livers compared with controls, whereas their levels are increased in the light mitochondrial fraction (Walter et al., 2014). Further purification of the light mitochondrial fraction by density gradient centrifugation and immunoblots of gradient fractions show that the OMM protein VDAC shifts toward lower density fractions. In summary, HIF-α signaling in the liver alters the ratio between heavy and light mitochondria, whereas mitochondrial protein levels are not changed in whole liver homogenates. It remains to be clarified if and how hepatic HIF-α signaling affects mitochondrial size, ultrastructure and function. Mitochondrial morphology and ultrastructure depend on mitochondria-shaping proteins that regulate organellar fusion and fission (Mishra and Chan, 2014). Rates of mitochondrial fission and fusion respond to changes in metabolism, and mitochondria regulate their shape to adjust their activity with metabolic conditions (Mannella, 2006).

Endoplasmic Reticulum and Hypoxia

ER Stress and Hypoxia

The ER is an extensive intracellular membrane network that extends throughout the cytoplasm and is essential for the translation and folding of membrane and secretory proteins (Gidalevitz et al., 2013). It is also a critical site of lipid and glucose metabolism, calcium homeostasis, and detoxification of drugs and metabolic byproducts. A biochemical process that is crucial for ER protein homeostasis is the formation of disulfide bridges, which is referred to as oxidative protein folding (Eletto et al., 2014). Disulfide bond generation by ER-localized protein

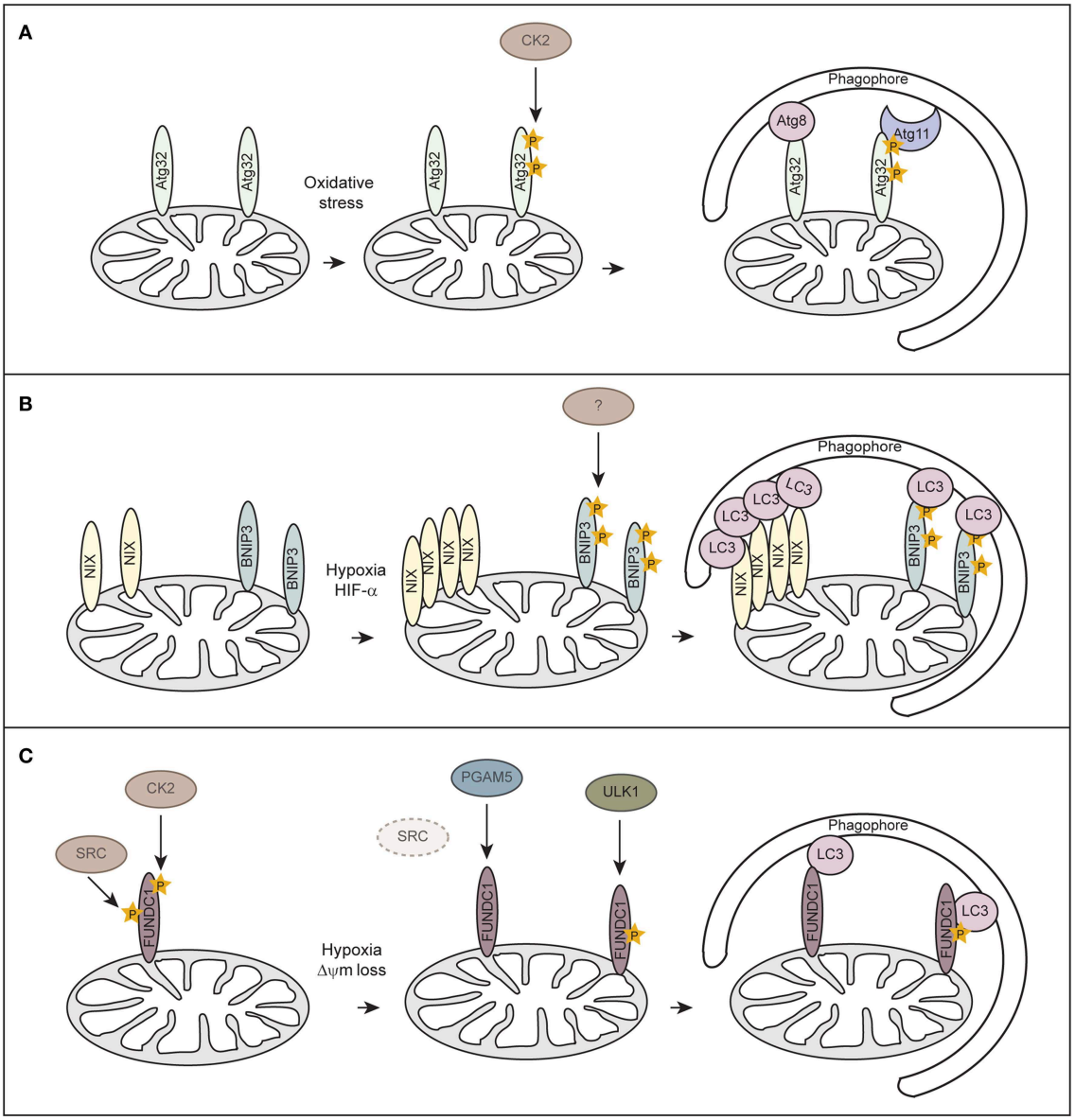

FIGURE 3 | Receptor-mediated mitophagy. (A) Atg32-mediated mitophagy in *S. cerevisiae*. Atg32 is an outer mitochondrial membrane protein whose expression is induced upon mitophagy-inducing conditions. Atg32 interacts with Atg8 and Atg11 via distinct domains. Casein kinase 2 (CK2) phosphorylates Atg32 upon mitophagy-inducing conditions, which is essential for the Atg11 interaction without affecting Atg32-Atg8 binding. **(B)** NIX/BNIP3-mediated mitophagy in mammalian cells. NIX and BNIP3 are outer mitochondrial membrane proteins that interact with LC3 through LIR motifs in their N-terminal region. Upon hypoxia, NIX and BNIP3 are transcriptionally induced in a HIF-α-dependent manner. Phosphorylation of BNIP3 promotes its binding to LC3 and subsequent mitophagy. The kinase for BNIP3 phosphorylation is unknown. **(C)** FUNDC1-mediated mitophagy in mammalian cells. FUNDC1 is an outer mitochondrial membrane protein that interacts with LC3 through a LIR domain at its cytosol-exposed N-terminus. Under normal physiological conditions, FUNDC1 is phosphorylated by SRC and CK2, thereby preventing LC3 binding. Upon hypoxia or loss of mitochondrial membrane potential ($\Delta\psi m$), the expression of SRC is strongly suppressed and PGAM5 dephosphorylates FUNDC1. Dephosphorylation of FUNDC1 enhances the interaction between FUNDC1 and LC3 and promotes mitophagy. Phosphorylation of FUNDC1 by ULK1 enhances its binding to LC3.

disulfide isomerases is an oxidative process, and molecular O_2 and H_2O_2 are the principal electron acceptors for oxidative folding in the ER (Eletto et al., 2014). Protein disulfide isomerases catalyze oxidation by coupling *de novo* disulfide formation to the reduction of O_2 to H_2O_2.

The term "endoplasmic reticulum stress" defines any perturbation that compromises the protein folding functionality of the ER (Walter and Ron, 2011). A number of biochemical and physiologic stimuli, such as perturbation in calcium homeostasis or redox status, elevated secretory protein synthesis, expression

of misfolded proteins, glucose deprivation, altered glycosylation, viral infection, and excess lipids can disrupt ER homeostasis and impose stress to the ER. These disturbances trigger an adaptive signaling pathway known as the unfolded protein response (UPR) that aims to restore ER homeostasis and function. Hypoxia is a physiologically important ER stress common to solid tumors. Several lines of evidence point to a strong relationship between hypoxia and the accumulation of misfolded proteins in the ER (Koumenis et al., 2007). In tumors, hypoxia is also associated with other conditions that can cause ER stress, such as glucose and amino acid deprivation, and oxidative stress.

The Unfolded Protein Response Signaling Pathways

Activation of the three canonical branches of the UPR is mediated by three stress-sensing ER transmembrane proteins: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) (**Figure 4**) (Walter and Ron, 2011; Faust and Kovacs, 2014). In a stress-free ER, these sensors are bound by the ER-resident chaperone glucose-regulated protein of 78 kDa (GRP78) in their intraluminal domains and rendered inactive. Upon ER stress, GRP78 dissociates from PERK, IRE1, and ATF6, leading to their activation (Bertolotti et al., 2000; Shen et al., 2002).

IRE1 is a serine-threonine kinase and endoribonuclease, which catalyzes the splicing of full-length *XBP1* (X-box-binding protein 1) mRNA to generate an active transcription factor, termed spliced *XBP1s*. XBP1s activates genes encoding proteins involved in protein folding, ER-associated degradation (ERAD) of misfolded ER proteins, and lipid synthesis.

ATF6 is comprised of two isoforms, ATF6α and ATF6β, and resides as transcriptionally inactive precursor protein in the ER membrane. ER stress leads to ATF6 translocation from the ER to the Golgi apparatus where it is cleaved sequentially by Site-1 and Site-2 proteases to produce an active transcription factor. ATF6 induces *XBP1* and genes mainly encoding ER chaperones and proteins involved in ERAD.

Dissociation of GRP78 from PERK leads to its homodimerization and activating autophosphorylation. PERK phosphorylates the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α) on serine 51. This phosphorylation event attenuates general translation, resulting in a reduced protein folding load of the ER (Baird and Wek, 2012), but it stimulates selective translation of the activating transcription factor 4 (ATF4), which plays a crucial role for the adaptation to stress. ATF4 target genes are involved in protein folding and assembly, metabolism, nutrient uptake, gene expression, alleviation of oxidative stress, autophagy, and the regulation of apoptosis. In addition to PERK, three other kinases induce eIF2α phosphorylation and preferential translation of ATF4: GCN2 (general control non-derepressible kinase 2), PKR (double-stranded RNA-activated protein kinase), and HRI (heme-regulated inhibitor kinase) (Baird and Wek, 2012). The PERK-eIF2α-ATF4 pathway is referred to as the integrated stress response (ISR), because divergent signals activate the four eIF2α kinases and the ISR, which seeks to remediate stress and restore cellular homeostasis.

Hypoxia and the Unfolded Protein Response

As protein synthesis and O_2-dependent protein folding are energy-intensive processes and chronic hypoxia markedly reduces intracellular ATP levels (Kim et al., 2006; Liu et al., 2006), control of mRNA translation is an important cellular response to hypoxia. Hypoxia activates PERK and thereby leads to eIF2α phosphorylation and global translation inhibition, whereas translation of ATF4 is increased in a PERK/eIF2α-dependent manner (Koumenis et al., 2002, 2007; Blais et al., 2004; Bi et al., 2005; Koritzinsky et al., 2006; Wouters and Koritzinsky, 2008). This is a rapid HIF-1α-independent response, occurring within minutes when cells are exposed to anoxic conditions and somewhat more slowly during moderate hypoxia. eIF2α phosphorylation is transient due to the negative feedback loop initiated by ATF4-dependent upregulation of GADD34 (growth arrest DNA-inducible gene 34), which dephosphorylates eIF2α (**Figure 4**).

Hypoxia increases intracellular ROS production in various cells to stimulate multiple biological responses, and mitochondria appear to be the primary source of hypoxic ROS (Liu et al., 2008). Mitochondrial hypoxic ROS activate the ISR to promote energy and redox homeostasis and to constitute an early adaptive response to hypoxia. Enzymatic antioxidants such as catalase and glutathione peroxidase reduce eIF2α phosphorylation caused by hypoxia, suggesting that H_2O_2 is a key biologically active form of ROS during hypoxia (Liu et al., 2008). ATF4 augments HIF-1α-mediated upregulation of its downstream targets to promote cell survival (Pereira et al., 2014).

Transient exposure to ER stress can condition and prepare cells for survival during a subsequent, more severe stress. This preconditioning is likely due to induction of pro-survival genes, and the ISR is an important prosurvival mechanism under hypoxia. Tumor cells in the primary tumor are exposed to hypoxia and might be preconditioned to survive the subsequent metastatic process. Indeed, cells with compromised PERK-eIF2α-ATF4 signaling are more sensitive to hypoxic stress *in vitro* and they form slower growing tumors *in vivo*, indicating that the PERK-eIF2α-ATF4 pathway confers a survival advantage for tumor cells under hypoxia (Fels and Koumenis, 2006). Severe hypoxia (<0.01%) and ER stress induce in cancer cells the transcription of *ULK1*, *LC3B*, and *ATG5* through the activity of ATF4 (Rouschop et al., 2010; Pike et al., 2013), and this up-regulation is crucial for maintaining high levels of autophagic flux to survive intratumoral hypoxia, metabolic stress, and starvation.

Analysis of gene expression changes during hypoxia indicated that UPR genes, including genes specifically regulated by XBP1, were most robustly induced during severe hypoxia/anoxia (Romero-Ramirez et al., 2004). Hypoxia induced in a HIF-1α-independent manner *XBP1* expression and activated splicing of its mRNA, resulting in increased levels of XBP1s (Romero-Ramirez et al., 2004). XBP1s colocalizes with hypoxia markers in tumors, and loss of *XBP1* increases the sensitivity of transformed cells to hypoxia-induced apoptosis and inhibits tumor growth (Wouters and Koritzinsky, 2008; Spiotto et al., 2010).

XBP1 is activated in triple-negative breast cancer (TNBC)—a type of breast cancer that does not have estrogen, progesterone and HER2 receptors—and has a pivotal role in the tumorigenicity

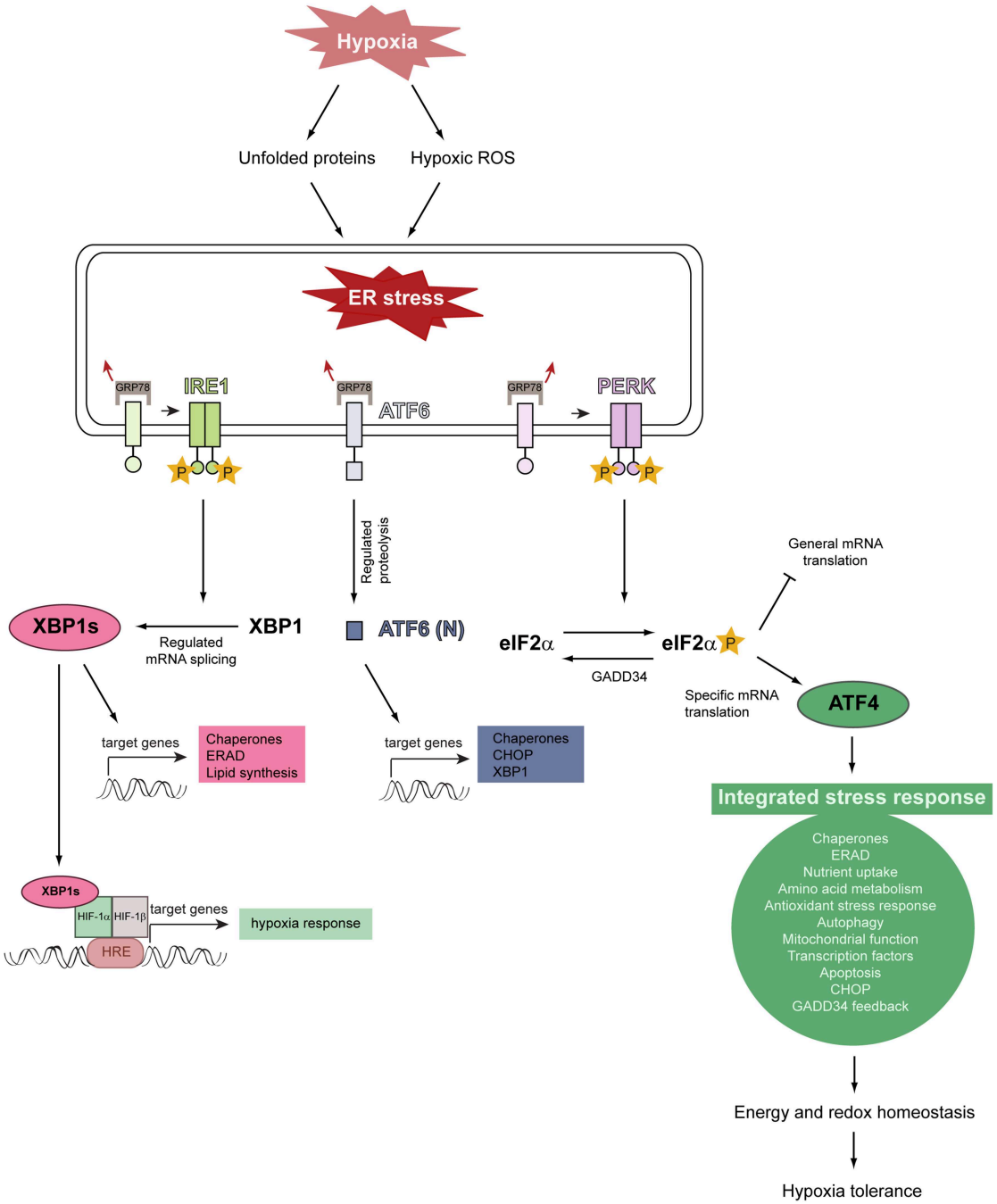

FIGURE 4 | Model illustrating the relationship between hypoxia, ER stress, and activation of the unfolded protein response. Severe hypoxic stress perturbs and reduces O_2-dependent protein folding capacity, resulting in the accumulation and aggregation of misfolded proteins in the ER lumen. Hypoxia increases intracellular ROS production and ROS stimulate multiple biological responses during O_2 deprivation. Unfolded proteins and hypoxic ROS trigger ER stress which leads to HIF-α-independent activation of the UPR. The UPR is initiated by the stress-sensing ER transmembrane proteins PERK, IRE1, and ATF6. The chaperone GRP78 is normally bound to these ER stress sensors and keeps them inactive, but dissociates from them under ER stress conditions. This dissociation leads to the activation of the three UPR pathways. GRP78 dissociation allows PERK to homodimerize, which facilitates autotransphosphorylation and kinase domain activation. Activated PERK phosphorylates eIF2α which decreases general translation while increasing the preferential translation of specific proteins, such as the transcription factor ATF4. ATF4 triggers the activation of a gene expression program referred to as the integrated stress response. ATF4 induces the expression of *GADD34*, which acts as a negative regulator of the PERK pathway by dephosphorylating eIF2α. The integrated stress response promotes energy and redox homeostasis and is an important prosurvival mechanism under moderate hypoxia. IRE1 homodimerization, followed by autotransphosphorylation, triggers its RNase activity. IRE1-mediated splicing of full-length *XBP1* mRNA generates *XBP1s*, which encodes an active transcription factor. ER stress leads to the translocation of ATF6 to the Golgi, where it is cleaved by regulated intramembrane proteolysis to produce the active transcription factor. Modified from Faust and Kovacs (2014).

and progression of TNBC (Chen et al., 2014b). HIF-1α is hyperactivated in TNBCs, but *XBP1* splicing is not directly regulated by HIF-1α. XBP1 drives TNBC tumorigenicity by assembling a transcriptional complex with HIF-1α that augments HIF-1α activity and regulates the HIF-1α transcriptional program, and *XBP1* knockdown reduces mammosphere formation in hypoxic conditions (Chen et al., 2014b). The XBP1 gene expression signature of TNBC patients correlates with HIF-1α and hypoxia-driven signatures and is associated with poor prognosis.

Although it is expected, a connection between ATF6 and hypoxia has not been reported yet and remains largely unexplored. One study showed that ATF6 is activated independently of HIF-1α by simulated ischemia (0.1% O_2) in a primary cardiac myocyte model system and inactivated upon reperfusion (Doroudgar et al., 2009). The absence of HIF-1α activation at 0.1% O_2 is consistent with other studies showing that HIF-α activation is maximal at 0.5% O_2 but decreases to nearly basal levels at lower O_2 concentrations (Jiang et al., 1996). Furthermore, while PERK and XBP1 activation occur in a HIF-1α-independent manner, a possible involvement of HIF-2α in UPR activation has not been addressed yet.

Peroxisomes and HIF Signaling

Peroxisomal Metabolism and Oxygen

Peroxisomes are ubiquitous and highly dynamic organelles whose number, size, and function are dependent on cell type and metabolic needs. They play key roles in the degradation of fatty acids [i.e., very long-chain fatty acids (VLCFAs), branched-chain FAs, polyunsaturated FAs (PUFAs)], ether lipid synthesis, cholesterol and bile acid synthesis, and metabolism of ROS (**Figure 5A**) (Van Veldhoven, 2010; Fransen et al., 2012; Faust and Kovacs, 2014). They also act as intracellular signaling platforms in redox, lipid, inflammatory, and innate immunity signaling (Dixit et al., 2010; Nordgren and Fransen, 2014; Odendall et al., 2014). The importance of peroxisomes for cellular metabolism is illustrated by the marked abnormalities in brain and systemic organs in peroxisome biogenesis disorders of the Zellweger spectrum in which functional peroxisomes are absent and disorders caused by single peroxisomal enzyme deficiencies (Raymond et al., 2009). Lack of peroxisomal metabolism creates severe biochemical abnormalities, leading to a variety of clinical symptoms both in patients with peroxisomal disorders as well as peroxisome-deficient mice (Kovacs et al., 2002; Raymond et al., 2009; Baes and Van Veldhoven, 2012; Faust and Kovacs, 2014).

Peroxisomal function depends highly on molecular O_2 due to its oxidative type of metabolism (**Figure 5A**). In fact, peroxisomes may be responsible for as much as 20% of O_2 consumption and 35% of H_2O_2 production in tissues such as the liver (Fransen et al., 2012). The number of peroxisomes is approximately 10–15 times less than that of mitochondria (De Duve and Baudhuin, 1966); therefore, on a per unit basis, peroxisomes may consume a significant amount of O_2 as compared to mitochondria. However, so far there has been no evidence linking HIF signaling to peroxisomes. We hypothesized that to minimize O_2 consumption under hypoxic conditions, HIF-α signaling may inhibit O_2-dependent peroxisomal metabolism and/or decrease peroxisome abundance. Since peroxisomes are highly abundant in the liver and liver-specific loss of *Vhl* causes severe lipid accumulation, we investigated peroxisome homeostasis and metabolism in the liver of control and liver-specific *Vhl*, *Vhl/Hif1*α, and *Vhl/Hif2*α knockout mice and explored the role of HIF-1α and HIF-2α in this context.

Peroxisome Biogenesis and Hepatic HIF-α Signaling

Peroxisome homeostasis is maintained by balancing biogenesis and degradation of peroxisomes. Peroxisomes can either multiply by growth and fission of pre-existing ones (Schrader et al., 2012) or develop *de novo* from the ER (Tabak et al., 2013). Proteins involved in peroxisome biogenesis, the peroxins, are encoded by *PEX* genes (Hasan et al., 2013; Smith and Aitchison, 2013). In mammalian cells, peroxisome proliferation is triggered by lipids which are substrates of peroxisomal metabolism and ligands of PPARα (Schrader et al., 2012). The majority of peroxins is not induced transcriptionally through peroxisome proliferators. The peroxins PEX11α, β, and γ are involved in the regulation of peroxisome size and number in mammalian cells (Schrader et al., 2012), but only *PEX11*α is a PPARα target gene. Their overexpression increases peroxisome number in the absence of extracellular stimuli or peroxisome metabolism (Li and Gould, 2002; Schrader et al., 2012). Recently, we showed that activation of HIF-α signaling in the liver does not affect the expression of *Pex* genes (Walter et al., 2014). *Pex11*α is the only peroxin that is transcriptionally induced in response to HIF-2α activation in $Vhl^{-/-}$ and $Vhl^{-/-}/Hif1\alpha^{-/-}$ livers (Walter et al., 2014). The peroxisome biogenesis machinery in $Vhl^{-/-}$ livers is functional, because peroxisome proliferation can be induced by treatment with PPARα-dependent and -independent peroxisome proliferators. However, HIF-2α signaling has a repressive effect on ligand- and fasting-induced PPARα activation (**Figure 5B**) (Walter et al., 2014).

Pexophagy

Three mechanisms for mammalian peroxisome degradation have been described, which include selective autophagy (pexophagy), proteolysis by peroxisomal Lon protease 2 (LONP2), and 15-lipoxygenase-1 (ALOX15)-mediated autolysis (Till et al., 2012). Studies using liver-specific $Atg7^{-/-}$ mice suggest that 70–80% of excess liver peroxisomes are degraded by pexophagy, while the remaining 20–30% are degraded via the action of LONP2 and ALOX15 (Till et al., 2012). A general rule applicable for both yeast and mammalian cells is that environmental conditions that require peroxisomal metabolism lead to peroxisome proliferation, followed by pexophagic degradation when the organelles are no longer required (Iwata et al., 2006; Farré et al., 2008; Motley et al., 2012).

The pexophagy receptors Atg30 and Atg36 were identified in *P. pastoris* and *S. cerevisiae*, respectively, and their overexpression stimulates pexophagy even under peroxisome-inducing conditions (Farré et al., 2008; Motley et al., 2012). Their synthesis is upregulated in peroxisome proliferation conditions, they

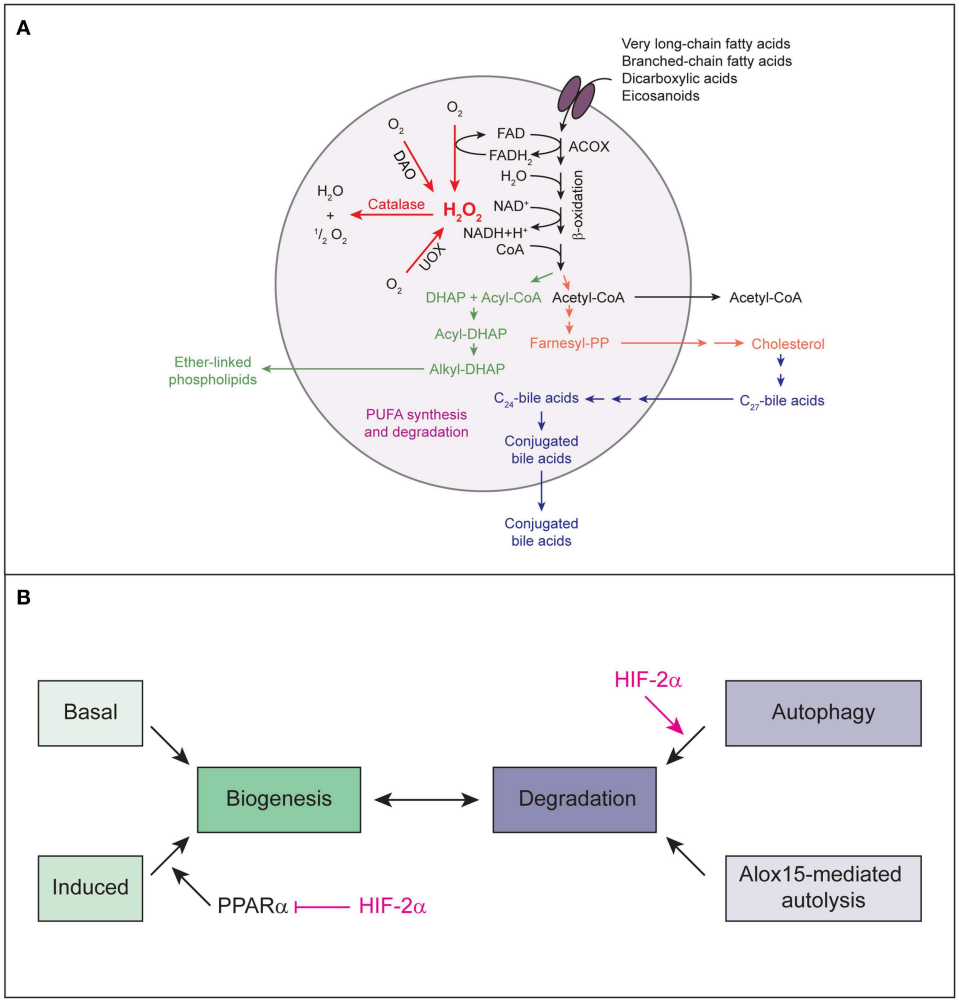

FIGURE 5 | (A) The major metabolic pathways in peroxisomes of the mammalian liver. Various lipids are transported by PMPs (e.g., the ABC transporter proteins ABCD1, ABCD2, ABCD3) into the peroxisomal matrix, where they are oxidized by the β-oxidation enzymes. The products of the β-oxidation can serve as substrates for the biosynthesis of ether-linked phospholipids, cholesterol and bile acids or may exit the peroxisome for further oxidation in mitochondria. With regard to PUFAs, peroxisomes not only degrade these compounds but are also involved in their formation through retroconversion of PUFAs by catalyzing the chain-shortening steps. Peroxisomal function depends highly on molecular O_2 due to its oxidative type of metabolism. Peroxisomal β-oxidation and the activity of other peroxisomal oxidases (e.g., UOX, DAO) result in the production of H_2O_2, which is decomposed by catalase. Modified from Schrader and Fahimi (2008). **(B)** Model for HIF-2α-mediated decrease in peroxisome abundance. Peroxisome homeostasis is achieved by balancing biogenesis and degradation of peroxisomes. HIF-2α signaling promotes degradation of peroxisomes by pexophagy. Reduced peroxisome abundance and the ensuing deficiency in peroxisomal function leads to major changes in the lipid profile, such as accumulation of VLCFAs. VLCFAs are activating ligands for the transcription factor PPARα. HIF-2α represses ligand-induced PPARα-mediated peroxisome proliferation and consequential restoration of peroxisome homeostasis. Thus, by simultaneously inducing pexophagy and counteracting PPARα, HIF-2α ensures efficient depletion of the peroxisome pool.

localize to the peroxisome membrane and bind to Pex3, and they depend on phosphoregulation for their interactions with components of the autophagy machinery (i.e., Atg8, Atg11) during pexophagy conditions (Farré et al., 2008, 2013). Pex3 acts as a docking station for several proteins involved in peroxisomal biogenesis and its interaction with Atg30 regulates the phosphorylation status of Atg30 by a yet unknown kinase (Burnett et al., 2015). Atg30 interacts also with Pex14, the scaffold protein Atg17, and the acyl-CoA binding protein Atg37 (Nazarko et al., 2014). The human ortholog of Atg37, acyl-CoA-binding domain containing protein 5 (ACBD5), is also peroxisomal and required for pexophagy (Nazarko et al., 2014). Despite their functional similarities Atg30 and Atg36 do not display any significant sequence homology and they are conserved only among a few yeast species.

There are no orthologous genes of *Atg30* and *Atg36* in mammals. Overexpression of *NBR1* and *SQSTM1*, which are autophagy receptors of ubiquitinated targets, induces clustering and degradation of peroxisomes in cell lines (Deosaran et al., 2013). SQSTM1 is not required for pexophagy when NBR1 is in excess, but its binding to NBR1 increases the efficiency of NBR1-mediated pexophagy (Deosaran et al., 2013). Artificial mono-ubiquitination of peroxisomal membrane proteins (PMPs) in mammalian cells causes peroxisome turnover

by pexophagy in a SQSTM1-dependent manner (Kim et al., 2008). However, it is unknown if a PMP is ubiquitinated under pexophagy-inducing conditions and whether subsequent interaction with NBR1 and/or SQSTM1 links ubiquitinated peroxisomes to the autophagic machinery.

In Chinese hamster ovary (CHO) cells, it has been suggested that under nutrient starvation PEX14 is involved in pexophagy by interacting with the lipidated form of LC3 (Hara-Kuge and Fujiki, 2008). Cell-free synthesized lipidated LC3 interacts in an *in vitro* assay with the transmembrane domain of recombinant PEX14, although PEX14 does not contain a LIR sequence that could ensure LC3 binding (Jiang et al., 2015). PEX14 is an essential component of the peroxisomal translocon complex (Hasan et al., 2013), and it has been proposed that the PEX14-LC3 and PEX14-PEX5 interactions are mutually exclusive (Hara-Kuge and Fujiki, 2008). This competitive interaction might ensure functional segregation of metabolically active and degradation-prone peroxisomes.

Overexpression of *Pex3* in CHO cells and mouse embryonic fibroblasts induced clustering of peroxisomes and NBR1-mediated pexophagy, albeit no direct interaction between PEX3 and NBR1 could be detected (Yamashita et al., 2014). Interestingly, ubiquitin signals were observed on peroxisomes upon *Pex3* overexpression, suggesting that a currently unidentified PMP is ubiquitinated in PEX3-mediated pexophagy and might function in NBR1 recruitment (Yamashita et al., 2014).

HIF-2α-Mediated Pexophagy in the Liver

We examined the effect of HIF-α signaling on hepatic peroxisome abundance in control and liver-specific $Vhl^{-/-}$, $Vhl^{-/-}/Hif1\alpha^{-/-}$, and $Vhl^{-/-}/Hif2\alpha^{-/-}$ mice. Peroxisome abundance is significantly decreased in livers of $Vhl^{-/-}$ mice (Walter et al., 2014). Reduction of peroxisome abundance is mediated by HIF-2α, because, with respect to the peroxisomal phenotype, we observe a striking rescue in $Vhl^{-/-}/Hif2\alpha^{-/-}$ but not $Vhl^{-/-}/Hif1\alpha^{-/-}$ mice. HIF-2α promotes pexophagy because peroxisome abundance is increased after inhibition of autophagy with 3-methyladenine (3-MA) in $Vhl^{-/-}$ mice. In addition, expression of a non-degradable active HIF-2α variant fails to decrease peroxisome abundance in liver-specific, autophagy-deficient $Atg7^{-/-}$ mice (**Figure 5B**) (Walter et al., 2014). In support of this finding super-resolution and electron microscopy demonstrated that both single and multiple peroxisomes, but no other cytoplasmic organelles, are sequestered in autophagosomes in $Vhl^{-/-}$ livers.

Peroxisome abundance and protein levels of NBR1 and SQSTM1 are concomitantly decreased in $Vhl^{-/-}$ and $Vhl^{-/-}/Hif1\alpha^{-/-}$ livers (Walter et al., 2014). Neither peroxisome abundance nor NBR1 and SQSTM1 levels decline in 3-MA-treated $Vhl^{-/-}$ mice, showing that the abundance of these receptors and peroxisomes are interconnected. Expression of a constitutively active HIF-2α variant results also in a concomitant decrease of peroxisome abundance and NBR1 levels. NBR1 and SQSTM1 colocalize with peroxisomes in $Vhl^{-/-}$ livers, but surprisingly NBR1 already localizes to peroxisomes in control livers (Walter et al., 2014). An intriguing feature of autophagy receptors is their tendency to oligomerize, which facilitates sequestration and clustering of the autophagic cargo. Indeed, treatment of $Vhl^{-/-}$ mice with 3-MA leads to a significant clustering of NBR1- and SQSTM1-positive peroxisomes, suggesting that binding of multiple NBR1 and SQSTM1 to peroxisomes and oligomerization of these receptors might induce peroxisome clustering and prime peroxisomes for pexophagy.

In summary, by simultaneously inducing pexophagy and counteracting PPARα, HIF-2α ensures efficient depletion of the peroxisome pool (**Figure 5B**). Our data show that the autophagy receptors NBR1 and SQSTM1 localize to peroxisomes and are degraded together with peroxisomes by HIF-2α-mediated pexophagy. However, it remains an open question how HIF-2α induces pexophagy at the molecular level, but several possibilities exist and are discussed below.

Peroxisome Abundance in Tumors

Peroxisome proliferation is a unique phenomenon generated by a broad spectrum of structurally diverse compounds, such as lipid-lowering drugs and plasticizers (Pyper et al., 2010; Misra et al., 2013). These compounds induce peroxisome proliferation in liver parenchymal cells of rodents, whereas no effects have been observed in non-human primates and humans. Prolonged exposure to peroxisome proliferators leads to the development of hepatocellular carcinomas in rodents (Misra et al., 2013). The mechanism(s) by which these non-mutagenic peroxisome proliferators induce liver tumors remains controversial. Evidence strongly implicates that hyperactivation of PPARα leads to disproportionate large increases in H_2O_2-generating enzymes, whereas the expression of H_2O_2-degrading enzymes is only moderately increased (Pyper et al., 2010). This imbalance increases the levels of H_2O_2 and other ROS in hepatocytes and contributes to oxidative stress, lipid peroxidation, and oxidative DNA damage (Pyper et al., 2010; Misra et al., 2013).

Information on the role of peroxisomes in human tumor development is scarce. It has been shown that the protein levels of peroxisomal branched-chain FA β-oxidation enzymes (i.e., α-methylacyl-CoA racemase, peroxisomal multifunctional protein 2) are upregulated in human prostate cancer (Zha et al., 2005) and that this pathway is essential for optimal proliferation of some prostate cancer cell lines (Zha et al., 2003). Recently, it has been shown that monocarboxylate transporter 2 localizes to peroxisomes in prostate cancer cells and that its expression increases from non-malignant to malignant cells (Valença et al., 2015). It would be important to know if these proteins are selectively upregulated or if peroxisome abundance is also increased in prostate cancer.

A decrease in peroxisome abundance has been observed in various tumor cells, including hepatocellular carcinoma (Litwin et al., 1999), colon carcinoma (Cable et al., 1992; Lauer et al., 1999), breast cancer (el Bouhtoury et al., 1992; Keller et al., 1993), and in renal cell carcinoma (Frederiks et al., 2010). However, so far the mechanism leading to reduced peroxisome abundance was unknown. We explored the relevance of HIF-2α-dependent pexophagy in human clear cell renal cell carcinomas (ccRCC), because loss of *VHL* function occurs in up to 90% of sporadic human ccRCC and HIF-2α is considered to be a driver

oncoprotein for ccRCC. Analysis of more than 200 ccRCC tissue samples revealed that peroxisome abundance is reduced in *VHL*-deficient ccRCC characterized by high HIF-2α levels (Walter et al., 2014), suggesting that HIF-2α-mediated pexophagy is relevant to human disease. Interestingly, peroxisome abundance is reduced more frequently in well-differentiated tumors, however, it remains to be determined if induction of pexophagy and subsequent loss of peroxisomes promotes or slows down tumor growth. Since HIF-2α stabilization is observed in the vast majority of solid tumors (Franovic et al., 2009; Qing and Simon, 2009), we propose that in addition to ccRCC HIF-2α-mediated pexophagy might also lead to reduced peroxisome abundance in other cancer types.

Metabolic Consequences of Reduced Peroxisome Abundance

Livers of $Vhl^{-/-}$ and $Vhl^{-/-}/Hif1\alpha^{-/-}$ mice are enlarged and display severe steatosis (Rankin et al., 2009; Walter et al., 2014). HIF-2α-mediated reduced peroxisome abundance leads to major changes in the lipid profile of $Vhl^{-/-}$ and $Vhl^{-/-}/Hif1\alpha^{-/-}$ livers, like accumulation of VLCFAs and VLC-PUFAs and depletion of docosahexaenoic acid (DHA) and arachidonic acid (Walter et al., 2014). Furthermore, the levels of the C_{27}-bile acid intermediates 3α,7α-dihydroxycholestanoic acid and 3α,7α,12α-trihydroxycholestanoic acid are significantly increased in the plasma of $Vhl^{-/-}$ and $Vhl^{-/-}/Hif1\alpha^{-/-}$ mice. These lipid changes are characteristic features of human patients and mice lacking peroxisomes (Raymond et al., 2009; Van Veldhoven, 2010; Wanders et al., 2010; Baes and Van Veldhoven, 2012). β-oxidation of VLCFAs and C_{27}-bile acid intermediates occurs only in peroxisomes, peroxisomes play a role in synthesis and degradation of PUFAs, and DHA synthesis requires one cyle of peroxisomal β-oxidation (Van Veldhoven, 2010). Since peroxisomes are essential for plasmalogen biosynthesis and substrates for peroxisomal β-oxidation also include branched-chain FAs, dicarboxylic FAs, and eicosanoids (Van Veldhoven, 2010), it is likely that additional changes in lipid metabolism result from reduced peroxisome abundance in response to HIF-2α activation.

Indirect consequences of reduced peroxisomal metabolism like activation of ER stress pathways and mitochondrial dysfunction might also contribute to alterations in lipid metabolism (Baumgart et al., 2001; Dirkx et al., 2005; Kovacs et al., 2009, 2012). Toxic effects of accumulation of peroxisomal β-oxidation substrates might damage the mitochondrial compartment by altering the lipid composition of mitochondrial membranes. It is well-known that free fatty acids act as potent detergents that can damage cellular membranes (Ho et al., 1995). Membrane properties (e.g., acyl chain order, fluidity, permeability, fusion events, lipid raft microdomains, protein activity) are affected by changes in VLCFAs, VLC-PUFAs, DHA, and plasmalogen levels, influencing secretory and vesicular trafficking pathways (Whitcomb et al., 1988; David et al., 1998; Gleissman et al., 2010; Obara et al., 2013). Hypoxia or loss of VHL function has been shown to delay endocytosis and thereby to enhance receptor tyrosine kinase-mediated signaling (Wang et al., 2009). Thus, lipid alterations as a result of HIF-2α-mediated pexophagy might affect endosomal trafficking and signaling pathways downstream of membrane receptors.

A hallmark of cancer is the reprogramming of metabolism, and recent data suggest that alterations in lipid metabolism play an important role in tumor development (Hirsch et al., 2010; Santos and Schulze, 2012; Currie et al., 2013). Fatty acids support cancer growth by providing substrates for energy production or by generating building blocks for membranes and signaling lipids in proliferating cells (Carracedo et al., 2013). VLC-PUFAs could be converted to eicosanoids, biologically active lipids involved in various pathological processes such as inflammation and cancer. Eicosanoids are degraded in peroxisomes, and loss of peroxisomes affects eicosanoid signaling. Peroxisomes are essential for the synthesis of ether lipids, which represent up to 20% of the total phospholipid mass in humans (Braverman and Moser, 2012; Lodhi and Semenkovich, 2014). Aggressive cancers have high levels of ether lipids, and the expression of the peroxisomal ether lipid synthetic enzyme alkylglyceronephosphate synthase (AGPS) is increased in various cancer cell lines and primary tumors (Benjamin et al., 2013). AGPS knockdown impairs cancer pathogenesis through not only lowering the levels of ether lipids, but also by altering fatty acid, eicosanoid, and glycerophospholipid metabolism, resulting in an overall reduction in the levels of several oncogenic signaling lipids (Benjamin et al., 2013).

Several environmental challenges including ischemia-reperfusion injury, obstructive sleep apnea, viral hepatitis, and alcohol-mediated liver injury are known to induce hepatic hypoxia signaling and are associated with changes in lipid metabolism (Nath and Szabo, 2012). It is tempting to speculate that HIF-2α-mediated pexophagy contributes, at least in some of these pathophysiological conditions, to alterations in lipid metabolism. Decreased plasma and hepatic levels of arachidonic acid and DHA have been observed in patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (Puri et al., 2007, 2009), suggesting impaired peroxisomal metabolism in their pathogenesis. An increasing number of studies suggest that peroxisome dysfunction may be a specific marker for Alzheimer disease. Kou et al. (2011) noted extensive peroxisome-related alterations in Alzheimer disease brains such as increased VLCFAs and decreased plasmalogens containing PUFAs. The question remains if the general loss of peroxisome functions in AD brains is due to pexophagy.

Models How HIF-2α Might Trigger Pexophagy

Since HIF-2α is a transcription factor, the most likely possibility would be that HIF-2α induces the expression of an autophagy receptor and subsequent clustering of peroxisomes via oligomerization of receptor-bound organelles, however, neither *Nbr1* nor *Sqstm1* are HIF-2α target genes (Walter et al., 2014). Ubiquitination of cargo prone for selective autophagic degradation is the most prevalent autophagy-targeting signal in mammals, and most of the currently known autophagy receptors harbor both ubiquitin-binding domains and LIRs (Kirkin et al., 2009; Stolz et al., 2014). HIF-2α might induce an E3 ubiquitin ligase that mediates the ubiquitination of a PMP (**Figure 6A**). We

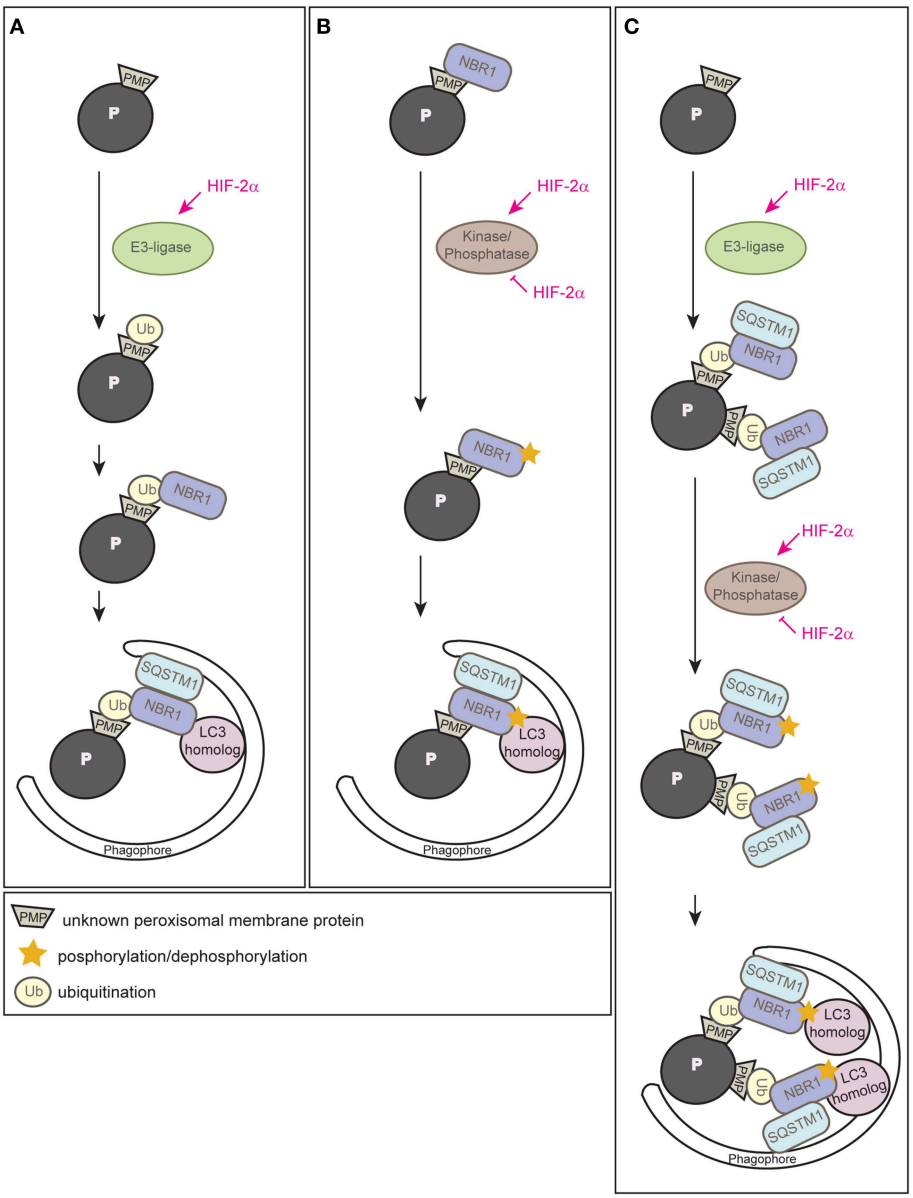

FIGURE 6 | Three alternative models illustrating how HIF-2α might trigger pexophagy. (A) HIF-2α might induce an E3 ubiquitin ligase that ubiquitinates a PMP that enhances the recruitment of the autophagy receptor NBR1 to the peroxisome surface. Accumulation of NBR1 on peroxisomes likely recruits SQSTM1, which was suggested to act as pexophagy co-receptor, and subsequently leads to clustering of peroxisomes via oligomerization of receptor-bound organelles. Accumulation of a critical mass of autophagy receptors might prime phagophore assembly at peroxisomes. **(B)** NBR1 could be recruited to peroxisomes independently of ubiquitin via its membrane-interacting amphipathic α-helical J domain. HIF-2α might induce or inhibit a kinase/phosphatase that leads to a change in the posttranslational modification of peroxisome-bound NBR1 and thereby triggers recruitment of the autophagic machinery. **(C)** HIF-2α-dependent activation of pexophagy might be a 2-step process and HIF-2α functions as master regulator that combines two layers of posttranslational modifications to trigger pexophagy. First, it induces an E3 ubiquitin ligase leading to an increased ubiquitination of PMP(s) and subsequent accumulation of NBR1 and SQSTM1 on peroxisomes. Second, HIF-2α activates or inhibits a kinase/phosphatase that leads to a change in the posttranslational modification of peroxisome-bound NBR1 and thereby enhances its binding affinity to a LC3 homolog that finally results in pexophagy. Modified from Schönenberger et al. (2015).

propose that HIF-2α signaling increases in this manner NBR1 accumulation on peroxisomes, which in turn serves as a platform for the recruitment of SQSTM1 to achieve a critical mass of autophagy receptors on peroxisomes required for pexophagy (Schönenberger et al., 2015). This might concentrate sufficient ubiquitin-like modifiers (e.g., LC3 and GABARAPs) in close proximity to peroxisomes to prime phagophore assembly. The peroxisomal membrane harbors three E3 ligases (PEX2, PEX10,

PEX12) that are essential for peroxisome biogenesis and involved in PEX5 receptor ubiquitination. Their transcriptional induction and concomitant increase of protein levels could increase their ubiquitination capability leading to enhanced ubiquitination of PMPs, but HIF-2α does not induce the expression of those E3 ligases (Walter et al., 2014). Thus, further studies are required to identify and characterize putative E3 ligases involved in HIF-2α-mediated pexophagy.

Why does NBR1 localize to peroxisomes in control livers where pexophagy is not induced? In fact, yeast Atg30 and Atg36 also localize to peroxisomes under peroxisome proliferation conditions, but they depend on phosphoregulation for their interactions with components of the autophagy machinery during pexophagy conditions (Farré et al., 2008, 2013). Posttranslational modifications (e.g., phosphorylation, ubiquitination, acetylation) of autophagy proteins are crucial for induction, inhibition, cargo-recognition, and fine-tuning of autophagy. We propose that additional protein modifications are very likely necessary to ultimately drive pexophagy by recruiting and tailoring the autophagic machinery to peroxisomes. For example, phosphorylation as an inducing event of autophagy is conserved from yeast to mammals and has already been discussed above in the context of pexophagy in yeast and mitophagy. Phosphorylation of SQSTM1 and optineurin increases affinity to ubiquitin chains and LC3 (Stolz et al., 2014), and NBR1 phosphorylation by glycogen synthase kinase 3 prevents the aggregation of ubiquitinated proteins and their selective autophagic degradation (Nicot et al., 2014). We propose that HIF-2α governs pexophagy by promoting posttranslational modifications of PMPs and/or autophagy receptors that enhance interactions of receptor-labeled peroxisomes with the autophagic machinery (**Figure 6B**) (Schönenberger et al., 2015).

Finally, one could envision that HIF-2α-dependent activation of pexophagy is a 2-step process that involves interplay between ubiquitination of a PMP(s) as well as phosphoregulation of autophagy receptors or a PMP(s) as a trigger of pexophagy (**Figure 6C**) (Schönenberger et al., 2015). Interestingly, a similar interplay promotes PINK1-Parkin-mediated mitophagy whereby phosphorylation of ubiquitin contributes to a feedforward mechanism for ubiquitination events on dysfunctional mitochondria (Ordureau et al., 2014).

A receptor protein complex (RPC) model has been proposed that encompasses the receptor protein as the key player that establishes interactions with ligands, scaffold, and phagophore proteins (Nazarko et al., 2014). The question remains which components of the RPC are involved in HIF-2α-driven pexophagy. Is there an Atg11 homolog in the mammalian liver that would act as a scaffold for HIF-2α-mediated pexophagy? Little is known about mammalian autophagy adaptor proteins that bind to LC3 family members and serve as an anchor point to regulate autophagosome formation around the specific cargo (Stolz et al., 2014). Similar to Atg11, ALFY (autophagy-linked FYVE protein) is a scaffolding protein implicated in aggrephagy that links cargo to the autophagic machinery (Isakson et al., 2013). Moreover, Huntingtin (HTT) has been proposed to serve as adaptor for any type of selective autophagy, because the domain of HTT shares structure similarities and binding activity with the yeast Atg11 protein and interacts with autophagic effector proteins (Ochaba et al., 2014). It is tempting to speculate that ALFY or HTT are part of the RPC mediating HIF-2α-induced pexophagy and thus, functioning as scaffold protein(s). In summary, the identification of HIF-2α as an inducer of pexophagy opens new avenues for studying the underlying molecular mechanism.

Concluding Remarks

We have described hypoxia signaling pathways that regulate function and abundance of mitochondria, ER, and peroxisomes under hypoxia or in response to loss of VHL function. There is emerging evidence that these O_2-related organelles exhibit a close functional interplay, and peroxisomal alterations influence mitochondrial and ER functions and vice versa. Although peroxisomal function depends highly on molecular O_2, there has been no evidence linking their abundance to O_2 availability and HIF-α signaling. In a recent study we identified a unique function of HIF-2α as promoter of pexophagy. An open question that remains to be answered is how HIF-2α induces pexophagy, and we discussed in this review alternative models for how it might trigger pexophagy. Posttranslational modification of autophagy-related proteins and receptors has emerged as an essential regulatory mechanism of selective autophagy. Future studies should address which posttranslational modifications regulate HIF-2α-mediated pexophagy and which components of the receptor protein complex are involved in HIF-2α-mediated pexophagy. In addition, it remains to be determined how HIF-α signaling affects mitochondrial size and ultrastructure and consequently their activity. PPARα modulates metabolic and inflammatory pathways by responding to nutritional signals through ligand activation of transcription, and it is a target of drugs in use and in development to treat diseases. We showed that HIF-α signaling has a repressive effect on ligand-dependent PPARα transcriptional activity, but the mechanism by which HIF-α exerts its inhibitory effect requires further studies. In the past the role of peroxisomes in the cell and in human disease apart from peroxisomal disorders has been grossly underestimated, but this might change given increasing appreciation for the complexity of their interactions with other organelles and the recent discovery of novel functions for peroxisomes. Reduction in peroxisome abundance by pexophagy might positively and negatively impact human disorders including cancer, inflammation, metabolic and neurodegenerative diseases. Along with mechanistic studies of HIF-2α-dependent regulation of pexophagy, the identification of pharmacological regulators of pexophagy might have practical health benefits.

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