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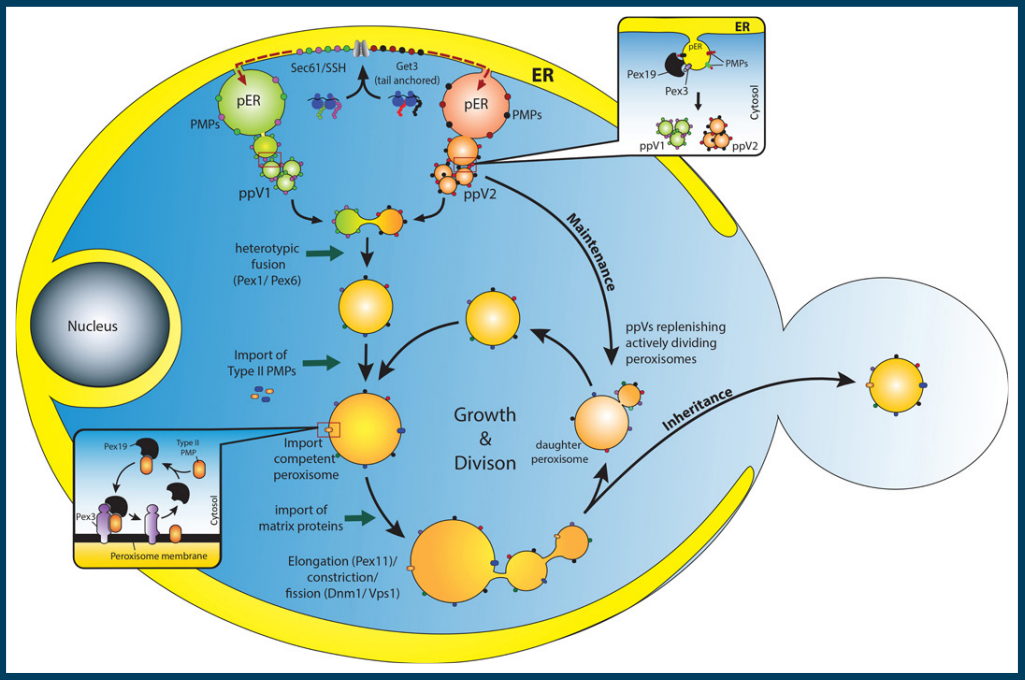

ORIGIN AND SPATIOTEMPORAL DYNAMICS OF THE PEROXISOMAL ENDOMEMBRANE SYSTEM

Topic Editors
Vladimir I. Titorenko and Richard A. Rachubinski

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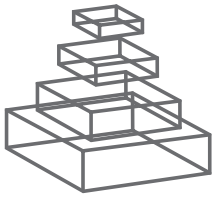

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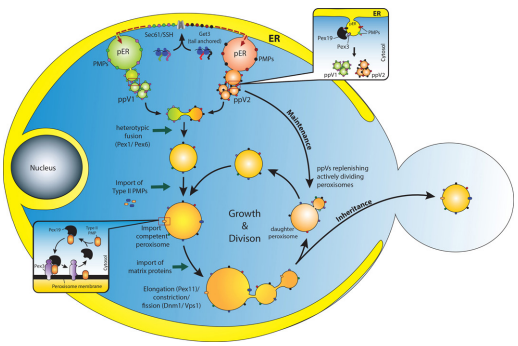

Agrawal, G., and Subramani, S. (2013). Emerging role of the endoplasmic reticulum in peroxisome biogenesis. Front. Physiol. 4, 286. doi: 10.3389/fphys.2013.00286.

The peroxisome is an organelle with essential roles in lipid metabolism, maintenance of reactive oxygen species homeostasis, and anaplerotic replenishment of tricarboxylic acid cycle intermediates destined for mitochondria. Peroxisomes constitute a dynamic endomembrane system. The homeostatic state of this system is upheld via two pathways for assembling and maintaining the diverse peroxisomal compartments constituting it; the relative contribution of each pathway to preserving such system may vary in different organisms and under various physiological conditions. One pathway begins with the targeting of certain peroxisomal membrane proteins to an endoplasmic reticulum template and their exit from the template via pre-peroxisomal carriers; these carriers mature into metabolically active peroxisomes containing the entire complement of membrane and matrix proteins. Another pathway operates via growth and maturation of pre-existing peroxisomal precursors that do not originate from the endoplasmic reticulum; mature peroxisomes proliferate by undergoing fission. Recent studies have uncovered new roles for the peroxisomal endomembrane system in orchestrating important developmental decisions and defining organismal longevity. This Frontiers Special Topic Issue is focused on the advances in our understanding of how evolutionarily distant organisms coordinate the formation, maturation, proliferation, maintenance, inheritance and quality control of the peroxisomal endomembrane system and how peroxisomal endomembranes communicate with other cellular compartments to orchestrate complex biological processes and various developmental programs from inside the cell.

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EDITORIAL
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Origin and spatiotemporal dynamics of the peroxisomal endomembrane system

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The peroxisome is an organelle with essential roles in lipid metabolism, maintenance of reactive oxygen species homeostasis, and anaplerotic replenishment of tricarboxylic acid cycle intermediates destined for mitochondria (Islinger et al., 2012; Beach and Titorenko, 2013; Wanders, 2014). Peroxisomes constitute a dynamic endomembrane system. The homeostatic state of this system is upheld via two pathways for assembling and maintaining the diverse peroxisomal compartments constituting it; the relative contribution of each pathway to preserving such system may vary in different organisms and under various physiological conditions. One pathway begins with the targeting of certain peroxisomal membrane proteins to an endoplasmic reticulum (ER) template and their exit from the template via pre-peroxisomal carriers; these carriers mature into metabolically active peroxisomes containing the entire complement of membrane and matrix proteins (Titorenko and Rachubinski, 2009; Hu et al., 2012; Tabak et al., 2013). Another pathway operates via growth and maturation of pre-existing peroxisomal precursors that do not originate from the ER; mature peroxisomes proliferate by undergoing fission (Nuttall et al., 2011; Hettema et al., 2014; Knoops et al., 2014). Recent studies have uncovered new roles for the peroxisomal endomembrane system in orchestrating important developmental decisions and defining organismal longevity (Titorenko and Rachubinski, 2004; Dixit et al., 2010; Beach et al., 2012). This Frontiers Research Topic is focused on the advances in our understanding of how evolutionarily distant organisms coordinate the formation, maturation, proliferation, maintenance, inheritance, and quality control of the peroxisomal endomembrane system and how peroxisomal endomembranes communicate with other cellular compartments to orchestrate complex biological processes and various developmental programs from inside the cell. Veenhuis and van der Klei (2014) provide insights into the mechanisms underlying the biogenesis of early peroxisomal precursors that do not arise from the ER. In the yeast *Hansenula polymorpha*, these vesicular precursors undergo multistep maturation into metabolically active peroxisomes only after acquiring the peroxin Pex3; the subsequent import of membrane and matrix proteins into such Pex3-containing peroxisomal precursors drives their multistep conversion into mature peroxisomes. Agrawal and Subramani (2013) discuss the relationship between two alternative routes of peroxisome biogenesis. One route involves the *de novo* formation of peroxisomes from an ER template, while the second involves the growth and division of pre-existing peroxisomes. The authors suggest that both routes operate simultaneously in organisms across phyla and propose a model that integrates the two routes into a single pathway for peroxisome assembly and maintenance. The model posits that a balance between progression rates of the two routes is modulated by various intracellular and extracellular signals; such modulation enables to preserve the peroxisomal endomembrane system under various physiological conditions. Kim and Mullen (2013) review the diverse ways through which the peroxin Pex16 can function to preserve the peroxisomal endomembrane system in such evolutionarily distant organisms as the yeast *Yarrowia lipolytica*, the plant *Arabidopsis thaliana*, and mammals. They dissect the mechanisms by which this peroxin orchestrates both routes of peroxisome biogenesis, i.e., the route of *de novo* formation of peroxisomes from an ER template and the route of growth and division of pre-existing peroxisomes, in these organisms. To explore the relationship between peroxisomes and the ER, Barton et al. (2013) concurrently visualized both organelles in living *A. thaliana* plants expressing differently colored peroxisome- and ER-targeted fluorescent proteins. The authors provide evidence that, although peroxisomes can be found in close contact with the ER, no luminal continuity exists between the two organelles. Mohanty and McBride (2013) evaluate evidence that the recently discovered vesicular flow from mitochondria to peroxisomes plays important roles in the assembly, maintenance, metabolic functions, and signaling activities of the peroxisomal endomembrane system in mammalian cells. They outline the molecular mechanisms underlying such a vesicular coupling of mitochondria to different compartments of the peroxisomal endomembrane system. Hasan et al. (2013) explore the molecular dynamics of a multistep process for protein import into the matrix of peroxisomes. They discuss the recent advances in our understanding of the mechanisms underlying the formation of a receptor/cargo-complex in the cytosol and its subsequent docking at the peroxisomal membrane, the translocation of the

cargo protein across the membrane and its release into the peroxisomal matrix, and the recycling of receptor molecules. Ast et al. (2013) discuss the molecular mechanisms by which isoforms of proteins known to be peroxisomal can also be actively sorted to the cytosol, mitochondrion, nucleus, or plastid. The authors hypothesize that such dual sorting of peroxisomal proteins within a cell could have an important role in extending the metabolic capacity of peroxisomes in response to specific changes in cell physiology and/or environmental conditions. Kunze and Hartig (2013) explore how different intermediates of the glyoxylate cycle are transported across the peroxisomal membrane and how individual enzymes of this cycle are distributed along both sides of the membrane. They suggest that the efficient and selective transport of glyoxylate and other metabolites across the peroxisomal membrane may be essential for the high functional adaptability of peroxisomes. Fujiki et al. (2014) discuss the multicomponent protein machineries that in mammalian cells orchestrate the assembly of membrane proteins in the peroxisomal membrane and the import of matrix proteins across this membrane. Nordgren et al. (2013) examine the mechanisms by which a selective degradation of dysfunctional or excessive peroxisomes in a mammalian cell enables it to maintain a healthy population of peroxisomes. They discuss the evidence supporting the essential contribution of defects in peroxisome degradation to human disease. Van Veldhoven and Baes (2013) review how mutations impairing peroxisome biogenesis affect organismal size, development, and longevity in various invertebrate and vertebrate models, including nematodes, fruit fly, zebrafish, and mouse. Their analysis implies that a reduced size at birth, delay in development, and shortened lifespan are the most common features of different peroxisome biogenesis deficiencies. Maruyama and Kitamoto (2013), as well as Peraza-Reyes and Berteaux-Lecellier (2013), explore mechanisms underlying the essential roles of peroxisomes in the morphogenetic program initiated by physical damage to hyphae, biosynthesis of biotin, and sexual development in fungi.

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A critical reflection on the principles of peroxisome formation in yeast

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We have evaluated the current knowledge on peroxisome proliferation in yeast. In wild-type cells, peroxisomes multiply predominantly by fission at conditions that require peroxisome function(s) for growth. In cells that lack peroxisomes, for instance in *pex3* and *pex19* mutants or in mutants that display inheritance defects, peroxisomes may form *de novo*. We propose a novel machinery for the *de novo* formation of peroxisomes in *pex3* cells, in which new peroxisomes do not arise from the endoplasmic reticulum. This machinery is based on the recent observation that membrane vesicles are present in *pex3* cells that display peroxisomal characteristics in that they contain specific peroxisomal membrane and matrix proteins. These structures are the source for newly formed peroxisomes upon reintroduction of Pex3. Furthermore, we critically evaluate the principles of sorting of other peroxisomal membrane proteins to their target organelle and the function of the endoplasmic reticulum therein.

Keywords: peroxisome, fission, endoplasmic reticulum, *de novo* peroxisome formation, yeast

INTRODUCTION

Peroxisomes are highly versatile organelles that readily adapt their numbers and physiological function in relation to metabolic needs. This functional flexibility requires a careful regulation of controlling organelle number and size. The factors controlling organelle size are still an enigma.

In yeast, low numbers of peroxisomes are normally present in cells grown at glucose excess conditions. However, when the cells are placed in media supplemented with carbon sources that require peroxisomal enzymes for growth (i.e., fatty acids, methanol, purines, and D-amino acids), organelle proliferation rapidly starts (Veenhuis et al., 1978). The mode of yeast peroxisome multiplication is still controversial. The current models range from the suggestion that in normal wild-type (WT) cells peroxisome multiplication exclusively results from fission to the view that all organelles form *de novo* from the endoplasmic reticulum (ER). Also combinations of these two modes have been suggested.

This contribution presents a critical overview of recent data on peroxisome multiplication in yeast and proposes possible novel directions aimed at resolving the molecular mechanisms of peroxisome formation.

PEROXISOME DEVELOPMENT

The origin of peroxisomes is still controversial. Following their discovery, the organelles were considered to bud from the ER based on the observations that peroxisomes—and in particular young developing ones—were invariably seen in close contact with the ER. Since direct contacts between the organelles were not observed, the ER theory was replaced by a model of development by growth and fission of pre-existing ones (Lazarow and Fujiki, 1985). The first morphological data that suggested growth and fission came from kinetic studies using the yeast *Hansenula polymorpha*, shifted from glucose to methanol, conditions that require peroxisome enzymes for growth (Veenhuis et al., 1978). During growth on glucose, *H. polymorpha* cells contain a single peroxisome. In the first 6–8 h after the shift alcohol oxidase and catalase, key peroxisomal enzymes of methanol metabolism, are synthesized and incorporate in the original organelles present in the glucose inoculum cells. After maturation, the organelle formed an extension that subsequently budded off and in turn grew (**Figure 1**). This way the cells formed 5–7 organelles of approximately equal size (**Figure 1**) within a period of 24 h of growth. These morphological data were subsequently reinforced by biochemical data which indicated that peroxisomal matrix proteins were synthesized on free ribosomes in the cytosol (Goldman and Blobel, 1978; Fujiki et al., 1985) and post-translationally incorporated in the organelle by a unique protein translocation machinery (Lazarow and Fujiki, 1985).

The first genetic support for the growth and fission model came from studies in the Tabak group, who identified the *Saccharomyces cerevisiae* dynamin-like protein (DLPs) Vps1 as a component involved in peroxisome fission together with actin and the class V myosin motor Myo2 being required for transport of newly separated organelles to the developing bud (Hoepfner et al., 2001). Studies in other yeast species, plant and mammals resolved another DLPs (designated Drp1, Dnm1 or DRP3 respectively) that is involved in peroxisome fission (Koch et al., 2003; Kuravi et al., 2006; Zhang and Hu, 2009). Interestingly, these proteins also play a role in mitochondrial fission. Subsequent studies identified additional components, namely the tail anchored protein Fis1 that, together with Mdv1 (only in yeast) and, unique for *S. cerevisiae*, Caf1, are required to bind Dnm1—but not Vps1—to the target membrane (Motley et al., 2008; Nagotu et al., 2008a). In addition, Mff1 and GDAP1 have been identified to control both mitochondria and peroxisome fission in mammalian cells

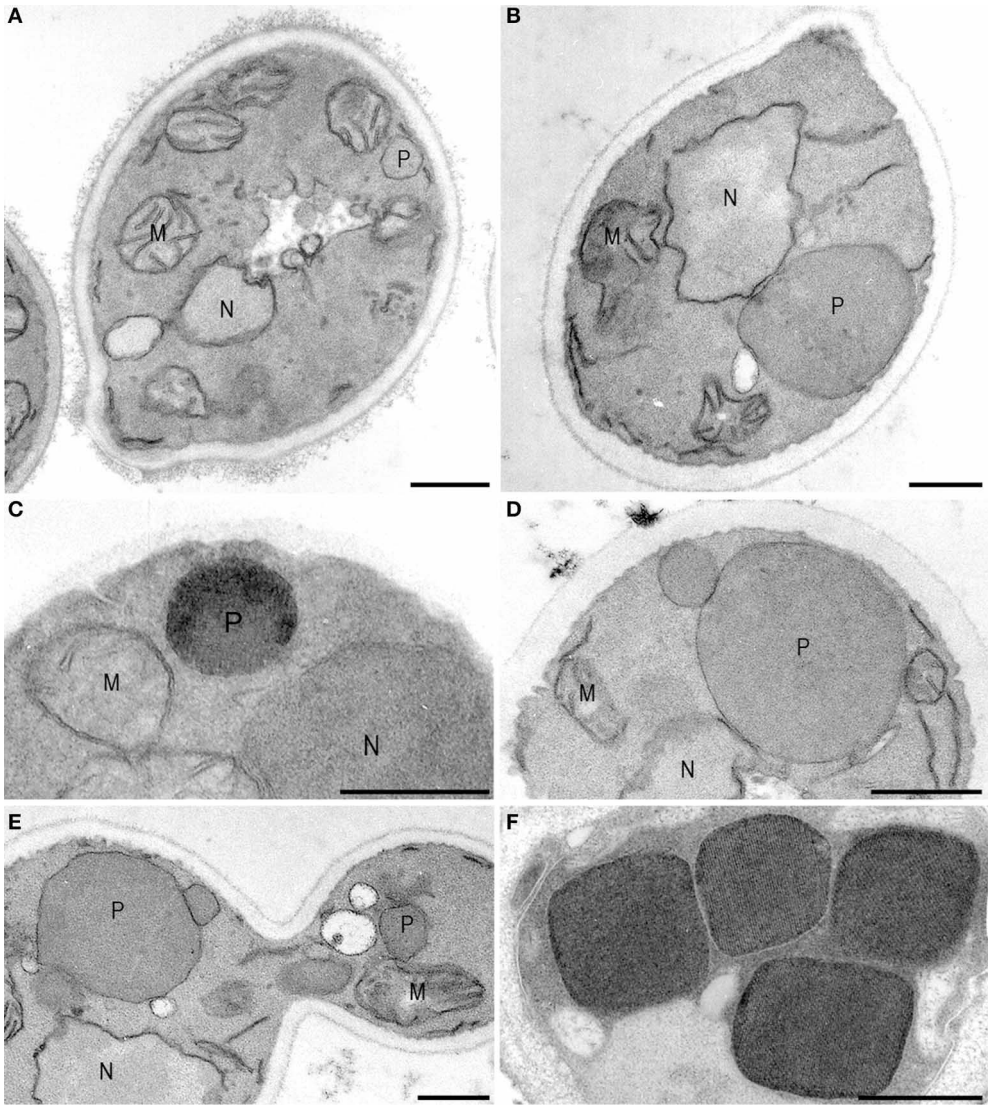

FIGURE 1 | Peroxisome multiplication imaged. *Hansenula polymorpha* cells are shifted from glucose- to methanol media, conditions that require peroxisome functions for growth. On glucose, characteristically a single peroxisome is present per cell **(A)** that—upon a shift to methanol containing media—incorporates alcohol oxidase **(C)**, catalase and dihydroxyacetone synthase proteins, which are essential for growth on methanol. As a result the organelle increase in size **(B)** and, after maturation, forms a new organelle by fission **(D)** that subsequently will grow. A similar a-symmetric fission machinery is responsible for the administration of small organelles to the yeast bud **(E)**. When the culture has reached the late exponential phase of growth, typically 4–6 organelles are present of comparable shape. The cuboid shape is due to the presence of large luminal alcohol oxidase crystals. M, mitochondria; N, nucleus; P, peroxisome. Cells are fixed with KMnO4, except **(C,F)**, which are glutaraldehyde fixed. In these cells alcohol oxidase activity is demonstrated using CeCl3.

(Gandre-Babbe and van der Bliek, 2008; Huber et al., 2013). With this, consensus had been reached in the field for the autonomous nature of peroxisomes for many years.

This view changed again when the first data came available on the reintroduction of peroxisomes in cells lacking the organelles due to a mutation in a gene essential for peroxisome membrane biogenesis. Bulk of these studies were conducted with *pex3* or *pex19* cells, in which the organelles reappeared after reintroduction of the corresponding deleted gene, by a process often referred to as "*de novo* peroxisome formation" (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Haan et al., 2006). It is commonly accepted that peroxisomes, which form *de novo*, are not created from scratch, but originate from another membrane in line with the proposition "Omnis membrana e membrana" (Günther Blobel, Nobel Prize 1999). Most of the available experimental data point to the ER as a template for this pathway.

With this, the question raised whether and in how far this process contributes to the total peroxisome population in WT cells. Indications for this came from studies in which *VPS1* and/or *DNM1* were deleted. In all yeast species studied this resulted in the

reduction of peroxisome numbers to generally only one organelle per cell. Under these conditions the *de novo* peroxisome formation machinery is normally active since Dnm1 and Vps1 are not involved in this process (Motley et al., 2008; Nagotu et al., 2008b). Very recently, it was shown that a peroxisome-deficient phenotype was obtained in mutant yeast cells in which both *de novo* synthesis and fission are blocked (in *H. polymorpha pex11 pex25* cells), but not when only one of these processes was blocked in *pex11* or *pex25* cells; (Saraya et al., 2011). This reinforces that in yeast the cellular peroxisome population can be maintained predominantly by fission (in *pex25* cells).

Taken all data together, fission appears to be the dominant mode of organelle maintenance in yeast although it cannot be excluded that few organelles are formed *de novo* too in WT cells. Evidence for the latter is however not observed. Possibly, the yeast model is not universal as data have been presented suggesting that in mammals *de novo* synthesis prevails in organelle formation (Kim et al., 2006). However, other studies indicate that mammalian peroxisomes also predominantly form by fission (Huybrechts et al., 2009; Delille et al., 2010).

Clearly, organelle multiplication in substrate induced yeast cells serves different functions: in WT cells new organelles will be formed that will stay in the mother cell and mature to support growth on the carbon source that is supplemented for growth (i.e., oleate or methanol). On the other hand, organelles multiply dependent of the cell cycle to administrate new organelles to the daughter cell (**Figure 2**). While mother organelles are hooked up in the mother cell via the function of Inp1, these newly formed organelles bind Inp2, which is required for binding of the organelle to Myo2 and subsequent transport to the developing bud (Fagarasanu et al., 2005, 2006; Knoblach et al., 2013). This suggests that upon peroxisome fission two types of organelles may form that biochemically differ. Possibly, this is related to the function of Pex19. This suggestion is based on the important observation that Inp2 is not the sole determinant in Myo2 binding in that Inp2 interacts with both Myo2 and Pex19 to serve the function in organelle transport to the bud (Otzen et al., 2012). Therefore, it may well be that the availability of Pex19 at the membrane (and thus Inp2 binding) is the key determinant that prescribes which organelle is donated to the bud and which one will stay in the mother cell to serve a function in optimal cell metabolism.

Obviously, peroxisome fission and partition have to be carefully controlled to sustain optimal cell health. Indeed, a mutation in human DLP1 results in a lethal phenotype (Waterham et al., 2007). The fission process can be divided into three steps, namely the initial organelle elongation step, growth and constriction of the elongated organelle followed by the actual fission process. Various proteins have been suggested to be involved in peroxisome fission, including members of the Pex11 and the Pex23 protein families (Kiel et al., 2006). In yeast the molecular function of most of these proteins is unknown except for Pex11 and Pex25 (Saraya et al., 2011). Opalinski and colleagues demonstrated that Pex11 is specifically involved in the initial membrane elongation process, a function that is mediated by an amphipathic α-helix located in the N-terminus of the protein and that is conserved between species (Opalinski et al., 2011). The principles of constriction are unknown but may, similar to mitochondria, be related to the function of the ER in conjunction with actin filaments. Scission is mediated by Dlp's, i.e., Vps1 and Dnm1 in baker's yeast (Hoepfner et al., 2001; Kuravi et al., 2006) and Dnm1 in *H. polymorpha* (Nagotu et al., 2008b).

Surprisingly, peroxisome fission is associated with a major rearrangement of various peroxisomal membrane proteins (PMPs) belonging to the importomer that are specifically donated to the developing small organelle (Cepinska et al., 2011). This process appeared to be dependent on the function of Pex11. In contrast to the bulk administration of PMPs to the small peroxisome, fluorescence microscopy analysis revealed that generally very low levels of matrix components, often below the limit of detection, are included in these structures. Apparently, during yeast fission the developing bud is administrated with new organelles that are optimally equipped for their function in matrix protein import (thereby determining their future function!) rather than having an immediate function in cell metabolism (**Figure 2**).

Obviously, peroxisome development requires functional interactions with other organelles, such as the ER, where most peroxisomal phospholipids are synthesized (Raychaudhuri and Prinz, 2008) as well as with mitochondria, which synthesize cardiolipin, a lipid present in the peroxisomal membrane (Wriessnegger et al., 2007), and heme (for catalase synthesis). So far, very little is known on possible physical interactions of peroxisomes and other organelles, which may contribute to various aspects of peroxisome proliferation.

De novo PEROXISOME FORMATION

PEROXISOME REINTRODUCTION IN *pex3* YEAST CELLS

The most extensively used experimental system to study *de novo* peroxisome formation in yeast is the reintroduction of peroxisomes in *pex3* strains in which a *PEX3-GFP* hybrid gene is placed under control of an inducible promoter (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Haan et al., 2006). Fluorescence microscopy (FM) analysis revealed that upon induction newly synthesized Pex3-GFP sorts to the ER, concentrates in foci followed by the formation of a pre-peroxisomal structure, which pinches off and develops into a nascent peroxisome. Alternatively two (*S. cerevisiae*; van der Zand et al., 2012) or multiple (*Y. lipolytica*; Titorenko et al., 2000) types of vesicles have been proposed to pinch off from the ER, which subsequently fuse to form a nascent peroxisome.

Invariably, these models predict that Pex3 initially sorts to the ER. This is strengthened by the finding that the extreme N-terminus of Pex3 proteins has characteristics in common with signal-anchor-like sequences, typical for authentic ER membrane proteins (Thoms et al., 2012; Fakieh et al., 2013). Another argument for ER sorting of Pex3 is the observation that peroxisome reintroduction in *S. cerevisiae pex3* cells is affected in a temperature sensitive *sec61* mutant strain or upon depletion of Sec61 (Thoms et al., 2012). However, opposite data were described by the Gould group (South et al., 2001) using a cold-sensitive *S. cerevisiae sec61* mutant.

Also, experiments reported by Kragt and colleagues suggest initial sorting of Pex3 to the ER (Kragt et al., 2005). In this study

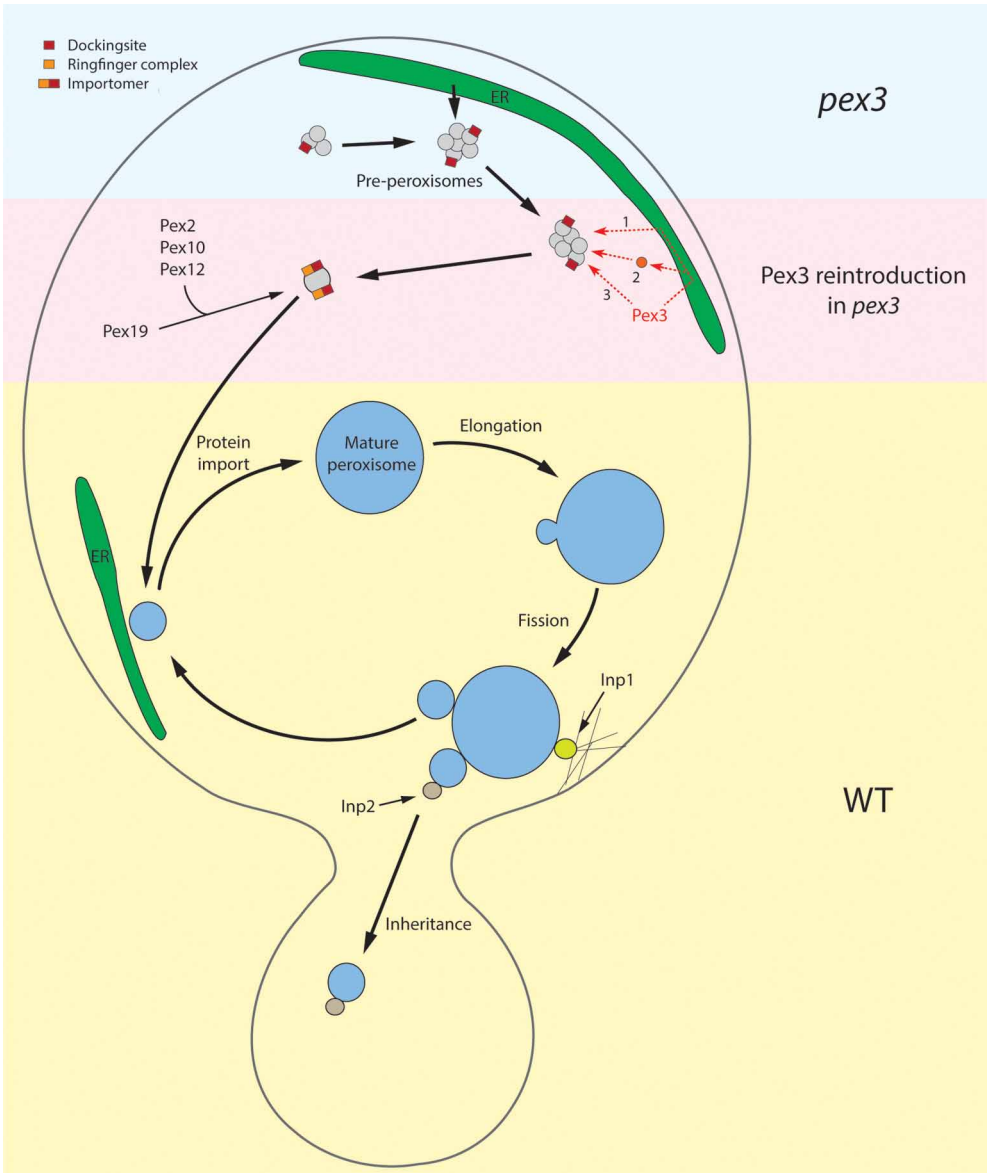

FIGURE 2 | Schematic overview of peroxisome re-introduction in *pex3* and peroxisome proliferation in WT cells. The upper part of the cell represents the situation in *pex3* cells. These cells contain vesicular structures that harbor Pex13 and Pex14, proteins of the receptor docking site. How these proteins are sorted to these membranes is unknown. These structures may derive by proliferation of pre-existing ones or be formed from the ER. The pre-peroxisomes are the target for re-introduced Pex3, which may reach these structures via the ER (1), upon incorporation in vesicles that derive from the ER and subsequently fuse with pre-peroxisomes (2) or sort directly to them (3). With the presence of Pex3 at the pre-peroxisome, other PMPs can subsequently be taken up via the Pex3/Pex19 machinery. These include the ring finger proteins Pex2, Pex10, and Pex12, leading to the formation of a functional importomer. This allows uptake of matrix proteins resulting in organelle maturation and subsequent multiplication by fission as depicted in the lower half of the picture (WT situation). During growth the organelle is invariably closely associated with strands of ER. During cell fission Inp1 is essential to dock the mother organelle to the cortex whereas Inp2 determines the delivery of newly formed organelles to the bud.

a Pex3 variant was used containing an artificial ER signal peptide and cleavage site, which functionally complemented *S. cerevisiae pex3* cells, whereas a similar construct in which cleavage of the signal peptide was blocked by a mutation did not. This result was interpreted by an inability of the uncleaved variant to exit the ER during *de novo* peroxisome formation.

However, these studies and other studies were conducted at conditions in which the N-terminus of Pex3 was modified or in which *PEX3* was not under control of its endogenous promoter, which could lead to artificial mislocalization.

Analysis of mRNA transcript levels demonstrated that the expression of genes encoding peroxins/PMPs hardly increased at peroxisome induction conditions (Kal et al., 1999). Hence, overproduction is readily achieved which may lead to mislocalization. This may also be true for Pex3, when produced under control of strong inducible promoters, even when the ultimate

Pex3 levels do not exceed WT levels. This is related to the fact that the initial kinetics of the P_{GAL} driven Pex3 synthesis strongly exceeds those normally occurring in WT cells via the endogenous promoter.

Indeed, also data have been presented suggesting that the ER is not the target membrane for authentic Pex3. For instance, studies by Matsuzaki and Fujiki, who analyzed *in vitro* insertion of Pex3 in different cellular fractions of Chinese hamster ovary cells, revealed that Pex3 only inserted in peroxisomal membranes, but not in microsomes or mitochondria (Matsuzaki and Fujiki, 2008). Similarly, *in vitro* experiments indicated that PMP24 was directly inserted in rat liver peroxisomes in a Pex3 and Pex19 dependent manner (Pinto et al., 2006).

Although the data reported by Kragt and colleagues, strongly suggest that Pex3 traffics via the ER (Kragt et al., 2005), a similar approach in the Erdmann group using a Pex3 variant containing artificial sorting information for the mitochondrial outer membrane protein, also resulted in functional complementation of *pex3* cells (Rucktaschel et al., 2010). This at least suggests that Pex3 not necessarily needs to sort via the ER for *de novo* peroxisome formation. Moreover, for both experiments it cannot be excluded that a minor portion of the modified Pex3 escapes from sorting to mitochondria or ER.

Moreover, two recent studies revealed that newly synthesized Pex3 targets to all pre-existing peroxisomes in WT yeast cells (Fakieh et al., 2013; Menendez-Benito et al., 2013). If under these conditions Pex3 would traffic via the ER, a vesicular transport pathway from the ER to pre-existing peroxisomes should exist. Indeed data have been presented supporting this possibility (Motley and Hettema, 2007). On the other hand, to the best of our knowledge Pex3-GFP has never been localized to the ER in WT cells.

As is clear from the above, both data indicating that Pex3 directly sorts to peroxisomes or traffics via the ER to these organelles have been presented. A model explaining these seemingly contradicting observations could be that both at the ER and peroxisomes a Pex3 insertion machinery exists. Assuming that the ER insertion machinery has a lower affinity for newly synthesized Pex3 relative to the peroxisomal one, Pex3 will predominantly sort to the high affinity site at peroxisomes in WT cells. However, in cells lacking peroxisomes or at Pex3 overproduction conditions Pex3 will then (also) be sorted to the ER.

In vitro protein insertion studies as well as *in vivo* experiments tracking (single) Pex3 molecules on their way to peroxisomes in WT cells (e.g., in pulse chase experiments and/or superresolution fluorescence microscopy), will help to resolve this urgent question.

PEX3 DEPENDENT PEROXISOME REINTRODUCTION: AN ALTERNATIVE PATHWAY

We recently re-investigated the location of Pex14 in *H. polymorpha pex3* cells using high resolution immune electron microscopy and observed that the Pex14-GFP spots in fact represent vesicular structures located at the vicinity of mitochondria or ER, but never were found connected to these organelles. Comparable observations were made using *H. polymorpha pex19* cells (Knoops et al., 2014). Using deconvolution microscopy, such structures previously were also observed in *P. pastoris pex3* cells.

These recent studies revealed that the Pex14-containing structures in *H. polymorpha pex3* cells contained, besides Pex14, also Pex8 and Pex13 and hence may contain a functional receptor docking site. However, none of the other PMPs tested (Pex10, Pmp47, Pex11) was observed at these structures, so a functional importomer was not formed. Hence, similar to matrix protein import mutants, *pex3* cells apparently contain peroxisome ghosts that do not harbor all typical marker PMPs, but only a subset. This may add to the explanation why they were overlooked in earlier studies. The origin of these structures is currently unknown. We speculate that they could proliferate from pre-existing structures, like assumed for other peroxisomal ghosts. Alternatively, they may form from the ER (**Figure 2**). If so, however, their formation is independent of Pex3.

Interestingly, the Pex14-containing structures, but not the ER, were the target for reintroduced Pex3-GFP after which these structures developed into normal functional peroxisomes. Moreover, we also observed that Pex25 and Pex19, two other peroxins proposed to be involved in the *de novo* peroxisome formation, are not involved in the formation of the vesicles in *pex3* cells. This new insight into peroxisome formation in *pex3* cells fundamentally differs from the generally accepted models and may stimulate further studies on the principles of *de novo* peroxisome formation.

PEROXISOME REINTRODUCTION IN INHERITANCE MUTANTS

Cells of mutants defective in peroxisome segregation (*inp1* or *inp2* deletion strains) temporarily lack peroxisomal structures detectable by FM (Fagarasanu et al., 2005, 2006). In these cells peroxisomes reappear shortly after budding is completed, presumably by *de novo* peroxisome formation (Motley and Hettema, 2007). So far, the reappearance of peroxisomes in *inp1* or *inp2* cells has only been studied using matrix marker proteins, but not PMP markers. Hence, it cannot be excluded yet that in these cells also small peroxisomal remnants occur, like in *pex3* cells. It is important to solve this issue, using PMP marker proteins also including Pex3. If ghosts are fully absent, these mutants would be the preferred model systems for analyzing *de novo* peroxisome formation.

Interestingly, upon deletion of *PEX25* in *S. cerevisiae inp2* cells, the cells become peroxisome deficient, indicating that the *de novo* peroxisome formation process is blocked in these cells (Huber et al., 2012). Pex25 is also required for *de novo* formation in yeast *pex3* cells upon reintroduction of the *PEX3* gene (Saraya et al., 2011; Huber et al., 2012). These data indicate that both *de novo* peroxisome formation processes depend on Pex25 and probably represent the same process.

THE DISPUTED PMP SORTING MACHINERY

As for the role of the ER in *de novo* peroxisome formation, also no consensus is reached on the involvement of the ER in trafficking of PMPs other than Pex3 to peroxisomes in WT cells. Current models range from PMP trafficking via the ER to direct post-translational insertion in peroxisomal membranes (Schliebs and Kunau, 2004; Menendez-Benito et al., 2013; Yagita et al., 2013).

In the first model Pex3 and Pex19 play a role in the exit of PMPs from the ER. In the second one Pex19 serves as a soluble receptor/chaperone that binds newly synthesized PMPs and is recruited to the peroxisomal membrane by Pex3 (Fang et al., 2004), followed by the insertion of PMPs by a yet unknown mechanism. According to this model PMPs are predicted to be cytosolic or mistargeted to other cellular membranes in the absence of Pex3 or Pex19. The fact that many PMPs physically interact with Pex19 strongly supports the second model. Also, the observations that the levels of many PMPs strongly drop in *pex3* cells, and often are below the limit of detection, are in favor of this model. However, also many data in support of the first model have been presented.

van der Zand and colleagues determined the localization of 16 PMPs using fluorescence microscopy upon pulsed induction using P_{GAL} which suggested that these proteins initially sorted to the ER (van der Zand et al., 2010). As indicated above, these experiments should be interpreted with care because of the strong, initial temporal overexpression due to using P_{GAL}. Moreover, also in these studies the limitations of the relatively low resolution of fluorescence microscopy can easily result in misinterpretation of the data. The same authors also analyzed the localization of PMPs in *pex3* cells and concluded that all accumulated at the ER. Careful re-inspection of the published images suggests that Pex8, Pex13 and Pex14 indeed were present in foci, whereas the other PMPs tested (Pex2, Pex6, Pex11, Pex15) showed a very low, dispersed localization. We recently observed that also *S. cerevisiae pex3* cells harbor Pex14-containing peroxisomal ghosts, like in *H. polymorpha pex3* cells (unpublished results). Hence, most likely also in *S. cerevisiae* Pex8, Pex13 and Pex14 are present at peroxisomal membrane structures, whereas the other PMPs are instable and located to the cytosol.

In line with initial ER sorting would be a role of the Sec complex in PMP routing. Indeed, upon *in vivo* depletion of Sec components, a portion of certain PMPs became soluble (van der Zand et al., 2010). Also, data have been presented showing that peroxisomal tail anchor proteins depend on the function of the Get complex. For instance, Schuldiner and colleagues showed that the tail anchored protein Pex15 mislocalized to mitochondria when a component of the GET complex was depleted. Moreover, a physical interaction between Pex15 and Get complex components has been demonstrated (Schuldiner et al., 2008). Conversely, however, the insertion of the mammalian homolog of Pex15, Pex26, depends of Pex19 (Halbach et al., 2006; Matsuzono and Fujiki, 2006) and is independent of TRC40, the mammalian homolog of Get2 (Yagita et al., 2013). Also, insertion of the tail anchored protein Fis1 in peroxisomal membranes was shown to depend on Pex19 (Delille and Schrader, 2008).

As argued before for *pex3* cells, in fact both pathways may exist simultaneously. In this view the final location of the PMP is determined by the affinity of it targeting information for either the ER or normal peroxisomes. An alternative may be that—at least in part—different pathways exist, depending on marker protein and model organism used.

For instance, our recent findings clearly show that the localization of Pex13 and Pex14 into peroxisomal membranes does not require Pex3. However, in the same cells other PMPs require the Pex3/Pex19 machinery for stability and insertion in these membranes. This is supported by various data previously reported for *S. cerevisiae* and *P. pastoris* (Hettema et al., 2000; Hazra et al., 2002).

Interestingly, also in human cells Pex13 was shown to be able to insert into peroxisomal membranes independent of Pex19, whereas in yeast and mammals Pex13 is essential for the association of Pex14 with the peroxisomal membrane (Fransen et al., 2004). These data underscore that these two peroxins do not require the Pex3/Pex19 machinery for proper membrane insertion.

CONCLUSIONS

Peroxisome proliferation at inducing conditions is heavily debated but in yeast consensus is achieved that in these organisms fission is the main mode of organelle multiplication rather than *de novo* synthesis. Also in mammals the major mode of peroxisome proliferation is most likely fission, although *de novo* synthesis may occur as well.

De novo synthesis in yeast is observed in cells that lack peroxisomes. This process is in particular studied in *pex3* strains upon reintroduction of the *PEX3* gene. Recent data however indicated that *pex3* cells contain peroxisomal vesicles that form in the absence of Pex3 (Knoops et al., 2014). It was shown that in *pex3* cells not the ER but in fact these peroxisomal vesicles were the target for Pex3 and the subsequent formation of peroxisomes. However, various questions remain. For instance, it is unknown where the vesicles in *pex3* cells originate from. They may arise by fission of existing structures but also form from the ER (**Figure 2**). In the latter view these *in vivo* data may complement recent *in vitro* studies in which pre-peroxisomes were formed from microsomal fractions (Lam et al., 2011) or in permeabilized cells (Agrawal et al., 2011). Clearly, the *in vivo* data suggest a novel concept of peroxisome reintroduction in *pex3* cells and as such may promote future studies in this field. One approach may involve searching for novel proteins involved in *de novo* synthesis. A recent model that genetically separates *de novo* synthesis from fission may be useful in this respect. Two independent studies convincingly showed that mutants affected in fission or *de novo* synthesis do not display a peroxisome-deficient phenotype (Saraya et al., 2011; Huber et al., 2012). Only the combination of the two mutations as in a *pex11 pex25* double mutant leads to the absence of peroxisomes. With this, an elegant screen is now available for identifying novel components involved in *de novo* synthesis by creating double mutants in a *pex11* strain and select for peroxisome-deficient mutants.

Finally, the principles of PMP sorting are far from solved and change from the view that all PMPs travel via the ER to the assumption that PMPs travel directly to the target organelle. Considering the current literature, it is likely that both pathways in fact exist simultaneously. In this respect it is relevant to study the effect of manipulating modulation the affinity of the two sorting signals proposed (either for the ER or the intact peroxisome) for their substrate organelle. This may help in understanding why the protein travels to the ER in peroxisome-deficient mutants but to the intact organelle at WT conditions.

So far, most FM approaches used suffer from distinct drawbacks (i.e., overexpression effects) that do not allow drawing

unequivocal conclusions for WT conditions. Clearly, novel techniques are required, like pulse chase experiments to show the transient ER location of specific PMPs in conjunctions with high speed microscopy techniques to track the routing of these proteins.

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Emerging role of the endoplasmic reticulum in peroxisome biogenesis

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During the past few years, we have witnessed a paradigm shift in our long-standing concept of peroxisome biogenesis. Recent biochemical and morphological studies have revealed a primary role of the endoplasmic reticulum (ER) in the *de novo* formation of peroxisomes, thus challenging the prevalent model invoking growth and division of pre-existing peroxisomes. Importantly, a novel sorting process has been recently defined at the ER that segregates and assembles specific sets of peroxisomal membrane proteins (PMPs) into distinct pre-peroxisomal vesicular carriers (ppVs) that later undergo heterotypic fusion to form mature peroxisomes. Consequently, the emerging model has redefined the function of many peroxins (most notably Pex3, Pex19, and Pex25) and assigned them novel roles in vesicular budding and subsequent peroxisome assembly. These advances establish a novel intracellular membrane trafficking route between the ER and peroxisomes, but the components remain elusive. This review will provide a historical perspective and focus on recent developments in the emerging role of the ER in peroxisome biogenesis.

Keywords: peroxisome, intracellular protein trafficking, organelle biogenesis, ER involvement in peroxisome biogenesis, vesicle budding, peroxisomal ER

INTRODUCTION

The peroxisome, along with glycosomes and glyoxysomes, is a member of the microbody family of subcellular organelles. Peroxisomes are ubiquitously present in all eukaryotes. Their primary function is to sequester several metabolic enzymes that are involved in the β-oxidation of long-chain fatty acids, formation of bile acids, dolichol, and cholesterol (Van Veldhoven, 2010). In methylotropic yeasts, peroxisomes are essential for the metabolism of methanol (Subramani, 1998). In plants, peroxisomes house the glyoxylate cycle enzymes and also participate in photorespiration. In humans and other mammals, peroxisomes are required for the synthesis of plasmalogens that are vital membrane components of the heart and brain (Brites et al., 2009). Peroxisomes share several steps of these metabolic pathways with mitochondria, chloroplasts, ER, and cytosol through redox shuttles.

BACKGROUND

Early enzyme distribution studies led to the discovery of peroxisomes. In early 1950s, cellular fractionation of tubular cells of mouse kidney and liver parenchymal cells revealed dense, single membrane cytoplasmic bodies with a granular matrix (Rhodin, 1954; Bernhard and Rouiller, 1956). These structures were introduced in the electron microscopy literature as "microbodies." Toward the early 1960s, an extensive characterization for the enzymatic content of microbodies revealed the abundance of a variety of oxidases and catalase, among other enzymes. The association of these enzymes in a single organelle was biologically meaningful, particularly for the disposal of hydrogen peroxide, which is highly injurious to cell components. Thus, the segregation of the enzymes producing hydrogen peroxide together with an enzyme, catalase that effectively metabolizes it could be viewed as having an essentially protective function. Hence the term "peroxisome" was proposed (de Duve, 1960, 1965). However, since the discovery of peroxisomes, their cellular origin has been actively debated.

The prevailing view of the cellular origin of peroxisomes has been evolving and has narrowed to two alternative routes, one apparently more prevalent in mammalian cells (growth and division model) and the other in yeasts and plants [*de novo* model involving the endoplasmic reticulum (ER)]. However, this review will focus on how the field is converging toward a more generalized paradigm for mammalian and yeast systems, where both routes could be operating simultaneously. If true, understanding the environmental cues that shift this balance in favor of one model over the other will be very important. As mentioned above, the initial biochemical characterization has revealed a strong metabolic role of peroxisomes. This lead de Duve and others to suggest that peroxisomes, like mitochondria and chloroplasts, could be an autonomous organelle and endosymbiont in origin and multiply through growth and division of pre-existing organelles (de Duve, 1982; Lazarow and Fujiki, 1985). However, an ER origin of peroxisomes was proposed based on parallel observations of Novikoff and colleagues, that the peroxisomal membrane has continuities with the smooth ER and could be conceived as buds forming at the terminal ends of certain specialized areas of the ER (Rhodin, 1954).

Abbreviations: pER, pre-peroxisomal ER; ppVs, pre-peroxisomal vesicles; PMP, peroxisomal membrane protein.

They observed that a stalk-like structure attaches the peroxisomes to these ER regions that could eventually bud off into the cytoplasm.

SUPPORT FOR THE GROWTH AND DIVISION MODEL AS AN EXCLUSIVE ROUTE FOR PEROXISOME BIOGENESIS

In the 1980s, Lazarow and colleagues (Rachubinski et al., 1984; Lazarow and Fujiki, 1985; Lazarow, 1989) made observations that supported the growth and division model of peroxisome biogenesis in mammalian cells. Their key observations were, firstly, that matrix and membrane peroxins were synthesized on free ribosomes in the cytosol and were then sorted to pre-existing peroxisomes. Secondly, in mutants in which matrix protein import is impaired, membrane "ghosts" or peroxisomes remnants were present and these could provide a structural scaffold to reassemble functional peroxisomes once the missing peroxin is reintroduced by genetic complementation (Santos et al., 1988). Supporting this view was the observation that vesicles and tubular structures, possibly corresponding to peroxisome remnants, were observed with deconvolution microscopy in *pex3*Δ cells of *P. pastoris* (Hazra et al., 2002). Similarly, in *H. polymorpha*, vesicular membrane structures formed by the expression of a 50aa N-terminal fragment of Pex3 could act as precursors for reforming normal peroxisomes, when cells were complemented with the full-length Pex3 construct (Faber et al., 2002). It was noted that there are no peroxisomal remnants or "ghosts" in mutants in which the genes for two or more essential peroxins are deleted (e.g., *pex3*Δ, *pex16*Δ, *pex19*Δ). A major flaw in the hypothesis that growth and division is the only process for peroxisome biogenesis is that these cells are capable of regenerating peroxisomes when the missing genes are reintroduced, despite the observation that pre-existing peroxisomes are undetectable in the mutant cells.

Arguments against the *de novo* peroxisome biogenesis and the involvement of the ER came from the following negative observations. Firstly, inhibitors of COPI and COPII vesicle formation failed to inhibit peroxisome biogenesis in mammalian cells (South et al., 2000; Voorn-Brouwer et al., 2001). Secondly, in mutants with an inactive Sec61, a protein that forms the ER translocon essential for the entry of proteins into the ER, peroxisome biogenesis was unaffected (South et al., 2001). However, in hindsight these observations only suggested that the components of the standard secretory pathway were not required for peroxisome biogenesis. Lastly, if peroxisomes were to be formed from the ER, peroxins like Pex3, Pex16, and Pex19 that are essential for the assembly of peroxisomal membrane proteins (PMPs) should be localized to the ER (Lazarow, 2003). However, the majority of researchers never found Pex3, Pex16, and Pex19 or other PMPs localized to the ER in mammalian cells, even when peroxisomes are absent, perhaps due to the instability or aggregation of these PMPs in mutant cells (Voorn-Brouwer et al., 2001; Fang et al., 2004; Hunt and Trelease, 2004). In contrast, recent reports have emerged that show a transient localization of certain PMPs to the ER (Geuze et al., 2003; Kim et al., 2006; Yonekawa et al., 2011) and there is clear evidence in yeast that many PMPs do transit to peroxisomes via the ER (Titorenko and Rachubinski, 1998; Hoepfner et al., 2005; Yan et al., 2008; Agrawal et al., 2011; Joshi et al., 2012).

The growth and division model involves Pex19 and Pex3 in performing the post-translational insertion of PMPs into the peroxisomal membrane (Jones et al., 2004; Matsuzono and Fujiki, 2006; Matsuzaki and Fujiki, 2008). Earlier, using pulse-chase experiments, PMP70 was chased from the cytosol to mammalian peroxisomes without transiting any ER-like compartment (Imanaka et al., 1996). Similarly, another mammalian protein, PMP22, was post-translationally incorporated *in vitro* into purified peroxisomes (Diestelkotter, 1993). PMPs that depend on Pex19 for their targeting to the peroxisomal membrane are classified as Type I PMPs, whereas those that do not require Pex19 are termed as Type II PMPs (Jones et al., 2004). Except a few PMPs (Pex3 and Pex22), all other studied are either Type I or tail-anchored (TA) PMPs. In mammalian cells, Pex19 binds and stabilizes newly synthesized PMPs through their hydrophobic domains in the cytoplasm and acts as a chaperone and an import receptor to insert them into the peroxisomal membrane. The translated PMPs, which are soluble when Pex19 is present, form aggregates in its absence (Shibata et al., 2004; Kashiwayama et al., 2005). Pex19 binds to specific "*cis-acting*" peroxisome targeting signals within PMPs called mPTSs (Jones et al., 2001, 2004; Rottensteiner et al., 2003), which are important for their targeting to the peroxisomal membrane. PMPs are mislocalized either when their mPTSs are mutated or when Pex19 is missing, as in *pex19*Δ cells (Sacksteder et al., 2000; Jones et al., 2004; Halbach et al., 2009). After Pex19 has bound the mPTS domain/s of PMPs, it binds to Pex3 present on the peroxisomal membrane, thereby inserting the bound PMP into the membrane. For accomplishing these tasks, Pex19 uses non-overlapping binding sites that recognize Pex3 and mPTSs (Fransen et al., 2005; Sato et al., 2010; Schueller et al., 2010). In addition, Pex19 has been also shown to incorporate TA proteins directly into the peroxisome membrane (Fujiki et al., 2006; Matsuzono and Fujiki, 2006; Halbach et al., 2009) independent of the classical TRC40 pathway (Yagita et al., 2013). Otherwise, the TRC40/GET pathway is widely accepted as the dominant pathway for targeting and inserting TA proteins into cellular membranes, including the ER (Borgese and Fasana, 2011). Upon accomplishing membrane biogenesis, peroxisomes acquire import competence for matrix enzymes and eventually grow and will undergo division to meet the metabolic requirements of the cell. The growth and division model side-steps the issue of where membrane lipids are derived from for peroxisome growth and how they are inserted into the membrane. However, very different roles of Pex19 and Pex3 are proposed in the *de novo* peroxisome biogenesis model (see below).

THE ER AS A PRECURSOR FOR PMP BIOGENESIS

Though there were intermittent reports (Gonzalez and Beevers, 1976; Ohno and Fujii, 1990), renewed focus on the ER did not occur until the late 1990s. In *S. cerevisiae,* Pex15, a TA protein, was suggested to traffic from the ER to the peroxisomes (Elgersma et al., 1997). An ER targeting signal overlapping with its mPTS was found in Pex15, and its overexpression caused profound proliferation of the ER membrane. In the following year, another study, this time in *Y. lipolytica* showed a more direct involvement of the ER in peroxisome biogenesis. The temperature-sensitive mutants of *SEC238* and *SRP54,* whose genes products are involved

in the secretory pathway, not only inhibited the exit of an alkaline extracellular protease from the ER, but also lead to temperature-sensitive growth of cells in peroxisome proliferating conditions (Titorenko and Rachubinski, 1998, 2000). In addition, this study also showed two other peroxins, Pex2, and Pex16, which were delivered to the peroxisomes via the ER. The two peroxins were pulse-labeled and were imported from the cytosol to the ER, N-glycosylated in the ER-lumen and then chased to the peroxisomes. Unlike some of the previous studies (Baerends et al., 1996; Komori et al., 1997; Kammerer et al., 1998), these observations were more relevant physiologically since the PMPs were not overexpressed. Additional reports for the involvement of the ER came from yeast and plant cells treated with Brefeldin A (BFA; a fungal toxin that inhibits vesicle transport from the ER). In *H. polymorpha* cells treated with BFA, several peroxins accumulated in a structure resembling the ER (Salomons et al., 1997). In plants, a peroxisomal isoform of ascorbate peroxidase (APX) was localized to the reticular ER, in addition to the peroxisomes (Mullen et al., 1999). But treatment with BFA restricted the localization of APX to the ER-like structures and this could be reversed by removal of BFA. These ER-like structures lacked typical ER resident proteins, such as BiP2, calnexin, and calreticulin. In addition, the *in vitro* translated APX could only be incorporated into the ER membranes and not into any other organelle membranes (including peroxisomal membranes), suggesting that the ER hosts the protein before it is trafficked to the peroxisomes. In addition, other studies identified several ER-associated proteins of the secretory pathway that are necessary for peroxisome assembly. Previously the *SEC238* and *SRP54* genes in *Y. lipolytica* were found to be essential for the exit of Pex2 and Pex16 from the ER and for peroxisome assembly (Titorenko and Rachubinski, 1998). More recently, it was reported that repression of other ER proteins, Sec20, Sec39, and Dsl1, causes the mislocalization of Pex3 and Pot1 (Perry et al., 2009). Collectively, these studies provide compelling evidence for the ER as the precursor for peroxisomes, at least in yeast and plants. These studies were viewed with skepticism initially because they were counter to the widely accepted growth and division model (Lazarow, 2003) and negative experiments ruling out a role for certain components of the ER-secretory pathway (South and Gould, 1999; Voorn-Brouwer et al., 2001). However, this view has shifted over the past decade.

DE NOVO PEROXISOME BIOGENESIS IN YEAST

The reappearance of peroxisomes in cells completely lacking pre-existing peroxisomes presented the most relevant argument against the growth and division model. During the discovery of the genes essential for peroxisome biogenesis, mainly *PEX3* and *PEX19*, the reintroduction of a functional copy of these genes restored peroxisome biogenesis in the mutant cells (Hohfeld et al., 1991; Erdmann and Kunau, 1992; Baerends et al., 1996; Wiemer et al., 1996). However, this was not the case with mitochondrial biogenesis mutants, where reintroduction of the corresponding functional gene could not rescue the organelle (Ryan and Hoogenraad, 2007). Later in 2005, Tabak and colleagues (Hoepfner et al., 2005) provided more conclusive proof for the involvement of ER in peroxisome biogenesis. They followed the intracellular route for the newly-synthesized, YFP-tagged Pex3 and Pex19 in *pex3*Δ, *pex19*Δ, and wild-type cells using advanced real-time fluorescence microscopy and biochemical experimentation. Pex3 first appears at the perinuclear ER and can be followed to punctate structures coinciding with the ER. These dot-like structures later detach in a Pex19-dependent manner from the ER and start to co-localize with matrix proteins representing import-competent peroxisomes. In addition, Pex3 remains trapped in the ER in the absence of Pex19; and without Pex3, Pex19 never localizes to the ER membrane (Hoepfner et al., 2005). These data suggest that metabolically-active peroxisomes are formed *de novo* from the ER through the recruitment of Pex19 by ER-localized Pex3.

Subsequent studies established PMP traffic to peroxisomes via the ER as a rule, rather than as an exception, not only in *S. cerevisiae*, but also in other yeasts (Kragt et al., 2005; Tam et al., 2005; Yan et al., 2008; Knoblach and Rachubinski, 2010; van der Zand et al., 2010; Huber et al., 2011; Saraya et al., 2011; Joshi et al., 2012) (**Figure 1**). At least 20 different PMPs have been followed from the ER to the peroxisomes irrespective of their membrane topologies or function in peroxisome biogenesis. Interestingly, van der Zand et al. found that the ER-routed trafficking of more than 15 different PMPs was not restricted only during the *de novo* formation of peroxisomes, but was detected in wild-type cells already containing peroxisomes (van der Zand et al., 2010). The authors thus suggest a unified PMP biogenesis route in both wild-type and mutant cells. However, Motley and Hettema suggested that peroxisomes are formed *de novo* only when pre-existing peroxisomes are absent, as seen in cells with a defect in peroxisome inheritance, whereas growth and division is the default pathway for peroxisome biogenesis in wild-type cells (Motley and Hettema, 2007). They also showed that newly formed Pex3-GFP is trafficked through ER in both wild-type and *pex3*Δ *cells*, but is targeted to the pre-existing peroxisomes in the wild-type cells (since they do not detect *de novo* formation of peroxisomes in wild-type cells), but localizes to new peroxisomes in *pex3*Δ cells. This extended the role of the ER from *de novo* peroxisome biogenesis to the maintenance of the pre-existing peroxisomes population (for renewing them with fresh PMPs). This would also explain how the pre-existing peroxisomes could repeatedly divide without being depleted of PMPs. However, in the alternative view, if *de novo* peroxisome biogenesis occurs only when pre-existing peroxisomes are absent (van der Zand et al., 2010) and the *de novo* pathway serves only as a back-up process that is not utilized in cells constantly, it would have been eliminated from the system. In support of their postulate, they recently reported observations where pre-peroxisomal vesicular carriers (ppVs) carrying PMPs from the ER fuse only with each other but never with the pre-existing peroxisomes (van der Zand et al., 2012). This has challenged the role of ER in the maintenance of the pre-existing peroxisome population (Motley and Hettema, 2007).

CONFLICTING REPORTS ON THE ROLE OF ER IN MAMMALIAN PEROXISOME BIOGENESIS

In yeast, in spite of certain differences among various groups regarding the extent to which the ER contributes to peroxisome biogenesis, it is now widely accepted as a source of PMPs and

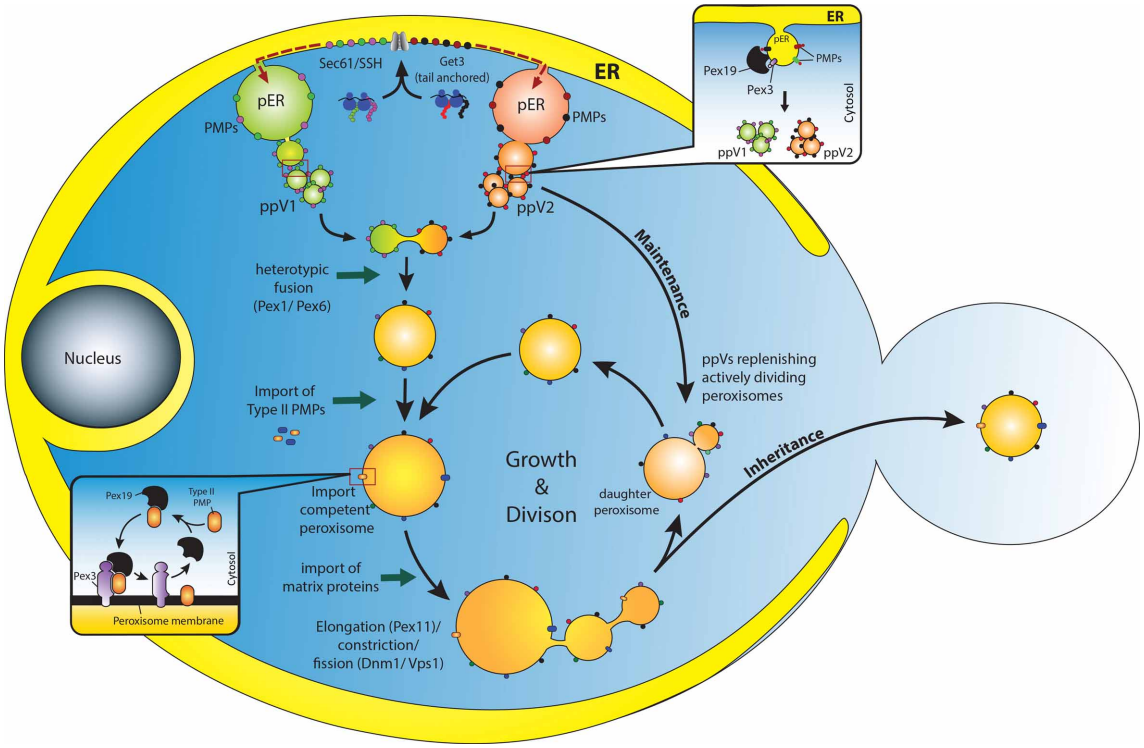

FIGURE 1 | Overview of peroxisome biogenesis: converging *de novo* and growth and division models. PMPs are translated in the cytosol on free ribosomes and are incorporated into the ER membrane through specific translocons (Sec61 or Get3) (van der Zand et al., 2010; Borgese and Fasana, 2011). It is assumed that the PMPs are segregated and sorted (presumably, through an intramolecular signal) into at least two different pre-peroxisomal compartments (pER) in the ER (Fakieh et al., 2013). These PMPs leave the ER through vesicular carriers that bud in a Pex19 dependent manner (upper inset) (Lam et al., 2010; Agrawal et al., 2011). These vesicles undergo heterotypic fusion to unite the components of the importomer to form import competent peroxisomes (van der Zand et al., 2012), a process that requires the Pex1 and Pex6 proteins (Titorenko et al., 2000). It is expected that Type II PMPs are incorporated at this stage since they are essential for the import of matrix proteins (Koller et al., 1999). Here Pex19 might act as an mPTS receptor that binds and stabilizes the Type II PMPs in the cytosol and incorporates them into the peroxisomal membrane upon docking to the membrane bound Pex3 (Schmidt et al., 2012) (lower inset). Upon subsequent import of matrix proteins, peroxisomes grow in size and undergo division to replenish cells with an adequate number of peroxisomes to meet metabolic requirements of the cells. This pathway could be the most plausible way by which peroxisomes are repopulated in cells lacking peroxisomes (such as *pex3*Δ/*pex19*Δ) when the missing gene is reintroduced (Hoepfner et al., 2005; van der Zand et al., 2010; Agrawal et al., 2011). However, in WT cells as well, ER might supply PMPs to replenish and maintain the actively dividing peroxisomes (Motley and Hettema, 2007). An active interaction between the two pathways would enable cells to adapt dynamically to changing environments.

membrane (including lipids) during *de novo* biogenesis of peroxisomes (Tabak et al., 2013; Theodoulou et al., 2013). However, such a consensus is not yet attained in mammalian systems. Several experimental setups that proved the involvement of ER in peroxisome biogenesis in yeast have yielded inconclusive results in mammalian cells. Firstly, while BFA did affect peroxisome biogenesis in yeast cells, it had no effect with mammalian cells (Salomons et al., 1997; Mullen et al., 1999; South and Gould, 1999; Voorn-Brouwer et al., 2001). In addition, there are inconsistent reports for the involvement of the components of the secretory pathway in peroxisome biogenesis in mammalian cells. Passreiter et al. reported the involvement of ARF and coatomer complex in peroxisome biogenesis in mammalian cells (Passreiter et al., 1998). This was contradicted in further reports showing that neither COPI, ARF1, or SAR1 (South et al., 2000; Voorn-Brouwer et al., 2001), nor Sec61 (South et al., 2001), are involved in peroxisome biogenesis in mammalian cells. Secondly, unlike yeast, mammalian cells with non-functional mutant of Pex3 or Pex19 or their knockdowns did not cause the ER accumulation of PMPs (South et al., 2000; Fang et al., 2004; Hunt and Trelease, 2004; Jones et al., 2004). In addition, several import assays were established in the mammalian cells for the *in vitro* import of PMPs (including TA proteins) to the peroxisomes directly from the cytosol (Diestelkotter, 1993; Imanaka et al., 1996; Matsuzono and Fujiki, 2006; Matsuzaki and Fujiki, 2008; Yagita et al., 2013).

Although, there are few independent reports suggesting a direct involvement of the ER in mammalian peroxisome biogenesis, a renewed focus on the contribution of ER in mammalian peroxisome biogenesis came when Gueze et al. convincingly showed the association of the ER with peroxisomes using advanced electron microscopy and three-dimensional image reconstruction in mouse dendritic cells (Geuze et al., 2003; Tabak et al., 2003), as proposed earlier(Rhodin, 1954; Ohno and Fujii, 1990). Using Immuno-gold labeling of mouse dendritic cells, it was found that lamellar structures enriched in Pex14 and PMP70 connected to the ER as its sub-domain (termed "specialized ER"), which is

remarkably different from the rough ER in that it is less enriched with typical luminal ER markers, like PDI and calreticulin, and is devoid of attached ribosomes. Using 3-D reconstructions, they found that mature peroxisomes were also associated with similar lamellar structures that were not connected to, but sometimes showed membrane continuities with, the ER (Tabak et al., 2003). These structures might be similar to the "stalks" through which peroxisomes are connected with the ER (Rhodin, 1954).

More recently Kim et al. provided further evidence for the involvement of the ER in peroxisome biogenesis in mammalian cells (Kim et al., 2006). Using a photo-activatable, GFP-tagged Pex16, they showed that it is routed through the ER to the peroxisomes. They also demonstrated that *de novo* peroxisomes biogenesis contributes significantly more toward the total cellular pool of peroxisomes compared to the peroxisome population arising through growth and division (fission). Furthermore, they showed that Pex16 is first incorporated into the ER, which further recruits Pex3 and other PMPs to the membrane. This eventually leads to the differentiation of a "peroxisome-like" domain in the ER similar to those observed in mouse dendritic cells (Geuze et al., 2003). These "specialized ER" domains can detach from the ER to form peroxisomes *de novo* through a fission event. However, Pex3 and Pex16 were not in the ER when BFA was used to inhibit ER-mediated vesicle transport (Voorn-Brouwer et al., 2001). Nonetheless, they never tested the localization of these PMPs in *pex3*Δ or *pex19*Δ mutants. Kim et al. cited unpublished data where they observed a redistribution of Pex16 to the peroxisomes when cells were treated with BFA (Kim et al., 2006). Additionally, there are distinct mechanisms reported for the secretion of various proteins from the ER that do not require the COPI or COPII machinery and are thus insensitive to BFA (such as fibroblast growth factor, interleukin-1b, HIV-tat, galectin-3, thioredoxin) (Nickel, 2010). A further indication for an involvement of the ER in the biogenesis of mammalian peroxisomes came when Sec16B, a protein that defines ER exit sites, was overexpressed in HeLa cells (Yonekawa et al., 2011). It was found that Pex3, Pex16 along with Sec16B were redistributed and colocalized to the entire ER. However, a knockdown of Sec16B caused the ER retention of Pex16 and a suppression of Pex3 expression with a prominent effect on peroxisome morphology. Perhaps Sec16B recruits essential coat components to the pre-peroxisomal compartment at the ER for budding. Together, these results support the view that peroxisomes are also formed *de novo* from the ER in mammalian cells, thus creating a unified theme for peroxisome biogenesis with yeast and plants.

PRE-PEROXISOMAL INTERMEDIATES AND COMPARTMENTS

Several studies in mammalian and yeast cells have identified a peroxisomal pre-compartment in the ER (Geuze et al., 2003; Hoepfner et al., 2005; Tam et al., 2005), as well as various transitional precursors (Titorenko et al., 2000; Lam et al., 2010; Agrawal et al., 2011; van der Zand et al., 2012) that eventually mature into import-competent peroxisomes. In yeast, Pex3 is sorted first to the ER, where it further recruits other PMPs and transforms the site into a distinct compartment, often referred to as the "pre-peroxisomal ER (pER)" (Agrawal et al., 2011) or the "specialized ER" in mammalian cells (Geuze et al., 2003). In *S. cerevisiae*, these compartments are seen as one or two bright dots on the ER when a fluorescently tagged Pex3 is reintroduced in *pex3*Δ cells (Hoepfner et al., 2005; Tam et al., 2005; Agrawal et al., 2011). With time, a transient co-localization of Pex19 is seen (Hoepfner et al., 2005) which could be facilitating the budding process (Lam et al., 2010; Agrawal et al., 2011). However, in mammalian cells, Pex16 was first sorted to the "specialized-ER." Pex16 is shown to be the anchoring receptor for Pex3 in the peroxisomal membrane, which further recruits Pex19 and other PMPs (Geuze et al., 2003; Tam et al., 2005; Kim et al., 2006; Schmidt et al., 2012). A localization of Pex19 to these structures on the ER is not yet detected. Interestingly, as mentioned before, repression of Sec16B expression restricts the Pex16 to the ER. Perhaps a role for Pex19 in recruiting Sec16B to these sites is an interesting possibility. Nonetheless, the mechanism that sorts PMPs after their import into the ER to the pER is still unknown although distinct signals have recently been found in yeast Pex3 for ER and pER sorting (Fakieh et al., 2013) (**Figure 1**).

In view of the observation that newly-synthesized Pex3 and other PMPs are trafficked through the ER to form mature peroxisomes, it was expected that the PMPs exit the ER in vesicular carriers. Previously, Titorenko and Rachubinki reported an extensive biochemical and morphological characterization of a multistep peroxisome maturation pathway in *Y. lipolitica* (Titorenko et al., 2000). Sucrose density gradient analysis identified five distinct precursor populations, each containing Pex2 and Pex16, but differing in their matrix enzyme compositions. It was found that a constant import of matrix enzymes and heterotypic fusion events result in the transformation of one vesicle type to another with higher density. However, it was unclear that whether they originated upon budding from the ER or through fission of pre-existing peroxisomes.

Recently, two independent studies performed in *S. cerevisiae* and *P. pastoris* identified vesicular carriers that bud from the ER carrying PMPs (Lam et al., 2010; Agrawal et al., 2011). In *P. pastoris*, we showed that budded vesicles carried two different PMPs, Pex3, and Pex11, co-packaged together in the same vesicle in an ATP-dependent manner. Importantly, the budding process required Pex19, but not Pex3 or other peroxins (Pex1, Pex5, Pex7, and Pex14). However, the budded vesicles detected in *pex3*Δ cells carried a very limited repertoire of PMPs and lacked matrix proteins. Nonetheless, this was a surprising result since Pex3 was believed to be critical for docking of Pex19 at the ER. It further raised the possibility that Pex19 could dock with other peroxins at the ER membrane for initiating the budding process, while Pex3 might be critical for maturation of ppVs into import competent peroxisomes. More recently, van der Zand et al. showed that the pre-peroxisomal vesicles that bud from the ER are of at least two types, each carrying subcomponents of the peroxisomal translocon complex (van der Zand et al., 2012). Import competent peroxisomes were formed with heterotypic fusion of these vesicles, which fuse only with each other, but not with the pre-existing mature peroxisomes. Segregation of the peroxisomal translocon complex components into distinct compartments also suggested a way to keep the ER from importing the peroxisomal matrix enzymes. However, the biochemical requirements for the

budding of these heterogeneous vesicles were not identified, leaving the possibility for the need for non-overlapping components specific for each type of vesicle (**Figure 1**).

REVISITING THE ROLE OF Pex19 IN PEROXISOME BIOGENESIS

Several studies during the last decade have bought a paradigm shift in our understanding of the mechanistic role of Pex19 in peroxisome biogenesis. Pex19 has been ascribed multiple roles in peroxisome biogenesis pathway including the PMP receptor, the budding of ppVs, peroxisome division, as well as inheritance. Most importantly, Pex19 is considered as a PMP-chaperone and a shuttling receptor. Because several PMPs contain one or more mPTS for binding Pex19 and these PMPs are unstable or aggregate in the absence of Pex19, the Pex19 protein is considered essential for binding and stabilizing PMPs in the cytosol (chaperone like activity) (Sacksteder et al., 2000; Jones et al., 2004; Shibata et al., 2004; Kashiwayama et al., 2005). Moreover, it is presumed that Pex19 delivers the bound PMPs to the peroxisome by docking with Pex3 at the peroxisomal membrane (Fang et al., 2004; Rottensteiner et al., 2004; Hoepfner et al., 2005; Matsuzono and Fujiki, 2006). The role of Pex19 is often extended to insertion of PMPs into peroxisomal membrane as well (Sacksteder et al., 2000; Jones et al., 2004), where it is presumed to incorporate PMP. Following this step, Pex19 is recycled to the cytosol for the next round of insertion (Schmidt et al., 2012).

Most of the studies cited above are performed in mammalian cells, where growth and division is the more prevalent model. In yeast, the role of Pex19 in the direct insertion of PMPs into the peroxisomal membrane has been actively debated (Snyder et al., 2000; Hoepfner et al., 2005; van der Zand et al., 2010) with an alternative emerging role for Pex19 in the budding of ppVs (Lam et al., 2010; Agrawal et al., 2011). Presumably, Pex19 docks on Pex3 through its Pex3-binding or PMP-binding domains to recruit other components of the budding machinery (still unidentified). However, since components of the conventional secretory pathway are not involved in the budding process, Pex19 could be speculated to assemble a machinery similar to that for peroxisome division at the pER. The action of such membrane fission machinery, in concert with the Pex11-like proteins that cause membrane tubulation (Opalinski et al.), could result in the budding of ER-derived ppVs. This is a conceivable scenario since Pex19 interacts with several proteins involved in the peroxisome division process including Vps1, Fis1, Pex11, and Pex25 (Rottensteiner et al., 2004; Vizeacoumar et al., 2006; Delille and Schrader, 2008; Tarassov et al., 2008; Rucktäschel et al., 2009). This idea remains to be tested experimentally.

CONVERGING PATHWAYS

Our view of peroxisome biogenesis is now being transformed by multiple studies either supporting the *de novo* pathway for peroxisome biogenesis (van der Zand et al., 2010, 2012) or by those depicting peroxisomes as autonomous organelles that replenish themselves by growth and division (Lazarow, 2003; Nagotu et al., 2008). However, a handful of studies have suggested that both pathways might operate simultaneously (Kim et al., 2006; Huber et al., 2011), or could be conditionally segregated (Motley and Hettema, 2007). Since both pathways eventually lead to peroxisome biogenesis, it is natural to see them as two sides of the same coin. Evidently, when key components of one pathway are blocked, the other pathway takes charge to replenish the organelle supply, but when key components of both the pathways are blocked, the lack of peroxisome biogenesis is evident (Huber et al., 2011; Saraya et al., 2011). In addition, studies with an impaired division or inheritance machinery reveal a slowed biogenesis process (Kim et al., 2006; Motley and Hettema, 2007; Joshi et al., 2012). Nonetheless, cells form functional peroxisomes presumably through the *de novo* pathway. This could also suggest that both pathways need to operate simultaneously to make the organelle regeneration and maintenance kinetically efficient. If true, the alternative models of peroxisome generation described herein might not be mutually exclusive, but rather redundant mechanisms evolved for infallible organelle regeneration. Crosstalk between the two pathways might be essential for achieving dynamic peroxisome homeostasis (**Figure 1**). These remain as interesting topics for further exploration.

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PEX16: a multifaceted regulator of peroxisome biogenesis

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Peroxisomes are formed by two distinct pathways: the growth and fission of mature peroxisomes and *de novo* synthesis at the endoplasmic reticulum (ER). While many of the molecular mechanisms underlying these two pathways remain to be elucidated, it is generally accepted that their relative contribution to peroxisome formation may vary depending on the species, cell type and/or physiological status of the organism. One pertinent example of the apparent differences in the regulation of peroxisome biogenesis among evolutionarily diverse species is the involvement of the peroxin PEX16. In *Yarrowia lipolytica*, for instance, PEX16 is an intraperoxisomal peripheral membrane protein that participates in peroxisomal fission. By contrast, Human PEX16 is an integral membrane protein that is thought to function at the ER during the early stages of *de novo* peroxisome formation and also recruits peroxisomal membrane proteins directly to mature peroxisomes. Similarly, PEX16 in the plant *Arabidopsis thaliana* is speculated to be a PMP receptor at the ER and peroxisomes, and is also required for the formation of other ER-derived organelles, such as oil and protein bodies. Here we briefly review the current knowledge of *Y. lipolytica*, human and *A. thaliana* PEX16 in the context of our overall understanding of peroxisome biogenesis and the role of the ER in this process in these three divergent species.

Keywords: endoplasmic reticulum, organelle biogenesis, peroxin, peroxisome, PEX16

INTRODUCTION

Peroxisomes are found in virtually all eukaryotic organisms and while they possess a somewhat simple architecture consisting of a nonhomogenous matrix enclosed by a single membrane, their metabolic functions are highly complex (Islinger et al., 2010). For instance, peroxisomes in plants participate in a remarkable array of processes, including the glyoxylate cycle and the synthesis of phytohormones (Hu et al., 2012), while in humans the organelle is involved in cholesterol and bile acid biosynthesis, and defects in the organelle result in a number of lethal genetic disorders (Waterham and Ebberink, 2012). In yeasts, peroxisomes are required for metabolizing nonfermentable carbon sources such as methanol and oleate (Van Der Klei and Veenhuis, 2006). Notably, this metabolic feature has been readily exploited for the identification of yeast mutants with defects in the biogenesis of peroxisomes and the subsequent identification of the corresponding genes and their protein product (collectively referred to as peroxins or PEX proteins) [reviewed in Distel et al. (1996); see also Tower et al. (2011)].

To date, over 30 peroxins involved in the key steps underlying peroxisome biogenesis in yeast have been identified, many of which are also present in other eukaryotes, including mammals and plants (Hayashi and Nishimura, 2006; Kiel et al., 2006). Pertinent examples of these conserved peroxins include those involved in peroxisomal matrix protein import (PEX5, 7, 10, 12, 13, etc.) and those that help orchestrate the growth and division of peroxisomes (e.g., PEX11 protein family). For more detailed information regarding these processes and the peroxins involved, we refer the reader to several recent reviews (Hu, 2010; Koch and Brocard, 2011; Liu et al., 2012; Schrader et al., 2012).

PEX3, PEX16, and PEX19 are another important set of peroxins that are generally referred to as "early" peroxins because of their essential roles in the initial steps of peroxisome biogenesis (Schliebs and Kunau, 2004). However, the precise roles of these peroxins appear to vary considerably depending on the organism. For instance, PEX19 serves in all peroxisome-containing species as a soluble receptor for nascent peroxisomal membrane proteins (PMPs) by binding and targeting them to the peroxisomal membrane (Ma et al., 2011; Theodoulou et al., 2013), but, in the yeast *Saccharomyces cerevisiae*, PEX19 functions also in peroxisome inheritance (Otzen et al., 2012). Likewise, PEX3 is a conserved membrane-bound docking receptor for incoming complexes of PEX19 and its PMP cargo (Sato et al., 2010; Schmidt et al., 2010), yet yeast PEX3 serves also in peroxisome inheritance and in the degradation of peroxisomes (Munck et al., 2009; Motley et al., 2012; Nordgren et al., 2013). PEX16 seems to possess the most diverse set of functions, ranging from a matrix-localized, peripheral membrane protein involved in peroxisomal fission in the yeast *Y. lipolytica* (Guo et al., 2007), to an integral membrane-bound PMP receptor at the ER and peroxisomes in mammals (Kim et al., 2006; Matsuzaki and Fujiki, 2008), and perhaps also in plants (Karnik and Trelease, 2007). Notably, PEX16 homologs are absent in some well characterized model organisms, including *S. cerevisiae* (Kiel et al., 2006) and *Caenorhabditis elegans* (Thieringer et al., 2003).

Interestingly, the results obtained from studies of PEX16 have been instrumental in the development of our current working models for peroxisome biogenesis, and have shed significant light on the role that ER plays in this process in evolutionarily distinct organisms (**Figure 1**) (Titorenko and Rachubinski, 2009; Hu et al., 2012; Dimitrov et al., 2013; Tabak et al., 2013). There is also a growing appreciation that there are differences in the relative contribution of these two pathways, as well as their underlying molecular mechanisms, to the biogenesis of peroxisomes in different organisms (Koch and Brocard, 2011; Islinger et al., 2012). Thus, it is not always appropriate to extrapolate the knowledge gained from one organism to another, and a unified model of peroxisome biogenesis, for either pathway, may not be feasible.

Here we briefly highlight the functional properties and intracellular trafficking pathways of PEX16 from the three species wherein this peroxin has been the best studied—*Y. lipolytica*, human, and *A. thaliana*—and, in doing so, how this knowledge has been incorporated into the models for peroxisome biogenesis among these evolutionarily diverse species.

YARROWIA LIPOLYTICA PEX16P

The PEX16 protein was first described in *Y. lipolytica* (Eitzen et al., 1997). In this study, a *Ylpex16* mutant strain was identified based on its inability to use oleate as a sole carbon source and subsequent cloning of the *YlPEX16* gene revealed it encoded a protein that had no obvious structural/functional domains and no significant sequence homology with any other functionally characterized protein. Phylogenetic analysis of sequences present in extant genome databases, however, reveals that PEX16 homologs exist in most, but not all, eukaryotes and that they share approximately 15–25% sequence identity (**Figure 2A**). PEX16 homologs from metazoans, yeasts and plants are also separated into distinct clades (**Figure 2B**), indicating early diversification and perhaps functional specialization.

The initial study of YlPex16p revealed that the protein is peripherally associated with the inner surface of the peroxisomal membrane (Eitzen et al., 1997) and that overexpression of *YlPEX16* yielded a reduced number of larger peroxisomes compared to those in wild-type cells, revealing that YlPex16p is required for peroxisomal fission. Additional studies on YlPex16p, as well as other studies aimed at deciphering how peroxisomes are formed and maintained in *Y. lipolytica,* have since led to the development of a sophisticated model of peroxisome biogenesis in this organism where YlPex16p plays a critical role in peroxisome division (Titorenko and Rachubinski, 2001; Boukh-Viner and Titorenko, 2006) As depicted in **Figure 1A**, this model includes six distinct peroxisomal subcompartments, termed P1–P6, which are organized into a multi-step biogenetic pathway. The

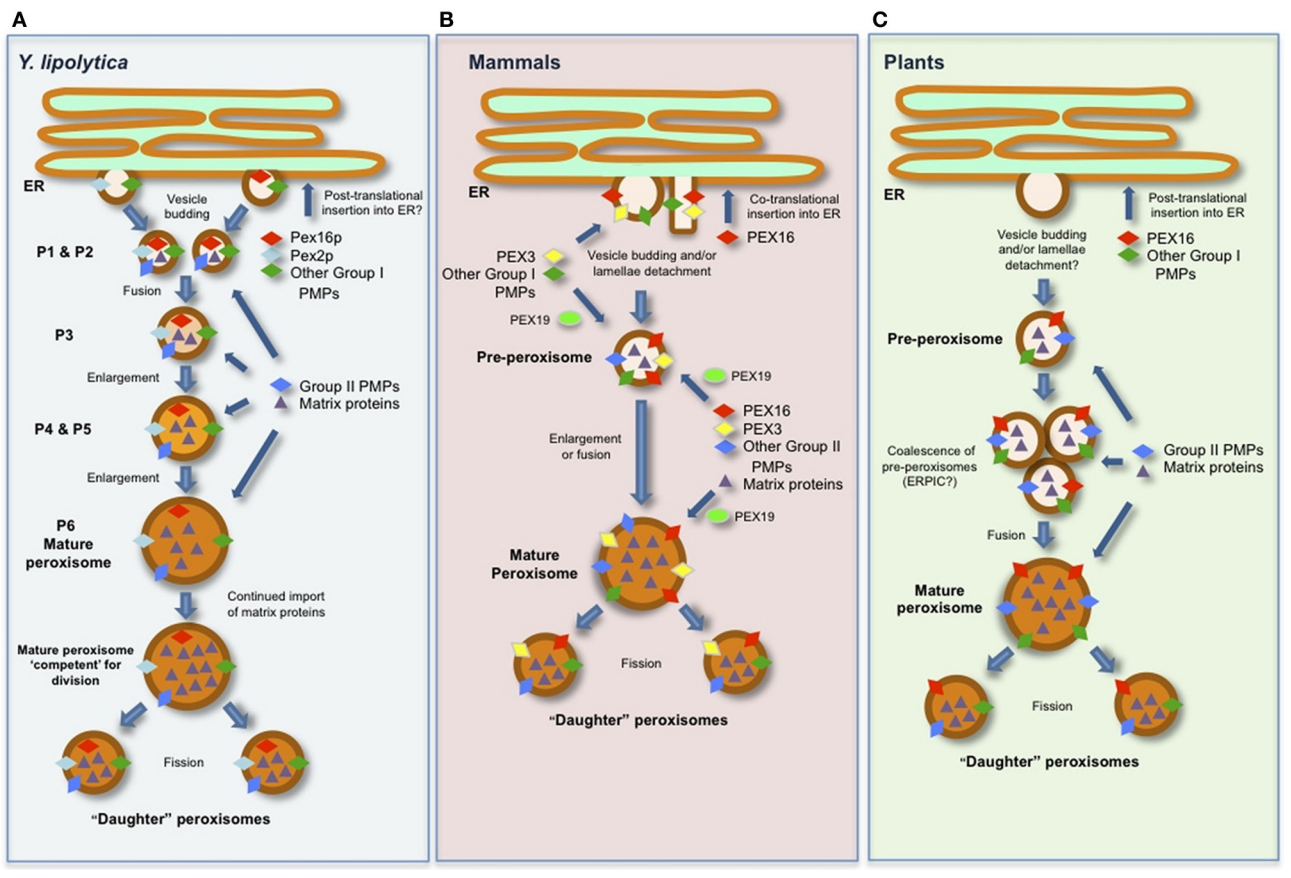

FIGURE 1 | Schematic representations of generalized models for the biogenesis of peroxisomes and the role(s) of PEX16 in (A) *Y. lipolytica,* (B) mammals (human), and (C) plants (*Arabidopsis*). See text for details and references.

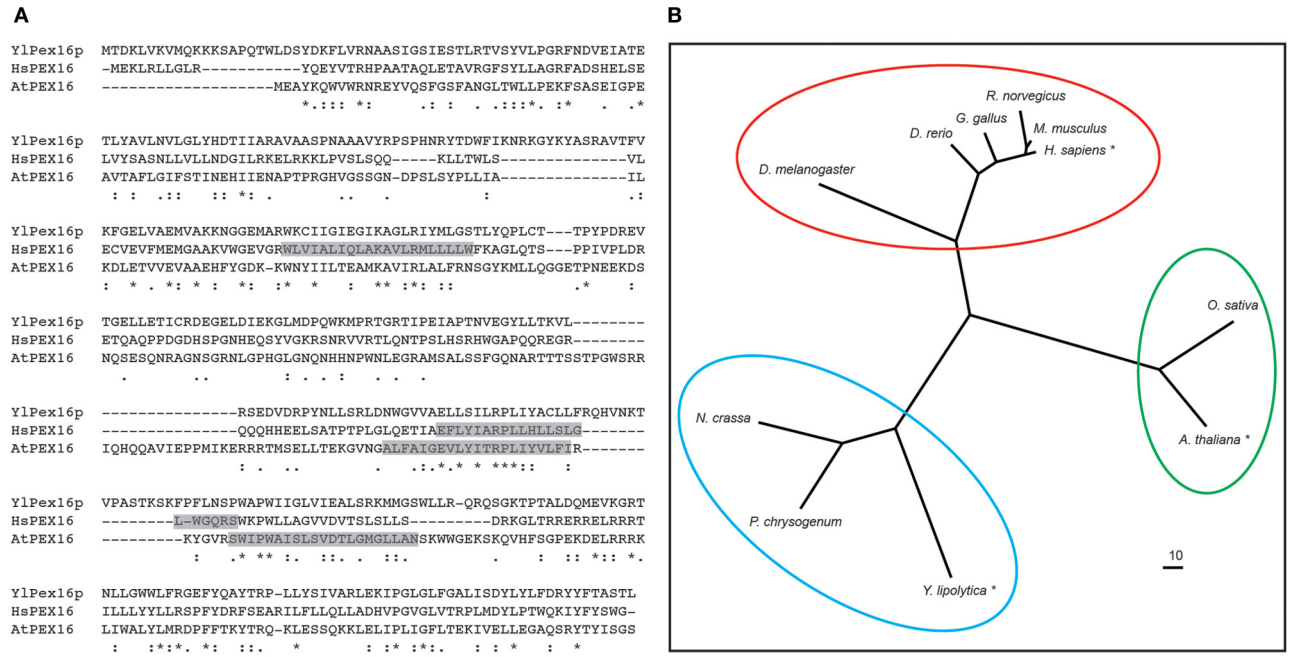

FIGURE 2 | Polypeptide sequence alignment and phylogenetic analysis of various PEX16 proteins. (A) Deduced amino acid sequence alignment of *Y. lipolytica* Pex16p (YlPex16p), human (*Homo sapiens*) PEX16 (HsPEX16), and *A. thaliana* PEX16 (AtPEX16). Identical residues are indicated with asterisks, strongly and weakly similar residues are indicated with a colons and periods, respectively. Predicted membrane-spanning sequences in HsPEX16 and AtPEX16 are shaded and based on Honsho et al. (2002) and Karnik and Trelease (2007), respectively. **(B)** Phylogenetic analysis of PEX16 sequences from selected evolutionarily diverse species. Each protein is labeled based on its respective Genus and species, and those shown in **(A)** are indicated with an asterisk, and circles represent PEX16 proteins of the metazons, yeasts (fungi), and plants that form distinct clades. Branch lengths of the tree are proportional to divergence with the "10" scale bar representing a 10% change. Sequence alignments were carried out using either CLUSTALW (Larkin et al., 2007) and the phlyogram was generated using the program TreeView (v1.6.6). Genbank® accession numbers are as follows: *H. sapiens* (BAA88826.1), *Rattus norvegicus* (NP_001012088.1), *Mus musculus* (NP_660104.2), *Drosophila melanogaster* (NP_649252.1), *Neurospora crassa* (XP_963884.2), *Danio rerio* (NP_001020340.1), *Gallus gallus* (XP_421125.3), *Penicillium chrysogenum* (ABH11422.1), *Y. lipolytica* (AAB41724.1), *A. thaliana* (NP_566053.1), *Oryza sativa* (EEC72380.1).

earliest of these subcompartments, the so-called pre-peroxisomes P1 and P2, are considered to bud as vesicles from a specialized region of the ER and contain a unique subset of PMPs, including YlPex16p, which are collectively known as group I PMPs, i.e., PMPs that sort initially to the ER and then to peroxisomes. Thereafter, P1 and P2 are thought to fuse to form the P3 subcompartment, which in turn enlarges due to the continual import of matrix proteins and/or group II PMPs directly from the cytosol to form P4, then P5, and eventually a mature peroxisome (P6), which can subsequently divide into new "daughter" peroxisomes.

YlPex16p is thought to function by binding the membrane lipid lyso-phosphatidic acid (LPA) in the matrix-facing leaflet of the P1–P5 membranes (**Figure 1A**), thereby inhibiting fission of the P1–P5 subcompartments by suppressing the synthesis of LPA-derived diacylglycerol (DAG), a unique cone-shaped lipid that induces membrane curvature (Guo et al., 2003). In a mature (P6) peroxisome, however, the continued import of nascent matrix proteins eventually results in the organelle being "overloaded" with matrix protein constituents and, thus, competent for division (**Figure 1A**) (Guo et al., 2007). At this point, the enzyme acyl-CoA oxidase is thought to relocalize from the matrix to the membrane where it binds to YlPex16p and stimulates a decrease in its affinity for LPA. This leads in turn to an increase in the formation of DAG from LPA, which, along with phosphatidylserine (PS), "flips" between the leaflets of the peroxisomal membrane bilayer, causing lipid asymmetry that leads to bending of the membrane and the subsequent division of the organelle upon the recruitment of the peroxisome division machinery (Guo et al., 2007).

Besides its unique role in peroxisome division, YlPex16p is perhaps best known as one of the first PMPs experimentally shown to target indirectly to peroxisomes via the ER (Titorenko and Rachubinski, 1998), and thus, early evidence for a role of the ER in formation and maintenance of peroxisomes. However, the nature of the molecular machinery and targeting signals responsible for the ER-to-peroxisome sorting of YlPex16p are unknown.

HUMAN PEX16

Unlike YlPex16p, human PEX16 (HsPEX16) is an integral membrane protein containing at least two transmembrane domains (TMDs) (**Figure 2A**) and a topological orientation whereby both the N and C termini face the cytosol (Honsho et al., 2002). The *Y. lipolytica* and human proteins also differ in that YlPex16p presumably inserts into the ER in a post-translational manner, as do all PMPs that sort to peroxisomes via the ER in *S. cerevisiae* (Van Der Zand et al., 2010), while the insertion of HsPEX16 into the ER

occurs in a co-translational manner (Kim et al., 2006). HsPEX16 is also distinct from YlPex16p in that it does not appear to be directly involved in regulating peroxisome division, but, instead, functions as a PMP receptor during the early stages of *de novo* peroxisome formation at the ER, as well as in mature peroxisomes (Kim et al., 2006; Matsuzaki and Fujiki, 2008). Consistent with this, the loss of HsPEX16, unlike YlPex16p, results in the complete absence of any peroxisomal structures (Honsho et al., 2002).

The precise role of HsPEX16 during the *de novo* synthesis of peroxisomes seems to be as a receptor responsible for the integration of the peroxin PEX3 into the ER and, thus, possibly the subsequent insertion of other PEX3-dependent group I PMPs at the ER (Fransen et al., 2001; Kim et al., 2006; Matsuzaki and Fujiki, 2008). As mentioned previously, most nascent PMPs in the cytosol are recognized and bound by PEX19, a soluble receptor/chaperone that delivers its PMP "cargo" to the membrane-bound docking receptor PEX3. While the details of how the PEX3 receptor mediates the integration and assembly of PMPs into membranes are largely unknown, the way in which PEX3 itself is inserted into membranes seems to also vary depending on the organism. For instance, in *S. cerevisiae*, which lacks a Pex16p homolog, Pex3p is inserted post-translationally into ER membranes via the SEC61 complex (Van Der Zand et al., 2010; Thoms et al., 2012). In mammals, however, the import of PEX3 does not appear to rely on SEC61 (South et al., 2000), but does rely on HsPEX16 (Kim et al., 2006).

Based on these and other findings (Huybrechts et al., 2009), the working model for peroxisome biogenesis in mammals (**Figure 1B**) includes HsPEX16 serving as the receptor for PEX19-independent insertion of PEX3 at the ER. Thereafter, PEX16, PEX3, and the group I PMPs, which are subsequently recruited to the ER by PEX3, are incorporated into a pre-peroxisome in a process that appears to require the ER export factor SEC16B (Yonekawa et al., 2011). The structure of a pre-peroxisome in mammals, however, appears to vary, since some evidence suggests they are small vesicles, as in yeasts, while in certain specialized cells they resemble a lamellar extension that detaches *en block* from the ER (Geuze et al., 2003). Regardless, pre-peroxisomes in mammals are considered to be competent for nascent PMP import (i.e., group II PMPs) and matrix proteins from the cytosol to either enlarge into a new mature peroxisome or fuse with a pre-existing mature (or "daughter") peroxisome in order to promote its growth (**Figure 1B**) (Kim et al., 2006).

Similar to its role at the ER, HsPEX16 appears to function as a receptor for PEX3 in mature peroxisomes as well, and, in doing so, facilitating the subsequent PEX3-dependent import of group II PMPs into more mature organelles (Matsuzaki and Fujiki, 2008) (**Figure 1B**). However, the molecular mechanism underlying the import of PEX3 by PEX16 at peroxisomes seems to be distinct from that at the ER (Kim et al., 2006), since it is dependent on PEX19 (Matsuzaki and Fujiki, 2008). Conversely, HsPEX16 also targets directly to peroxisomes and does so in a post-translational, PEX3- and PEX19-dependent manner (Matsuzaki and Fujiki, 2008). These findings, and those discussed above for the role of HsPEX16 as a receptor for PEX3 at both the ER and peroxisomes, has led to the suggestion of a "chicken-or-the-egg" dilemma for how these two PMP receptors operate in a spatiotemporal manner (Matsuzaki and Fujiki, 2008). However, given that HsPEX16 is inserted into the ER via the SEC61 co-translational import pathway and that PEX3 is only found at the ER in the presence of HsPEX16 (Kim et al., 2006), it seems that HsPEX16 acts as the "master" peroxin responsible for the initiation of peroxisome biogenesis at the ER in mammals.

ARABIDOPSIS THALIANA PEX16

While the *de novo* synthesis of peroxisomes is a possibility in plants, there is almost no direct evidence in support of this pathway. Rather, the role of the ER in peroxisome biogenesis in plants is thought to serve strictly as the site from which group I PMPs and phospholipids are trafficked (via pre-peroxisomes) to mature peroxisomes (**Figure 1C**). For a more comprehensive discussion on the role of the ER in plant peroxisome biogenesis refer to Trelease and Lingard (2006) and Hu et al. (2012).

Among the plant PMPs that sort to peroxisomes via the ER is *Arabidopsis* PEX16, a membrane protein that, like HsPEX16, possesses two predicted TMDs (**Figure 2A**) (Karnik and Trelease, 2007). AtPEX16 was initially identified in a study of the *shrunken seed 1* (*sse1*) mutant in *Arabidopsis*. Herein, SSE1 was re-annotated as PEX16 based on its sequence similarity to *Yl*Pex16p (**Figure 2A**) and its ability to complement, albeit partially, the *Ylpex16* mutant (Lin et al., 1999). While the latter observation implies that AtPEX16 and YlPex16p operate in a similar manner during peroxisome division, which is supported by the observation that *Atpex16* knockdown cells possess fewer and enlarged peroxisomes (Nito et al., 2007), similar to *Ylpex16* mutant yeast cells, other studies indicate that AtPEX16 plays additional roles during plant peroxisome biogenesis. For instance, similar to *Hspex16* mutant cells, the *Atpex16* null mutant is devoid of normal peroxisomes (Lin et al., 2004), implying that AtPEX16 functions at the early stages of peroxisome biogenesis. Whether the *AtPEX16* gene can functionally complement the *Hspex16* mutant, or vice versa, has not been reported.

The intracellular localization and trafficking mechanisms of AtPEX16, including its molecular targeting signals, have been relatively well studied (Karnik and Trelease, 2007). Overall, these findings have not only helped to develop ideas on the possible roles of AtPEX16 in peroxisome biogenesis, but have also helped formulate the larger models for overall peroxisome biogenesis in plants, particularly for how the ER participates in this process (**Figure 1C**). For instance, that AtPEX16 localizes to both the ER and peroxisomes or to peroxisomes only depending on the tissue/cell type (Lin et al., 2004; Karnik and Trelease, 2005) supports the idea that this protein serves more than one function in the plant peroxisome biogenetic pathway, e.g., at the ER, AtPEX16, like HsPEX16, may act as a PMP receptor and help orchestrate the sorting of these PMPs into pre-peroxisomes. Notably, *Atpex16* mutant plants have defects not only in peroxisomal biogenesis, but also in the formation of other ER-derived organelles, such as oil and protein bodies (Lin et al., 1999), suggesting that the roles of PEX16 at the ER in plants may actually extend beyond those ascribed to its mammalian counterpart.

As depicted also in **Figure 1C**, the localization of AtPEX16 at mature peroxisomes and perhaps at pre-peroxisomes enroute to peroxisomes has been attributed to the protein's potential role as a

receptor for PEX3 and other group II PMPs (Karnik and Trelease, 2007). It is not known, however, whether AtPEX16 can target directly to pre-peroxisomes and/or mature peroxisomes in a post-translational manner, similar to HsPEX16, although it does target post-translationally to the ER (Karnik and Trelease, 2007).

The ER-to-peroxisome trafficking relies on two sets of overlapping molecular targeting signals: (i) those responsible for directing the protein from its sites of synthesis in the cytosol to the ER and (ii) those that direct it from the ER to peroxisomes (Karnik and Trelease, 2007). While the precise nature of these signals in AtPEX16 is an open question, the trafficking of AtPEX16 from the ER to peroxisomes appears to involve a so-called ER-peroxisome-intermediate-compartment (ERPIC), which is postulated to comprise ER-derived pre-peroxisomes that have coalesced prior to their fusion with mature peroxisomes (Karnik and Trelease, 2007). ERPIC-like compartments have been also identified in certain yeast and mammals (Titorenko and Mullen, 2006), although in no case, including in plants, have these been thoroughly investigated.

CONCLUSIONS AND PERSPECTIVES

One of the key regulators of peroxisome biogenesis is PEX16, a peroxin that, depending on the organism, functions in remarkably diverse ways, including the control of peroxisome fission [e.g., *Y. lipolytica* Pex16p (Guo et al., 2003, 2007)], or the de *novo* synthesis of peroxisomes [e.g., human PEX16 (Kim et al., 2006)]. On the other hand, it is equally remarkable that some organisms, such as *S. cerevisiae*, lack a PEX16 homolog (Kiel et al., 2006), yet their mode of peroxisome biogenesis is similar to Y. *lipolytica* (Van Der Zand et al., 2012), implying that they rely instead on an alternative mechanism(s), or other proteins that provide similar functions, for the control of key steps during peroxisome biogenesis. One possible explanation for this apparent loss of PEX16, at least in *S. cerevisiae*, is that all of the PMPs in this yeast are inserted into the ER via the SEC61 complex (Van Der Zand et al., 2010; Thoms et al., 2012). By contrast, in mammals, PEX16, not SEC61 (South et al., 2000), appears to mediate (presumably through PEX3) the insertion of PMPs that localize to peroxisomes via the ER (Kim et al., 2006), as well as PMPs that target directly to peroxisomes (Matsuzaki and Fujiki, 2008). Whether this premise holds true remains to be determined. Regardless, how PEX16 actually functions as a PMP receptor at the ER and peroxisomes, perhaps by forming part of a translocon analogous to the SEC61 complex, and how it regulates peroxisome division, which seems to rely on a dynamic interplay of peroxisomal proteins and lipids (Guo et al., 2007; Itoyama et al., 2012), will be fascinating subjects for future research. It is also conceivable that future cross complementation and heterologous expression studies between various yeast, mammalian, and plant species may reveal as-yet-unknown aspects of PEX16 in peroxisome biogenesis and, by doing so, will provide additional insight to the shared and/or unique spatiotemporal dynamics and molecular mechanisms that underlie the peroxisome biogenetic pathways in different organisms.

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Simultaneous live-imaging of peroxisomes and the ER in plant cells suggests contiguity but no luminal continuity between the two organelles

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Transmission electron micrographs of peroxisomes in diverse organisms, including plants, suggest their close association and even luminal connectivity with the endoplasmic reticulum (ER). After several decades of debate *de novo* peroxisome biogenesis from the ER is strongly favored in yeasts and mammals. Unfortunately many of the proteins whose transit through the ER constitutes a major evidence for peroxisome biogenesis from the ER do not exhibit a similar localization in plants. Consequently, at best the ER acts as a membrane source for peroxisome in plants. However, in addition to their *de novo* biogenesis from the ER an increase in peroxisome numbers also occurs through fission of existing peroxisomes. In recent years live-imaging has been used to visualize peroxisomes and the ER but the precise spatio-temporal relationship between the two organelles has not been well-explored. Here we present our assessment of the peroxisome-ER relationship through imaging of living *Arabidopsis thaliana* plants simultaneously expressing different color combinations of fluorescent proteins targeted to both organelles. Our observations on double transgenic wild type and a drp3a/apm1 mutant Arabidopsis plants suggest strong correlations between the dynamic behavior of peroxisomes and the neighboring ER. Although peroxisomes and ER are closely aligned there appears to be no luminal continuity between the two. Similarly, differentially colored elongated peroxisomes of a drp3a mutant expressing a photoconvertible peroxisomal matrix protein are unable to fuse and share luminal protein despite considerable intermingling. Substantiation of our observations is suggested through 3D iso-surface rendering of image stacks, which shows closed ended peroxisomes enmeshed among ER tubules possibly through membrane contact sites (MCS). Our observations support the idea that increase in peroxisome numbers in a plant cell occurs mainly through the fission of existing peroxisomes in an ER aided manner.

Keywords: peroxisome, ER, drp3a, membrane-contact-sites, live-imaging, EosFP

INTRODUCTION

Single membrane bound, spherical-ovoid 0.1–1.5 μm organelles that appeared closely associated with the ER were first reported at the ultra-structural level in the proximal convoluted tubules of mouse kidney by Rhodin (1954) and in hepatic parenchymal cells by Rouiller and Bernhard (1956). Soon after these microbodies were reported in plants (Porter and Caulfield, 1958; Mollenhauer et al., 1966; Frederick et al., 1968; Frederick and Newcomb, 1969). The discovery that certain microbodies contain catalase and other hydrogen-peroxide producing oxidases resulted in their being named peroxisomes (de Duve and Baudhuin, 1966; de Duve, 1969). Peroxisomes are now recognized as major producers as well as scavengers of reactive oxygen and nitrogen species (del Río et al., 2003; Palma et al., 2009).

While the biochemical characterization of peroxisomes progressed rapidly (reviewed in Tolbert and Essner, 1981) their cell biological dissection also kept pace through the development of several cytochemical procedures for staining peroxisomes using 3,3′ diaminobenzidine (DAB; Graham and Karnovsky, 1966; Fahimi, 1969; Hirai, 1969; Novikoff and Goldfischer, 1969). The DAB staining method localized the catalase in peroxisomes and greatly facilitated their identification in cells, and the evaluation of their relative abundance and relationship to other organelles, specially the endoplasmic reticulum (ER) (Frederick and Newcomb, 1969; Vigil, 1970).

The microbody–ER association noted in early electron microscopy based studies [reviewed by Hruban and Rechcigl (1969)] suggested that the semi-opaque material characteristic of microbodies is deposited within dilated portions of the ER that enlarge to form microbodies (Novikoff and Shin, 1964). Indeed ER like projections and continuities were observed so often in the rat liver cells that Novikoff and Shin (1964) considered microbodies to be always attached to the ER. Observations on absorptive cells of the mammalian small intestine also allowed small peroxisomes called micro-peroxisomes to be considered as localized dilations of the smooth ER that retain numerous

continuities (Novikoff and Novikoff, 1972; Novikoff et al., 1973a). Since peroxisome isolation was a major contributor in their biochemical characterization Novikoff and Novikoff (1972) speculated that the connections between peroxisomes and the ER might be broken during homogenization and subsequent isolation through centrifugation. The combined observations from microscopy and pulse chase experiments resulted in the vesiculation model wherein peroxisome biogenesis was proposed as taking place through a budding mechanism from the ER (Beevers, 1979). Whereas a complete list of publications providing documentation of associations between microbodies/peroxisomes and the ER is not presented here some outstanding contextual publications are Svoboda and Azarnoff (1966), Essner (1967), Essner (1969), Svoboda et al. (1967), Magalhaes and Magalhaes (1971), de Duve (1973), Novikoff et al. (1973a), Novikoff et al. (1973b), Reddy and Svoboda (1973), Frederick et al. (1975) and Hirai et al. (1983). Early studies clearly pointed to an ER based origin and intimate connectivity between peroxisomes and the ER.

However, the realization that peroxisomes do not possess their own DNA or protein synthesis machinery had already made it apparent that most of their membrane and matrix proteins are imported post-translationally from the cytosol (Lazarow and de Duve, 1973; Goldman and Blobel, 1978; Lazarow et al., 1980, 1982; Koster et al., 1986). The possibility that peroxisomes did not have to be created from the ER but could actually be formed from pre-existing peroxisomes was raised (Legg and Wood, 1970). The dominant peroxisome vesiculation model was rigorously tested by Poole et al. (1970), who searched for gradual dilation of ER tubules to form peroxisomes but were unable to find them. Subsequently Lazarow and Fujiki (1985) assessed the existing ultra-structural and morphological evidence as compared to the biochemical information and laid down a stringent criterion that sought direct luminal connectivity between the ER and the peroxisome.

Today there is increasing appreciation that peroxisomes are endomembrane derivatives (South and Gould, 1999; Geuze et al., 2003; Kunau, 2005; Tabak et al., 2008, 2013). It is believed that while *de novo* biogenesis of peroxisomes can occur directly from the ER, existing peroxisomes in a cell can also undergo fission to form more peroxisomes (Motley and Hettema, 2007). These recent molecular genetic and biochemical evidence have been taken into account in recent reviews (Tabak et al., 2003, 2013; Titorenko and Mullen, 2006; Fagarasanu et al., 2007) and resulted in models such as the "ER semi-autonomous peroxisome maturation and replication" for peroxisome biogenesis in plants (Mullen and Trelease, 2006; Trelease and Lingard, 2006) and for yeasts (Titorenko and Rachubinski, 2009). Additional detailed discussion on peroxisome biogenesis can be found in recent reviews by Hu et al. (2012), Tabak et al. (2013), and Theodoulou et al. (2013). The transit and accumulation of specific peroxisomal proteins such as peroxin 16 (pex16) (Kim et al., 2006), pex3 and pex19 (Hoepfner et al., 2005; Kragt et al., 2005), provide convincing evidence that favors peroxisome biogenesis from the ER in yeasts and mammals (van der Zand et al., 2010, 2012; Lam et al., 2010; Agrawal et al., 2011; Theodoulou et al., 2013). However, there is no clear evidence for the formation of peroxisomes directly from the ER in plants (Trelease and Lingard, 2006). The formation of an ER-peroxisome intermediate compartment (ERPIC) has been proposed but its actual relationship with the ER has not been adequately demonstrated (Mullen and Trelease, 2006; Trelease and Lingard, 2006). Despite the early micrographs suggesting ER-microbody associations (Reddy and Svoboda, 1973; Shio and Lazarow, 1981; Gorgas, 1984, 1985; Yamamoto and Fahimi, 1987) at best the ER in plants is viewed as a source of membrane components, which are delivered in some sort of membrane carrier to pre-existing peroxisomes (Titorenko et al., 2000; Mullen and Trelease, 2006; Hu et al., 2012).

While ultrastructural, biochemical and molecular-genetic approaches to understanding the peroxisome-ER link have been commendable, the direct and simultaneous visualization of the two organelles has not been carried out in plants. Nevertheless over the past years many fluorescent protein probes, mainly based on green fluorescent protein (GFP) and its color variants, that highlight peroxisomes and the ER separately have been developed for living plant cells (Mathur, 2007; Illuminated Plant Cell http://www.illuminatedcell.com/cytomembranes.html). Fluorescent highlighting of the 0.4–1.5 μm diameter peroxisomes shows their hitherto unexplained erratic motility that includes stop and go motion, sudden twists and turns including U-turns, and an almost individualistic manner of movement where one peroxisome might remain almost static while others around it move at varying velocities (Collings et al., 2002; Jedd and Chua, 2002; Mano et al., 2002; Mathur et al., 2002; Rodríguez-Serrano et al., 2009). In contrast to microtubule dependent movement of peroxisomes in mammalian cells (Wiemer et al., 1997; Schrader et al., 2003) their motility in plant cells takes place along F-actin strands in a myosin dependent manner (Collings et al., 2002; Jedd and Chua, 2002). By combining fluorescent probes for peroxisomes and the ER into one plant it is possible to look at both organelles in living plant cells simultaneously without the encumbrance and possibility of creating fixation induced artifacts, the need for sectioning and the limitation of single snapshots.

Here we report observations on peroxisomes and the ER obtained through simultaneous visualization of both organelles in double-transgenic plants of *Arabidopsis thaliana*. Our investigations have been extended to two alleles of the *drp3A/apm1* mutant of Arabidopsis (Mano et al., 2004), which offer aberrantly elongated peroxisomes and thus raise the chances of assessing peroxisome-ER connectivity or continuity. The live imaging study is complemented by 3D iso-surface rendering of confocal derived image stacks that provide more insight through a volume rendered version of the digital images. Our observations in living plant cells strongly support peroxisome–ER contiguity but have been unable to find evidence of luminal continuity between two inter-twined peroxisomes as well as between peroxisomes and the ER.

RESULTS

THE ERRATIC MOVEMENT OF PEROXISOMES CLOSELY SIMULATES THE DYNAMIC BEHAVIOR OF NEIGHBORING ER TUBULES AND CISTERNAE

As noted earlier by Jedd and Chua (2002), Mano et al. (2002), Mathur et al. (2002) and Collings et al. (2002) the basis for erratic

movement of peroxisomes in plants has remained unexplained. The seemingly individualistic patterns of peroxisomal motility include staying in one subcellular location for varying durations, bi-directional movements, cyclic revolution within a small region of the cell, U-turns, and sudden lateral or tangential forays (Mano et al., 2002; Mathur et al., 2002). Much of the straight axial movement can be attributed to direct peroxisomal dependence on myosin motors and the underlying F-actin cytoskeleton as part of the main cytoplasmic stream (Grolig and Pierson, 2000; Jedd and Chua, 2002). However, neither myosin motors nor the existence of fine F-actin tracks directly elucidate the mechanism underlying sudden stops, oscillations and other peculiarities of peroxisomal motility (Mathur et al., 2002). Interestingly the fine cortical F-actin mesh in a plant cell also provides a structural basis for cortical ER organization in plants (Quader and Zachariadis, 2006; Runions et al., 2006; Sparkes et al., 2009) and thus we speculated that observing the simultaneous behavior of peroxisomes and the ER might provide insights on the erratic motility of peroxisomes.

The cortical ER in plant cells displays a dynamic pattern of non-uniform polygons created by anastomosing cytomembranes wherein smooth tubules and lamellar segments undergo constant rearrangement, sliding, branching and fusion. Sub-cortically, the ER forms long tubules and fenestrated sheets that enmesh different organelles and form a major component of cytoplasmic strands stretching across the large, vacuolated plant cell (Quader and Zachariadis, 2006). Observations on dual marker lines co-expressing ss-mGFP5-HDEL and YFP-PTS1 as well as the YFP-RFP dual combination revealed many instances of forward movement followed by a U-turn with constant variation in the rate of motility. A representative time-lapse series is provided (**Figures 1A,B**) where the movement of a single peroxisome (**Figure 1C**—peroxisome with red spot) is charted (**Figure 1A** frames 1–22) and presented as a merged image (**Figure 1B**). The variable distance moved between each frame is presented in **Figure 1C** whereas **Figure 1D** follows the movement carried out over 120 s for 6 independent peroxisomes. Clearly, different peroxisomes move differently and there is a range of variation in their rate of their movement (**Figure 1D**). As observed in the images (and the **Movie S1**) the motility of the peroxisome closely followed the patterns of the neighboring ER (**Figure 1A**). Another representative series of 13 sequential images (**Movie S2**) each separated by ca. 8 s (**Figure 1E** frames 1–13) follows the movements of 3 peroxisomes (**Figure 1E**; 1-a, b, c) and reveals that to-and-fro oscillations ranging between 2 and 8 μm usually occur along short ER-tubules extending and retracting from an ER junction (**1E**; peroxisomes a, b frames 1–8). As shown in **Figure 1E** (frames 8–10) such movements continue until the ER tubule harboring a peroxisome fuses with another ER tubule to create the familiar ER polygon, whereupon the peroxisome moves again in the pattern defined by the newly organized polygon (**Figure 1E**; peroxisome b; frames 7–10). Finally the oscillating peroxisomes get drawn into a fast moving cytoplasmic ER strand and move away rapidly from their previous locations at velocities approaching $4 \pm 1.5\,\mu ms^{-1}$ (**Figure 1E** frames 9–13).

The cortical ER also consists of large membrane patches that constitute ER islands. When observed using only YFP-PTS1 many peroxisomes appear moribund or execute only Brownian movement (Mathur et al., 2002). When co-visualized with the ER these peroxisomes were confined to the ER islands (e.g., peroxisome-c in **Figure 1E**). These observations of individual peroxisomal activity within different regions of the interlinked and constantly rearranging ER suggested an explanation for the seemingly erratic peroxisome motility. The observations also suggested how, depending upon the rate of ER motility, up to $65 \pm 3\%$ ($n = 250$) peroxisomes within a cell might appear to be arrested in a particular sub-cellular location while other peroxisomes move past them at varying speeds. We further investigated this line of thought by observing peroxisomal motility under conditions that are known to affect ER dynamics.

EFFECT OF LOW TEMPERATURE ON ER-PEROXISOME DYNAMICS

Low temperature, around 4°C slows down ER motility, leads to the disappearance of long tubular ER strands and causes a distinct increase in short ER tubules and wider cisternae (Quader et al., 1989). Seedlings expressing RFP-ER and YFP-PTS1 were cold treated for 6 h and analyzed for motility. **Figure 2A** shows the change in peroxisome motility over 12 min as the ambient temperature around the cold treated seedlings returned to 23°C. Hypocotyl epidermal cells visualized within 3 min of removal from ice-cold temperatures displayed short cortical ER tubules, slow tumbling movements of spindle shaped ER bodies and a low motility of the ER in cytoplasmic strands. Only $2.79 \pm 0.9\%$ peroxisomes ($n =$ total 100 peroxisomes observed in 10 different cells in 10 seedlings) displayed short oscillations in these cells with the rest exhibiting Brownian movement. By 6 min the number of motile peroxisomes had risen to $35 \pm 2.8\%$, while ER-tubules started exhibiting remodeling and new areas of anastomosis to form the typical polygonal ER mesh. By 12 min the ER exhibited normal cortical and sub-cortical dynamics and $67 \pm 5\%$ peroxisomes had achieved motility with normal speeds of $4 \pm 1.5\,\mu ms^{-1}$ in sub-cortical strands. It was concluded that while slowing down the dynamic behavior of the ER results in slowing peroxisome motility an increase in the movement rate of the ER results in a concomitant change in peroxisomal movement.

EFFECT OF THE ACTIN POLYMERIZATION INHIBITOR LATRUNCULIN-B

Both actin and myosin inhibitors have previously been shown to interfere with peroxisome (Collings et al., 2002; Jedd and Chua, 2002; Mano et al., 2002; Mathur et al., 2002) and ER (Liebe and Quader, 1994) motility. Both organelles respond similarly by displaying an arrest of motility that is reversible upon washing out the inhibitor. The visualization of their concomitant response to 1 μM Latrunculin-B is shown in **Figure 2B**. In all hypocotyl cells ER and peroxisome motility ceased within 5 min of being exposed to the inhibitor. Both organelles froze in place but did not lose their form. However, in some areas peroxisomes were seen aggregating around large ER blobs (**Figure 2B**; asterisk). After 10 min the inhibitor was washed away through 5 sequential washes with 10 ml water. Within 5 min of the washing clear indications of ER recovery including the extension-retraction of cortical ER tubules, the resumption of sub-cortical-ER flow in cytoplasmic strands and the stirring of ER bodies, were observed.

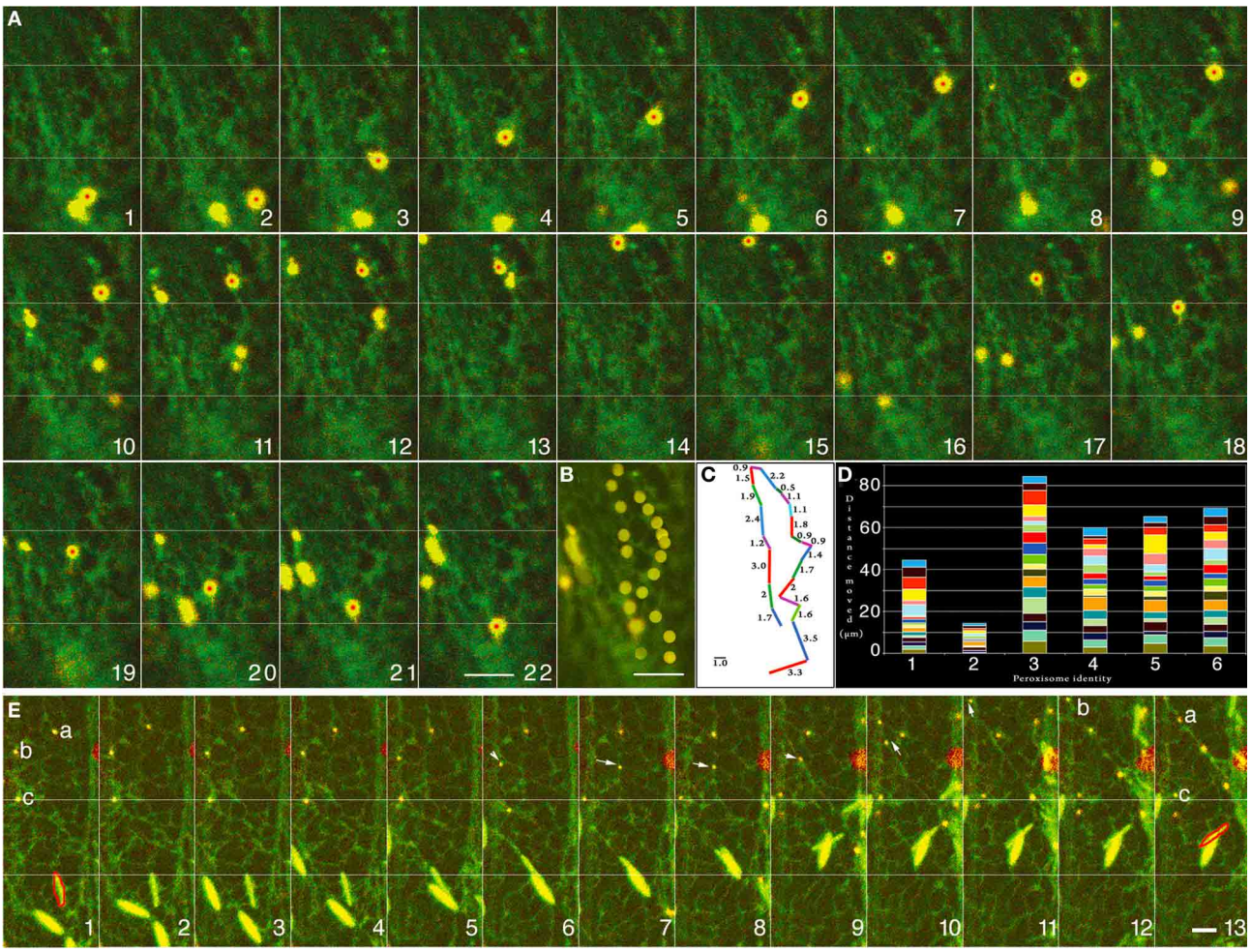

FIGURE 1 | Representative images of cells from double transgenic Arabidopsis plants expressing peroxisome and ER-targeted probes that suggest correlative behavior of both organelles. (A) Twenty-two time-lapse images that follows a single YFP-PTS1 highlighted peroxisome (with red dot) against the backdrop of green fluorescent ER (**Movie S1**). **(B)** A merge of all the frames from "**A**" traces the path of the single peroxisome. Every frame capturing the peroxisome movement has an accompanying subtle change in the subtending ER. **(C)** The movement per frame in the time-lapse image sequence in "**A**" and the path seen in "**B**" displayed as distance moved in μm shows the erratic nature of peroxisome motility. **(D)** Movement of six different peroxisomes followed over 120 s shows the range of variation in their rate of movement and suggests that no two peroxisomes move at the same rate or for the same distance. **(E)** The movement of three peroxisomes (a, b, and c) tracked along with changes in the organization of neighboring ER tubules over 13 sequential images from **Movie S2**. Frames are separated by ca. 8 s. In contrast the peroxisomes "a" and "b" exhibit oscillations ranging between 2 and 8 μm (frames 1–8) alongside short ER-tubules that extend and retract from an ER island. Arrows in frames 6–9 point to the movement of peroxisome "b" wherein the ER tubule harboring it fuses with another tubule to create the familiar ER polygon, whereupon the peroxisome moves again in the pattern defined by the newly organized polygon (frames 7–10). Finally the oscillating peroxisomes "a" and "b" are drawn into a cytoplasmic ER strand and move away rapidly from their previous locations (frames 9–13). Peroxisome "c" shows the least movement (white line across the 13 frames) and remains lodged on a broad patch of ER membrane. An ER body has been outlined in red (bottom half of frames) to provide a comparative estimation of ER reorganization in another area of the cell. Size bars = 2.5 μm.

Concomitantly, as shown through a representative series of time-lapse images the range of peroxisomal oscillations alongside ER tubules started increasing. In this early recovery period covering 3 min 80 ± 6% ($n = 200$) peroxisomes displayed relatively localized movements. These involved multiple circumambulations (path shown in **Figure 2C** frame 10) with diameters ranging from 10 to 15 μm before single peroxisomes moved away on tracks defined by tangential ER tubules (**Figure 2C** frames 1–10). By 10 min after inhibitor removal a majority of cells displayed normal ER and peroxisomal motility. The drug treatments in our experiments were restricted to 10 min since longer exposures to 1 μM Lat-B result in increased ER cisternae and aggregation (**Figure 2D**) where upon the associated population of peroxisomes becomes limited to Brownian movement. As estimated through observations carried out every 30 min more than 90% of such cells with ER globules did not recover complete organelle motility during 2 h. Similar ER-peroxisome aggregates were often observed in root cells that had been injured or been kept in water, presumably under hypoxic conditions for 2–3 h.

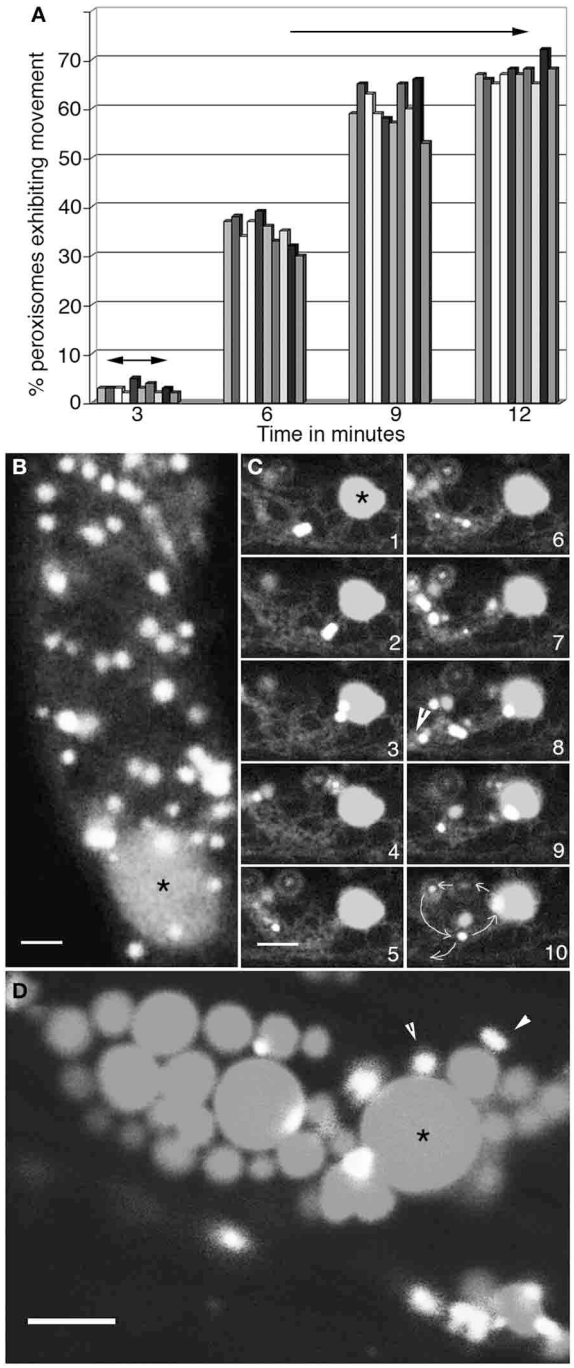

FIGURE 2 | Concomitant changes take place in peroxisome and ER motility. (A) Graphical representation of changes in motility of 10 peroxisomes over 12 min as the ambient temperature around cells in 4°C treated seedlings rises to about 23°C. At 3 min following exposure to room temperature peroxisomes begin to exhibit short oscillations (double sided arrows) in synchrony with extension-retraction of ER tubules. The range of movement increases over time (6- and 9-min time points) until by 12 min both the ER and peroxisomes exhibit normal motility including long saltations. **(B)** Both peroxisomes and the ER in cells treated with 1 μM latrunculin-B stop moving and large blobs of ER (*) surrounded by peroxisomes start appearing. **(C)** Sequential images taken at intervals *(Continued)*

FIGURE 2 | Continued
of after washing away latrunculin-B show the gradual recovery of the ER accompanied by the circumambulation of peroxisomes apparently embedded in the ER (arrowhead in panel 8; path shown by circular arrows in panel 10) during the first 3–4 min. **(D)** Treatment with lat-B for more than 10 min usually leads to the formation of large ER globules (*) surrounded by static peroxisomes. Such disorganized ER does not reorganize easily into normal cytoplasmic streaming of organelles. Size bars = 5 μm. *indicates an ER globule; arrowheads point to peroxisomes.

Our observations suggested a close correlation in the pattern of peroxisome motility and the dynamic behavior of ER tubules, but did not provide a sense of the physical relationship between the two organelles. In an earlier study (Sinclair et al., 2009) we have showed that thin tubules, called peroxules, are formed by peroxisomes exposed to subcellular oxidative stress. The peroxules are extended and retracted along ER tubules. We speculated that elongated peroxisomes would provide a better way of understanding the spatio-temporal relationship between peroxisomes and the ER in comparison to our observations on the small ca. 1 μm diameter organelles. Peroxisomes in the *apm1/drp3a* (Mano et al., 2004) mutant were visualized for this purpose.

UNLIKE ER TUBULES ABNORMALLY ELONGATED PEROXISOMES IN THE *apm1/drp3a* MUTANT DO NOT FUSE WITH EACH OTHER

Peroxisomes in the *apm1/drp3a* mutant of *Arabidopsis* are unable to undergo efficient fission and break into the typically spherical, ca. 0.4–1.5 μm diameter organelles. Consequently the *apm1* mutants display peroxisomes with an abnormally elongated morphology (Mano et al., 2004). Time lapse imaging of GFP-highlighted elongated peroxisomes in the apm1-1 mutant showed them morphing rapidly into various contorted forms that included polygons and extensions such as those displayed by the ER (**Figures 3A,B**). However, in the case of the ER the polygons are part of a continuous system and undergo constant fission and fusion (Quader and Zachariadis, 2006; Sparkes et al., 2009). The resemblance of peroxisomes in the apm1-1 mutant to ER polygons and reticulum made us wonder whether the peroxisomal tubules could actually be a part of the existing ER mesh. In such a situation, we reasoned, the tubules would also be able to fuse and exchange luminal proteins. In an earlier report on plastid stromules we have used photo-convertible protein based differential coloring to demonstrate mitochondrial fusion and plastid non-fusion (Schattat et al., 2012). Mitochondrial fusion resulted in their contents mixing and resulted in an intermediate color between red and green (Schattat et al., 2012). Therefore we introduced a green to red photo-convertible EosFP-PTS1 (Sinclair et al., 2009; **Figure 3C**) in the T-DNA insertional mutant allele of *apm1-1* (SALK_066958). The initially green fluorescent tubular peroxisomes in these lines could be readily photo-converted to red fluorescent ones through irradiation with a violet-blue light (355–425 nm; **Figure 3C**). We observed occasional fission of single red and green tubules but in 80 tubules from 10 different seedlings, where sometimes multiple tubules intermingled with each other for several min, we were unable to find even a single instance where a red tubular peroxisome fused with a green one. Representative frames from a time-lapse image sequence

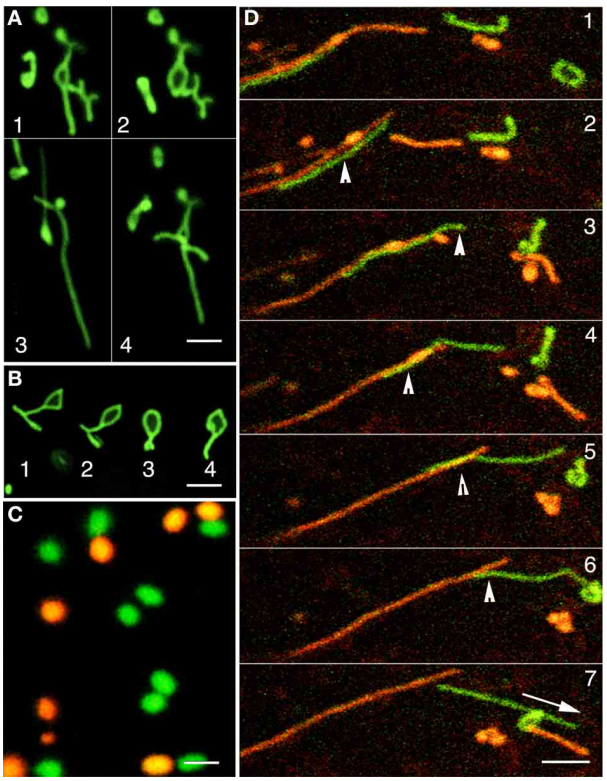

FIGURE 3 | The dynamic behavior of tubular green fluorescent protein highlighted peroxisomes in the apm1-1 mutant and differential coloring of peroxisomes using a green to red photoconvertible mEosFP. (A) GFP-highlighted abnormally elongated peroxisomes in the apm1-1 mutant organize into random shapes including open and closed polygons (sequential frames 1–4) within min. **(B)** A single tubular peroxisomes in the apm1-1 morphs sequentially (1–4) into a closed polygon that is highly reminiscent of shapes presented by polygons making up the cortical ER mesh. **(C)** The color of peroxisomes can be changed rapidly from green to red by using the photoconvertible mEos fluorescent protein and irradiating the organelles with violet-blue light. **(D)** A time-lapse image sequence shows the intermingling and subsequent separation of green (non-photo-converted) and red (photo-converted) tubular peroxisomes in a drp3-3 mutant line. The bottom left (Panel 1) shows laterally aligned green and red tubules. In subsequent panels 2–7 the green tubules glides over the red one until the two separate (panel 7). Arrowheads in panels 2–6 shows areas of overlap suggesting close interaction between the tubules. Note that the possible interactions do not appear to result in any exchange of fluorescent proteins. Other smaller tubules morph continuously, seem to interact transiently (panel 3), before separating but do not exchange fluorescent protein either. Scale bar: **(A,B,D)** = 5 μm; **(C)** = 2.5 μm.

are presented (**Figure 3D**). We concluded that individual peroxisomes, whether spherical or tubular, maintain a closed boundary and do not re-establish luminal continuity with other peroxisomes. Having established the individuality and closed nature of tubular peroxisomes, that displayed ER like behavior, we sought to investigate their spatial relationship with ER tubules.

ELONGATED PEROXISOMES IN THE *apm1/drp3a* AND ER TUBULES DISPLAY CONTIGUITY

Whereas observations of green fluorescent tubular peroxisomes in the *apm1-1* and its T-DNA insertional allele (SALK_066958) strongly suggested that one is observing an ER like compartment the simultaneous time-lapse observations of peroxisomes and the ER in double transgenics expressing GFP-PTS1 and RFP-ER showed several instances where peroxisomal tubules lay in ER lined channels (**Figure 4A**; **Movie S3**). The merged images clearly showed that the extension of tubular peroxisome closely followed the reorganization and dynamics of neighboring ER tubules (**Figure 4A**; frames 1–8). In other time-lapse images (such as **Figure 4B** frames 1–7; **Movie S4**) instances of a tubular peroxisome lodged on an ER island and rejoining a rapidly moving sub-cortical ER strand were noted (**Figure 4B**; **Movie S4**). These observations match the observations on motility of spherical peroxisomes in wild type plants (**Figures 1A–E**) but with the added advantage of being able to observe a larger area in which the two organelles maintain closeness. Occasionally the contiguity may extend to tubular peroxisomes wrapping around ER organelles such as large spindle shaped ER bodies as they pass by as part of the streaming cytoplasm (**Figure 4C**; **Movie S5**). In such cases the transient association, even if it occurs against the track being followed originally by the peroxisome (**Movie S5**), results in the peroxisomal shape tracing out the organelle surface (**Figure 4C** frames 1–7). In addition several instances were noted where a long tubular peroxisome broke into two unequal parts through the dynamic reorganization of the surrounding ER. The individual bits of tubular peroxisomes drew further apart as the neighboring ER polygons reorganized in different directions (**Figure 4D**; **Movie S6**). Observations on the pulling apart and breaking of a tubule through ER reorganization in different directions suggested that the tubular peroxisomes were somehow strongly tethered to the ER and not free to slip out of the ER mesh on their own. A software aided 3D volume rendering was carried out to get more insight into the relationship between the ER and the tubular peroxisomes.

TUBULAR PEROXISOMES APPEAR ENMESHED IN THE ENDOPLASMIC RETICULUM

Three-dimensional iso-surface rendering of confocal image stacks showed that peroxisomal tubules are closely inter-twined with tubules of the ER (**Figure 5A**; **Movie S7**). Such intertwining probably accounts for their fission as the ER undergoes reorganization in an actin-myosin dependent manner. Their position as embedded tubules in the ER also accounts for their dynamic ER-like behavior. Such coincidental behavior of contiguous ER and tubular peroxisomes suggests that they might have strong adherence to each other. Although the membrane contact sites (MCS) between peroxisomes and the ER are not readily visible in our rendered image the weaving of tubules between ER polygons suggests the possibility of such contact and adhesion sites. However, neither confocal imaging nor the iso-surface rendering suggests luminal connectivity between closed peroxisomal tubules and the ER.

DISCUSSION

Transmission electron microscopy and cytochemistry based publications in the early phase of research on microbodies/peroxisomes strongly suggested their association with the ER but the inability to actually track peroxisomes being born from the ER drew criticism for the ER-vesiculation model (Legg and

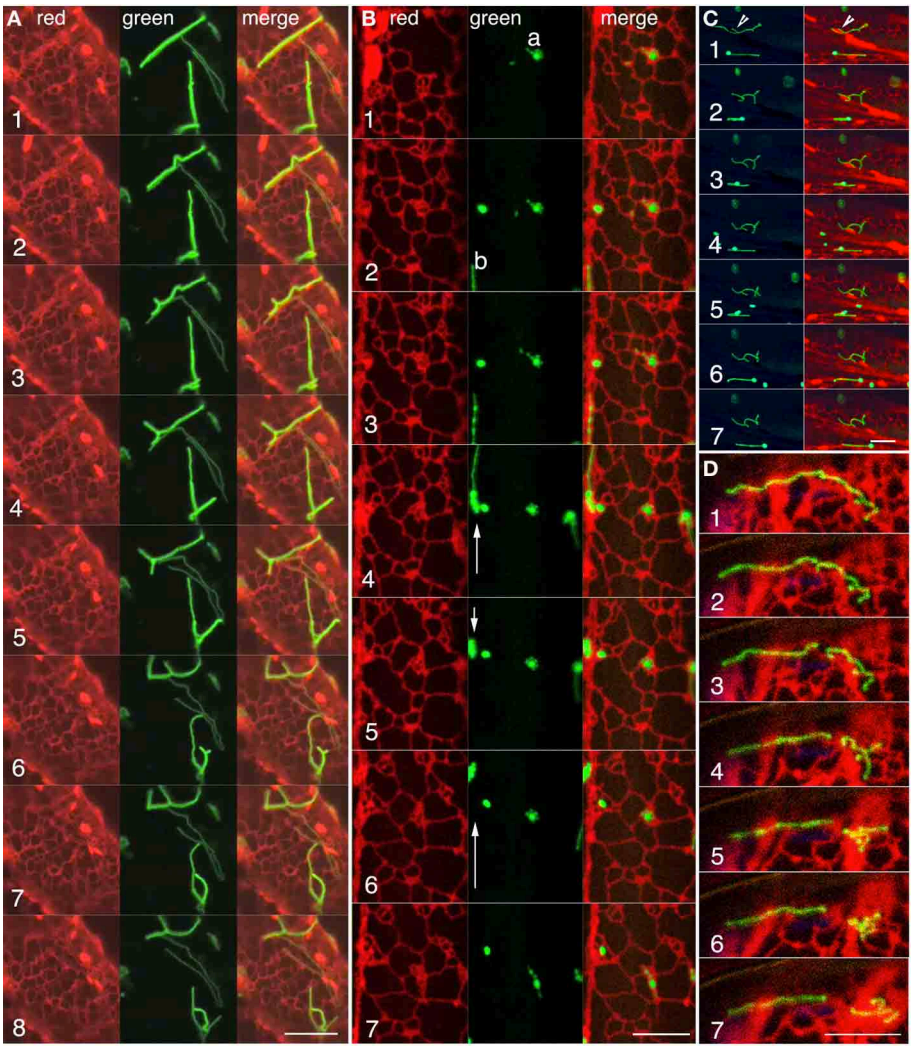

FIGURE 4 | Confocal visualization of green fluorescent tubular peroxisomes and red fluorescent luminal ER in living hypocotyl cells of the apm1-1 mutant of Arabidopsis. (A) Representative frames from a time lapse image sequence (**Movie S3**) showing the correlated behavior of red fluorescent cortical ER tubules around a tubular (green) peroxisome. The tubular peroxisomes lie in an ER lined channel and tubule extension and retraction (frames 2–5), formation of incomplete as well as complete polygonal arrangements (frames 6–8), appear to be defined by the surrounding ER (for animation see **Movie S3**). **(B)** The behavior of three peroxisome clusters and contiguous ER shows how tubular peroxisomes such as "a" undergo considerable contortions (frames 2, 6–7) while remaining confined to a small region of the ER. During the same period another elongated peroxisome "b" moves forward, retracts and moves again (arrows in green panels) along an ER strand. Note changes in ER organization concomitant with changes in peroxisome behavior (**Movie S4**). **(C)** A time-lapse sequence showing changes in the morphology of a tubular peroxisome (arrowhead frame 1) due to wrapping (frames 1, 2) and unfolding (frames 3–5) around a spindle shaped ER body and other neighboring ER tubules (see **Movie S5**). **(D)** Representative sequential images from a time-lapse series showing a tubular peroxisome (frame 1) extending over several ER polygons breaking (frames 2–3) and being pulled apart (frames 4–7) through the reorganization of its neighboring ER. Scale bars = 10 μm.

Wood, 1970; Poole et al., 1970; Beevers, 1979; Lazarow and Fujiki, 1985). Subsequent thinking considered peroxisomes as independent organelles with a possibly endosymbiont origin (Lazarow and Fujiki, 1985; de Duve, 1996) and radically polarized the field as biologists subscribed to one theory or the other. After more than half a century of debate our views on peroxisomes have come around a full circle and the majority of biologists now subscribe to a kind of status quo model which accepts *de novo* peroxisome biogenesis from the ER as well as the fission of existing peroxisomes as a means of increasing peroxisome numbers in a cell. Given that there are two possibilities for peroxisome proliferation it is likely that certain physiological conditions might favor one over the other. It is equally possible that both phenomena can occur simultaneously. Whereas the present models (Trelease and Lingard, 2006; Titorenko and Mullen, 2006; Fagarasanu et al., 2007; Hettema and Motley, 2009; Mast et al., 2010; Schrader et al., 2012; Tabak et al., 2013) rely heavily on experimental evidence from yeasts and mammals and there is general agreement that the ER plays an important role in the peroxisomal life cycle it is noteworthy that there is no clear proof for peroxisome

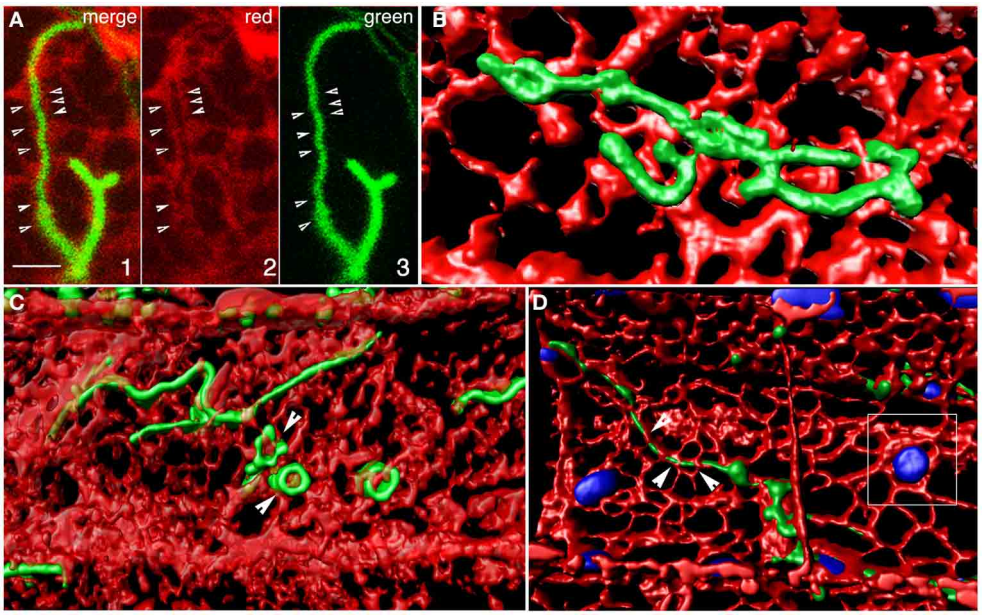

FIGURE 5 | Iso-surface rendering of confocal image stacks depicting the relationship between tubular peroxisomes and the neighboring ER tubules in the apm1-1 mutant of Arabidopsis. (A) A region of the cell showing a tubular peroxisome (panels 1, 3) extended within an ER-lined channel (panel 2). Arrowheads point to potential membrane contact sites. Scale bar = 5 μm. **(B)** A volume rendered stack of 12 images suggests that the tubular peroxisomes in the apm1-1 mutant are enmeshed and embedded in the ER. Changes in ER organization would be expected to create similar alterations in the morphology of the associated peroxisomal tubule. **(C)** Several tubules threaded between the tubules making u the cortical ER. Note that while some areas of the ER overlap regions of the tubule the tubular peroxisomes can also surround portions of the ER (e.g., arrowhead). **(D)**. A tubular peroxisome interwoven in the cortical ER mesh. Note embedded chloroplasts (blue; outlined square) and the possibility of finding membrane contact sites (arrowheads).

biogenesis from the ER in plants (Trelease and Lingard, 2006; Hu et al., 2012). Contrary to the convincing evidence of proteins such as the human peroxin16 labeling the entire ER in a cell upon over-expression (Kim et al., 2006), the plant pex16 homolog (e.g., Arabidopsis SSE1/At2g45690; Lin et al., 1999) does not localize in a similar manner (Karnik and Trelease, 2005, 2007). Instead AtPex16-GFP fusion protein accumulates in the form of reticulo-circular tubules that along with similar localization patterns for ascorbate peroxidase (APX; Mullen et al., 1999) are considered to suggest a pre-peroxisomal domain of the ER (Karnik and Trelease, 2005, 2007). Similar ER-like patterns are described by aberrantly elongated peroxisomes in the *apm1/drp3a* mutants of Arabidopsis (Mano et al., 2004). On the contrary, alignment of thin peroxules and the ER were observed and also suggested ER-like shapes (Sinclair et al., 2009). The rapid formation of peroxules and tubular peroxisomes gave rise to the conjecture that a subset of peroxisomes might exist as tightly pinched domains of the ER. It was speculated that the pinching activity might relax under certain stress conditions to allow peroxisomal contents to flow into connected tubules (Mathur, 2009). Here we used the hitherto unexploited technique of simultaneous live imaging of the two organelles to actually observe their relationships.

Our observations on cells with differently colored peroxisomes and the ER clearly show that irrespective of their morphology, peroxisomes closely align with the ER. Indeed when one considers the 3-dimensional aspect of a cell then peroxisomes can be considered enmeshed in the ER. This impression is reinforced through 3D volume rendering of image stacks (**Figure 5**) and provides a reasonable explanation for the erratic motility of peroxisomes observed in earlier studies (Jedd and Chua, 2002; Mathur et al., 2002). As shown by us, a slowing down of ER motility through cold treatment or treatment with an actin polymerization inhibitor results in a similar slow down of peroxisomes. Conversely an increase in ER motility is also matched by peroxisomes. Whereas observations on spherical peroxisomes and the ER mainly provided an idea of their correlated motility they did not provide a clear idea of spatial relationship. However, the use of tubular peroxisomes in the *apm1/drp3a* mutant clearly shows the intertwining of the two organelles and regions where the ER overlaps or surrounds the peroxisomes (**Figures 4**, **5**). As seen for plastids and mitochondria the ER-mesh around organelles acts as both reinforcement as well as a conduit for trafficking of proteins and lipids. Many such points of interaction between organelles are created through MCS (Levine, 2004; Michel and Kornmann, 2012). Although MCSs similar to those suggested for plastids and mitochondria have not been observed so far between peroxisomes and the ER it is known that these two organelles share a considerable degree of membrane homology (Schlüter et al., 2006) and therefore might have high chances of transient membrane contacts being formed. The presence of MCS with the ER also accounts for the fact that the motility of many organelles in the

cytoplasm appears very similar. In this context while considering the strong correlations between the ER and peroxisome activity this study does not overlook the fact that in plants the motility of both peroxisomes and the ER depends upon an acto-myosin system (Jedd and Chua, 2002; Hashimoto et al., 2005; Li and Nebenfuhr, 2007; Peremyslov et al., 2008, 2012; Ueda et al., 2010). Whether the coincidental behavior of the two organelles occurs along separate F-actin strands and involves independent motor molecules is unclear at this stage. There is also the possibility that both organelles become associated with their respective motors but use the same F-actin strands and bundles for movement. An experimental approach that focuses on unraveling the relationship between myosin motors, peroxisomes and the ER is being developed presently and will be reported independently.

The entanglement of peroxisomes in the ER mesh has an additional implication. This involves the moving apart of peroxisomes their fission in a sequential Pex11-DRP3A-DRP3B-FIS1A-FIS1B (Kobayashi et al., 2007; Zhang and Hu, 2009) mediated manner. Peroxisome attachment to the neighboring ER is apparently sufficiently strong to allow pieces of peroxisomes to be pulled apart as the ER polygons reorganize and separate from each other. While we are devising new fluorescent protein based tools to test this phenomenon it is clear that each separated fragment can continue growth by attracting fresh peroxisomal components from the surrounding cytoplasm. Thus our observations suggest that the ER, perhaps in its motor driven state provides the force to allow peroxisomes to undergo fission at a weak point. The weak point where breakage would be favored can be formed through the constrictase activity of a dynamin related protein and other helper proteins (Zhang and Hu, 2009; Hu et al., 2012).

Our present observations on co-visualized peroxisomes and the ER suggest contiguity but do not indicate any signs of luminal continuity between the two organelles. The observations favor the fission of existing peroxisomes in an ER aided manner to increase their numbers in a plant cell. Does this create a problem with the existing models that consider both *de novo* biogenesis as well as fission of existing peroxisomes for increasing the peroxisomal population? Where do the peroxisomes for fission come from in plant cells? The limitation of our present fluorescent protein based tools approach for answering this question is that the probes used for visualizing peroxisomes consist of matrix targeted fluorescent proteins. While existing peroxisomes are reliably highlighted through them these probes would not be expected to highlight precursors of peroxisomes on the ER or elsewhere in the cytoplasm. Moreover, several lines of evidence point that pre-peroxisomal ER derived vesicles (Titorenko et al., 2000) that might be biochemically distinct (van der Zand et al., 2012; Tabak et al., 2013) can fuse together in the general cytoplasm to assemble a peroxisome. If this is indeed the situation then the ER in plants can only be viewed as a source of membrane components and might not be the physical location for *de novo* peroxisome biogenesis.

MATERIALS AND METHODS

GENERATION OF FUSION CONSTRUCTS AND TRANSGENIC PLANTS

The creation of constructs YFP-PTS1 (Mathur et al., 2002), mEosFP-PTS1 (Sinclair et al., 2009), ss-RFP-HDEL (Sinclair et al., 2009) has been described earlier. The *apm1-1* mutant line carrying a GFP-PTS1 has been described (Mano et al., 2004) and was used as provided. Seeds of *drp3A-3* (SALK_066958) seeds were obtained from the Arabidopsis Biological Research Center (The Ohio State University, Columbus). Stable transgenic lines of wild type Arabidopsis and the apm1-1 mutant expressing one and two targeted fluorescent proteins were created through the floral dip method (Clough and Bent, 1998), and through crossings.

Arabidopsis plants in sterile culture were grown in petri dishes in an incubator maintained at 21 ± 2°C and a 16/8-h light/dark regime using cool-white light of approximately 80–100 μmol m^{-2} s^{-2}. To break dormancy *A. thaliana* seeds were incubated at 4°C for 48 h Growth medium for *A. thaliana* WT and mutant seedlings consisted of 1% agar-gelled Murashige and Skoog (1962) basal medium containing Gamborg vitamins (M404; PhytoTechnology labs) supplemented with 3% sucrose (pH 5.8). For obtaining etiolated *A. thaliana* seedlings, petri dishes were wrapped in two layers of aluminum foil right after the cold treatment and a pinhole created in the foil before placing the plates in an upright position. Seedlings were used between 8 and 11 days after sowing on plates for all experiments.

MICROSCOPY AND IMAGE RENDERING

Plant tissue and seedlings were mounted in tap water on a glass depression slide and placed under a coverslip. For plants expressing the photoconvertible mEosFP-PTS1 protein the photoconversion time was varied according to the brightness of the respective organelles. In general, exposure times for peroxisomes were between 3 and 6 s and resulted in bright red organelles. The light source for photo-conversion was a HBO 100 W/2 Mercury Short Arc lamp and the Leica fluorescence filter set "D" (Excitation filter: 355–425 nm; Dichromatic mirror 455 nm; Suppression filter LP 470 nm). The epi-fluorescence setup consisted of a Leica DM-6000CS microscope. Photo-conversion was performed manually by controlling the diaphragm as described earlier (Mathur et al., 2010). Simultaneous imaging of peroxisomal and ER probes was carried out using a Leica TCS-SP5 confocal laser-scanning unit equipped with a 488 nm argon and a 543 nm helium-neon laser. All images were captured using at a color depth of 24bit RGB.

All images and movies were cropped and processed for brightness/contrast as complete image or stacks using either Adobe Photoshop CS3 (http://www.adobe.com) or the ImageJ distribution Fiji (http://pacific.mpi-cbg.de/wiki/index.php/Fiji). Adobe Photoshop was used for annotation of movies. Imaris software (v. 6.4.0; Bitplane AG) was used to render iso-surface 3 D rendering of ER and peroxisomes from confocal image stacks and x-y-time series. All experiments were carried out at least five times.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Integrative_Physiology/10.3389/fphys.2013.00196/abstract

Movie S1 | Time-lapse sequence of 26 frames taken 1 s apart (played at 2X speed) showing simultaneously a peroxisome and the subtending ER suggests correlations between the movement of the two organelles. Note how the peroxisome moves forwards and then returns to nearly the same position after making a U-turn. (Basis for **Figures 1A–C**).

Movie S2 | Time-lapse sequence of 39 frames taken 1 s apart showing three different peroxisomes co-visualized with the ER. The sequence depicts how ER organization has a direct effect on peroxisomal positioning and relocation. (Basis for **Figure 1E**).

Movie S3 | Sequential time-lapse comprising 120 images taken at about 3 s intervals played over 50 s show red fluorescent ER tubules harboring green fluorescent tubular peroxisomes in the apm1-1 mutant of Arabidopsis. The time-lapse sequence shows tubule extension, retraction, branching, and polygon formation in tandem with contiguous ER tubules. At some stages the tubule appears to move within an ER lined channel. (Basis for **Figure 4A**).

Movie S4 | A 33 s time-lapse sequence comprising 165 frames of tubular peroxisomes and neighboring ER in the apm1-1 mutant of Arabidopsis suggests the ER as the basis for holding a peroxisome cluster in a particular location as well as its unfolding and extension. (Basis for **Figure 4B**).

Movie S5 | Co-visualization of RFP-highlighted ER and GFP-highlighted tubular peroxisomes in the apm1-1 mutant suggests how the shape of a peroxisome is molded through its transient adhesion with ER structures such as spindle shaped ER bodies and ER polygons. Note that the flow of the ER carrying the ER body is against the direction of flow for the tubular peroxisome, suggesting that transient contacts can occur even between organelles that are moving away from each other. (160 frames taken over 10 min played at 5× speed) (Basis for **Figure 4C**).

Movie S6 | Co-visualization of ER and tubular peroxisomes in the apm1-1 mutant show how long tubules might be broken and then draw apart through the reorganization of neighboring cortical ER. (40 frames covering ca. 3.5 min) (Basis for **Figure 4D**).

Movie S7 | An iso-surface rendering of a confocal image stack provides a 3D impression of green tubular peroxisomes enmeshed and embedded in the red colored cortical ER. (Basis for **Figure 5B**).

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Emerging roles of mitochondria in the evolution, biogenesis, and function of peroxisomes

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In the last century peroxisomes were thought to have an endosymbiotic origin. Along with mitochondria and chloroplasts, peroxisomes primarily regulate their numbers through the growth and division of pre-existing organelles, and they house specific machinery for protein import. These features were considered unique to endosymbiotic organelles, prompting the idea that peroxisomes were key cellular elements that helped facilitate the evolution of multicellular organisms. The functional similarities to mitochondria within mammalian systems expanded these ideas, as both organelles scavenge peroxide and reactive oxygen species, both organelles oxidize fatty acids, and at least in higher eukaryotes, the biogenesis of both organelles is controlled by common nuclear transcription factors of the PPAR family. Over the last decade it has been demonstrated that the fission machinery of both organelles is also shared, and that both organelles act as critical signaling platforms for innate immunity and other pathways. Taken together it is clear that the mitochondria and peroxisomes are functionally coupled, regulating cellular metabolism and signaling through a number of common mechanisms. However, recent work has focused primarily on the role of the ER in the biogenesis of peroxisomes, potentially overshadowing the critical importance of the mitochondria as a functional partner. In this review, we explore the mechanisms of functional coupling of the peroxisomes to the mitochondria/ER networks, providing some new perspectives on the potential contribution of the mitochondria to peroxisomal biogenesis.

Keywords: mitochondria, peroxisome, vesicle transport, biogenesis, evolution, contact site

THE URGENCY FOR A BETTER UNDERSTANDING OF PEROXISOMAL FUNCTION

Over the past decade we have learned a great deal about peroxisomal biogenesis and function, much of this using the genetic power of model organisms like yeast (Dimitrov et al., 2013; Tabak et al., 2013). However, peroxisomes are of critical importance to cellular homeostasis in mammalian systems, playing very specific and complex biochemical roles from myelination to the generation of bile (Wanders, 2013). Therefore, beyond their familiar and essential roles in beta-oxidation and the control of reactive oxygen species, peroxisomes contribute a host of specialized functions in mammalian systems. The devastating genetic diseases highlight this fact, with survival among some patients with errors in peroxisomal biogenesis between a few hours to a few years (Waterham and Ebberink, 2012). Peroxisomal genetic disorders were first defined in patients carrying mutations in the peroxisomal biogenesis/import machinery, leading to Zellweger syndrome. In these patients the primary effects is in neuronal survival, lack of myelination, and systemic muscle defects (Powers and Moser, 1998). When peroxisomes fail, there are also indirect effects on mitochondria, whose dysfunction amplifies the cellular damage (Baes et al., 1997; Baumgart et al., 2001; McGuinness et al., 2003; Dirkx et al., 2005). Exactly why mitochondria are so critically affected is unclear. However, the contribution of peroxisomal dysfunction to more common diseases like neurodegeneration, cardiovascular disease, cancer or immune disorders is only beginning to be appreciated (Fransen et al., 2013). Given the tight connection between mitochondria and peroxisomes, and the growing interest in the role of mitochondria in these diseases, it is of urgent importance that investigators examine the potential contribution of peroxisomal failure within these common human diseases. In this review we will reconsider the function and biogenesis of the peroxisomes in light of three emerging themes. First we will address their evolutionary origin, second we examine the current thinking of how peroxisomes are born in mammalian cells, and third, we focus on the functional contacts between mature peroxisomes, mitochondria, and ER in biochemical and signaling pathways. In all of these themes a common pattern emerges, where the peroxisomes have an obligate partnership with the mitochondria and the endoplasmic reticulum. We hope that a fresh look at the peroxisomes may help encourage researchers to look beyond the paradigms established from specialized, single cell experimental models and more carefully consider peroxisomal dysfunction in the etiology of complex disease pathologies.

THE EVOLUTIONARY LINKS BETWEEN MITOCHONDRIA, ER, AND PEROXISOMES

The evolutionary origin of peroxisomes may provide clues to help us understand the mechanisms of peroxisomal biogenesis that occur in cells today. Opinions on this subject have changed over the years, from a purely endosymbiotic origin (De Duve, 1969), to the current evidence that peroxisomes are derived from

the ER (Dimitrov et al., 2013). A bioinformatic analysis of the phylogeny of a number of peroxisomal proteins concluded that peroxisomal proteins fall into two major categories, prokaryotic and eukaryotic (Gabaldon et al., 2006). Peroxisomal proteins of eukaryotic origin (58% in yeast, 39% in rat) were primarily involved in peroxisomal biogenesis. Peroxisomal proteins with bacterial or archeabacterial ancestry included about 13–18% of the peroxisomal proteins. A large proportion of proteins were difficult to assign (∼25%), but these all had some homologies with prokaryotic proteins, although trees could not be constructed to distinguish bacterial or archeal origin. Of the assigned and unassigned proteins within the second category, all were functional enzymes. This suggests either that the perixosomes have evolved as endosymbionts, or that these enzymes evolved from mitochondrial proteins sometime after the last common ancestor. Since many of these enzymes remain dually targeted to both organelles, peroxisomal biologists generally suggest that these enzymes were most likely retargeted to peroxisomes from mitochondria (Gabaldon et al., 2006; Tabak et al., 2006). This would indicate that the peroxisome emerged as functionally specialized mitochondria. Since we now know that mitochondria are able to sort specific proteins into vesicular carriers, we can begin to imagine how functionally distinct mitochondria may have taken shape. These peroxisomal precursors would have housed enzymes responsible for breaking down a unique subclass of fatty acids, incorporated specialized enzymes regulating redox pathways, and other biochemical pathways like plasmalogen synthesis. Eventually, peroxisomes would have adapted protein import mechanisms, and new signal sequences could direct precursors directly to peroxisomes. Although the genetic expansion of the peroxisomal proteome provided a great deal of independence from the mitochondria, peroxisomes have retained the same mitochondrial machinery for their division, a central aspect of peroxisomal biogenesis (Schrader et al., 2012).

If the functions of the peroxisome are largely variants of those in mitochondria, why do they emerge from the endoplasmic reticulum? This should not be particularly surprising since the ER also provides the mitochondria with the bulk of it's lipid mass, and the ER and mitochondria are functionally and physically coupled in many ways (de Brito and Scorrano, 2010; Rowland and Voeltz, 2012). Therefore, the relationship of the peroxisome to the ER may also reflect an evolutionarily conserved variation on the mechanisms of mitochondria/ER coupling. In the phylogenic analysis of the peroxisomal proteins descended from a eukaryotic lineage, there was a clear relationship between the peroxisomal import machinery and the components of the endoplasmic reticulum associated degradation, or ERAD pathway (Gabaldon et al., 2006; Schluter et al., 2006; Schliebs et al., 2010). For peroxisomal import, the receptor Pex5 binds to cytosolic precursors to deliver them to the peroxisome in a cycle that involves ubiquitination and deubiquitination of Pex5 for the release of the substrate (for review see Schliebs et al., 2010). This appears to be analogous to the use of ubiquitin in the tagging and export of unfolded proteins within the ER, which are ultimately delivered to the proteasome. Indeed 5 of the 6 conserved Pex genes show homology with components of the ERAD machinery. Pex1 and Pex6 are homologous to Cdc48 and p97 [which are themselves of bacterial origin (Iyer et al., 2004)], whereby p97 is a AAA+ ATPase. Pex2 and Pex10 are similar to the ubiquitin E3 ligase Hrd1 enzyme that tags unfolded ER proteins. Hrd1 has a binding partner Hrd3, which shows homology to Pex5, and Pex4 resembles a ubiquitin E2 ligase. Therefore, the authors concluded that the biogenesis pathway of the peroxisomes evolved from the ER (Gabaldon et al., 2006; Schluter et al., 2006; Schliebs et al., 2010). The difference is, of course that the peroxisome system would deliver rather than extract proteins. However, the only ubiquitinated cargo in peroxisomal import is actually the receptor Pex5, which is ubiquitinated in order to be extracted and recycled, following the release of the Pex5-bound import substrates in the peroxisome. In this way, the Pex1/Pex6 complex is extracting Pex5, just as the ERAD machinery extracts ER proteins (Tabak et al., 2013).

Although it has been concluded that the similarly to the Cdc48/p97 infers an ER origin of the peroxisomal import machinery, it is important to note that this system has recently been demonstrated to have a clear role at the mitochondrial membrane as well (Heo et al., 2010; Tanaka et al., 2010; Chan et al., 2011; Xu et al., 2011; Esaki and Ogura, 2012). In this case, targeted substrates are ubiquitinated by E3 ligases such as Parkin, a protein that is mutated in familial cases of Parkinson's disease. p97 is required for the retrotranslocation of these tagged proteins, which are then targeted to the proteasome for degradation. Therefore, we would argue that this homology does not exclusively implicate the ER as the membrane of origin for the peroxisomal import machinery, and equally supports a mitochondrial origin.

BEYOND EVOLUTION: CELL BIOLOGY OF PEROXISOMAL BIOGENESIS TODAY

A number of studies using yeast as a model organism have unequivocally demonstrated that peroxisomes can be formed *de novo* from the endoplasmic reticulum (Dimitrov et al., 2013; Tabak et al., 2013). This information has effectively shelved the notion that peroxisomes evolved as endosymbionts. Unlike mammalian cells, yeast govern their peroxisomal numbers depending on the carbon source, for example in the presence of oleic acid (*Saccharomyces cerevisiae* or *Yarrow lipolytica*) (Trotter, 2001) or methanol (*Hansenula polymorpha* and *Pichia pastoris)* (Yurimoto et al., 2011). Since yeast mitochondria do not perform beta-oxidation, peroxisomes rapidly arise from the ER in order to catabolize these fats, or to metabolize methanol. In this way, fungi are highly specialized organisms where peroxisomal function has diverged between evolutionary lineages. On the other hand, the linkages to the mitochondria are much more obvious in multicellular organisms. For example, the transcriptional regulation of mitochondria and peroxisomal biogenesis is not coupled in yeast as it is in mammals (Issemann and Green, 1990; Mandard et al., 2004; Scarpulla et al., 2012). In addition, the shared roles of peroxisomes and mitochondria as signaling platforms (Dixit et al., 2010; Tait and Green, 2012) may not occur in yeast, and most obviously, the metabolic functions of peroxisomes have diverged significantly throughout evolution (Islinger et al., 2010; Pieuchot and Jedd, 2012; Wanders, 2013). Therefore, fungal lineages may have lost some of the linkages between the mitochondria and peroxisomes, instead developing closer ties to the ER. We consider that there is likely a great deal of plasticity in the evolution

of peroxisomes, depending on the specific functional role they play across diverse species. Given this divergence, we suggest that there may not be unified theory for peroxisomal biogenesis across species, where, for example, significant differences are likely to exist between yeast and mammalian mechanisms.

The most compelling evidence to demonstrate the contribution of the ER to peroxisomal biogenesis is the emergence of Pex-containing vesicles from the endoplasmic reticulum in yeast and mammals. A number of different experimental paradigms and model systems have proven this point. First, fluorescently tagged, membrane anchored Pex proteins, notably Pex3 and Pex16, have been observed emerging from the ER in conditions where peroxisomes are either induced by growth conditions or in pulse-chase type of rescue experiments (Titorenko and Rachubinski, 1998; Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Kim et al., 2006; Motley and Hettema, 2007). Second, cell free budding assays from isolated ER have established some of the machinery required to bud Pex-containing vesicles in yeast *Saccharomyces cerevisiae* (Lam et al., 2010). In this case, the authors showed both Pex3p and Pex15p emerging within vesicles in a manner that depended on ATP and Pex19p, but not Sar1, a GTPase essential for anterograde COPII budding events. The authors demonstrated a requirement for additional cytosolic factors that are yet to be identified. Using a semi-permeable cell system in *Pichia Pastoris*, the authors also demonstrated a Pex19 dependent, COPII-independent mechanism to generate Pex11-containing vesicles from the ER (Agrawal et al., 2011). These vesicles were generated even in the absence of Pex3, an essential component for peroxisome biogenesis. Similar data has shown an ER origin for mammalian peroxisomes. Using human fibroblasts lacking core proteins of the peroxisomal import machinery like Pex16 or Pex3, the reintroduction of GFP-tagged Pex16 or Pex3 can rescue the generation of new organelles from their ER localization (Kim et al., 2006; Toro et al., 2009; Yonekawa et al., 2011). This pathway was also shown to depend upon the ER budding factor Sec16b (Yonekawa et al., 2011). Although many peroxisomal proteins target the mitochondria in the absence of peroxisomes (see next section for further discussion), these data clearly establish the ER as a primary source of membrane in the generation of new peroxisomes.

How can you generate a mature peroxisome from an ER derived vesicle? Historically it has been assumed that the early peroxisomes would be import competent, and from there could mature through the targeting and import of all the required functional enzymes. This maturation model did not require any fusion events, instead all new peroxisomes would be formed from the growth and division of existing peroxisomes. However, now it is clear that small, vesicular carriers bud from the ER, which are termed "pre-peroxisomes." These vesicles must fuse with other pre-peroxisomes, or with more mature peroxisomes, to generate a larger, functional organelle (Boukh-Viner et al., 2005; van der Zand et al., 2012). Many studies have proven that mature peroxisomes do not fuse, both in mammalian or yeast cells (Motley and Hettema, 2007; Huybrechts et al., 2009; Bonekamp et al., 2012), raising the important question of specificity and regulation of fusion among/between pre-peroxisomes. Previous work in yeast *Yarrow lipolytica* demonstrated peroxisomal fusion *in vitro*, and it was suggested then that fusion was limited to an "early" pool of peroxisomes that would then mature into fully functional organelles (Titorenko et al., 2000; Boukh-Viner et al., 2005). In *Yarrow lipolytica*, peroxisomal fusion was dependent upon the import factors Pex1 and Pex6, of the Cdc48/p97 family (Titorenko et al., 2000; Boukh-Viner et al., 2005). Although p97 is a AAA+ATPase that functions in the ERAD pathway, p97/Cdc48 have also been shown to have an established role in ER and golgi membrane fusion (Latterich et al., 1995; Hetzer et al., 2001; Uchiyama et al., 2006; Totsukawa et al., 2013).

Another important question is how the fusion of two pre-peroxisomes would lead to a functional peroxisome. Recent work has answered this conundrum by revealing that the peroxisomal import machinery is sorted into two populations within the ER. They observed two distinct populations of vesicles budding from the ER, one containing the RING complex of the import machinery, and the second carrying the docking complex (van der Zand et al., 2012). Heterotypic fusion between these two distinct populations of pre-peroxisomes would then generate a functional import machine, and by definition, a functional peroxisome. This explains why peroxisomal import cannot occur into the ER, since the machinery remains segregated. The mechanisms for this segregation are not yet known. Consistent with previous work, the authors could not observe any fusion events between pre-peroxisomes and mature peroxisomes, or between mature peroxisomes, indicating a highly selective mechanism for pre-peroxisomal fusion (van der Zand et al., 2012).

IS THERE A POTENTIAL ROLE FOR MITOCHONDRIAL DERIVED VESICLES (MDVs) IN PEROXISOMAL BIOGENESIS?

Since the emergence of peroxisomes from the ER is so clearly demonstrated, is there any role for the mitochondria in the biogenesis of peroxisomes beyond their evolutionary links? Certainly our understanding of the flexibility of mitochondria has rapidly increased, and we appreciate how they fuse and divide in order to dynamically position themselves both functionally and spatially within cells. Our lab has also defined two distinct vesicular transport routes from the mitochondria (Neuspiel et al., 2008; Braschi et al., 2010; Soubannier et al., 2012a,b). Initially we described a route between the mitochondria and the peroxisome (Neuspiel et al., 2008), a pathway we also showed was dependent upon the retromer complex Vps35, Vps26 and Vps29 (Braschi et al., 2010). More recently we characterized another pathway between the mitochondria and the late endosome/multivesicular body. This latter pathway selectively targets oxidized or damaged protein and lipid (Soubannier et al., 2012b), removing them from the mitochondrial reticulum for degradation in the lysosome (Soubannier et al., 2012a). These two pathways open up new insights into how the mitochondria may deliver their contents to other cellular organelles.

What is the function of the vesicle transport route from the mitochondria to peroxisomes? So far only one cargo was identified in these vesicles, a membrane anchored protein called MAPL (mitochondrial anchored protein ligase, also called MUL1, GIDE, or HADES) (Neuspiel et al., 2008; Braschi et al., 2009). The carboxy-terminal of MAPL is exposed to the cytosol and contains a RING finger domain with strong SUMO E3 ligase activity.

Overexpression of MAPL drove massive mitochondrial fragmentation through the SUMOylation and activation of the mitochondrial fission GTPase Drp1 (Neuspiel et al., 2008). Mitochondrial vesicles carrying MAPL fused with a subset of peroxisomes, only about 10% of the total (Neuspiel et al., 2008; Braschi et al., 2009). These peroxisomes were able to import the transfected CFP-SKL marker, indicating that they had functional import machinery. Although we have not yet determined the function of this vesicular transport route, we offer three potential functions here. First, MDVs may contribute to peroxisomal biogenesis, fusing with the early, preperoxisomal population. Second, MDVs may carry metabolites and target a functionally distinct subset of peroxisomes, and third, MDVs may shuttle proteins that are not competent for peroxisomal import.

Given the evidence for a pre-peroxisomal population in cells, it is plausible that MAPL is targeted to this fusogenic population of peroxisomes and provides a mitochondrial component to the maturing peroxisomes. In evolutionary terms, a phylogenic analysis of MAPL indicates that it is of bacterial ancestry, with at least 5 prokaryotic domain structures (Andrade-Navarro et al., 2009). Therefore, this vesicle transport pathway may have played a role in the earliest segregation of specialized mitochondrial function. This possibility has not been previously considered due to the obvious assumption that the mitochondria were not competent to segregate cargo and bud vesicles. This assumption is fundamentally wrong. Our ongoing studies continue to characterize various classes of cargoes that are enriched in mitochondrial derived vesicles. For example, using an *in vitro* reconstitution system we demonstrated that the identity of the cargo within MDVs destined for the lysosome depends greatly on the nature of the insult (Soubannier et al., 2012b). We have a great deal of work ahead to identify the mechanisms and regulation of mitochondrial vesicle transport, but it is clearly a process that exists in steady-state conditions, suggesting a fundamental role for these vesicles in cellular homeostasis.

If mitochondrial vesicles play a role in peroxisomal biogenesis, why don't we observe peroxisomal membrane proteins targeting the mitochondria? Indeed, in mammalian cells, many peroxisomal proteins do default to the mitochondria when peroxisomes are absent. For example, Pex3, Pex14, Pex12, PMP70 and ALDP/ABCD1 were shown to target mitochondria in fibroblasts of patient cells with mutations in Pex3, Pex16 or Pex19 (Sacksteder et al., 2000; South et al., 2000; Kim et al., 2006; Toro et al., 2009). This has been generally discounted as an artifactual missorting, again, likely since it had been assumed that there was "no way out" for these mistargeted proteins. In contrast, yeast biologists did not consider sorting to the ER in peroxisome-deficient cells to be an artifact; rather it defined the ER as the site of peroxisomal biogenesis. In light of a vesicular transport route from the mitochondria to the peroxisomes, we should reconsider these older studies and realize that this supports the concept that mitochondria may contribute to the initiation of new peroxisomes in mammalian cells. The most consistent explanation is that both ER and mitochondrial derived vesicles could contribute to new peroxisomes (**Figure 1**). It is possible, for example that some pre-peroxisomes derived from the ER could fuse with pre-peroxisomes derived from the mitochondria.

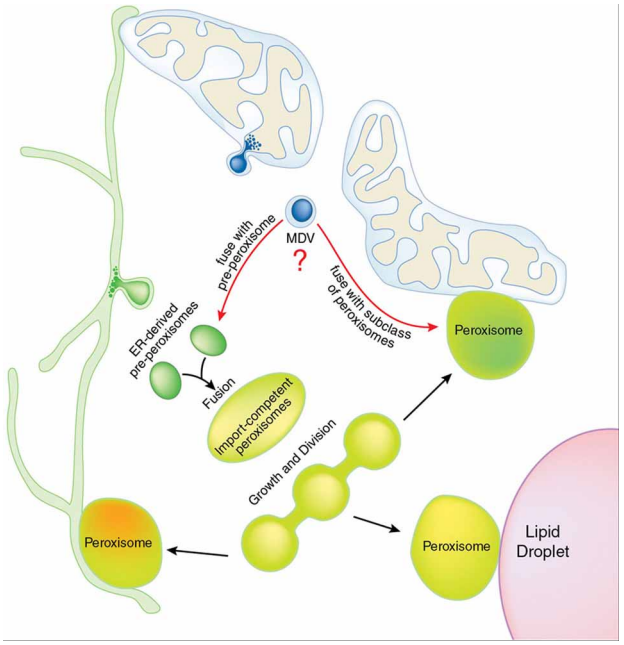

FIGURE 1 | Potential contribution of MDVs to peroxisomal biogenesis and functional specialization. Pre-peroxisomes bud from the endoplasmic reticulum (ER, in green), carrying subcomplexes of the import machinery. Fusion between ER-derived pre-peroxisomes results in an import competent peroxisome that continues to grow and mature. Elongation and division of peroxisomes occurs throughout the life of peroxisomes, in response to cellular conditions. Mitochondria are shown (blue) with a mitochondrial derived vesicle (MDV) emerging, containing both inner and outer mitochondrial membranes. Both membranes were observed in immuno-electron microscopy analysis of MAPL-positive vesicles, indicating that double-membrane bound vesicles target the peroxisome (Neuspiel et al., 2008). Since MAPL containing MDVs fuse with only a sub-population of peroxisomes, we envision two possible fates (indicated by red arrows). First, MDVs may fuse with an early pre-peroxisomal pool. MAPL was seen to fuse with peroxisomes containing CFP-SKL (Neuspiel et al., 2008; Braschi et al., 2010), indicating that MDVs fuse with an import-competent class of organelles. From this we predict that MDV fusion would occur downstream of ER-derived pre-peroxisomal fusion. Alternatively, MDVs could fuse with a functionally distinct subclass of peroxisomes, which are illustrated by different shading within mature peroxisomes. A number of direct contact sites are shown between organelles, which have been characterized in many cellular conditions (see text for details).

The plasticity of these pathways is illustrated by comparing rescue experiments in different systems. For example, the reintroduction of Pex3-GFP into a mammalian cell line lacking Pex3 led to the generation of new peroxisomes, which emerged via ER-localized Pex3-GFP, not the mitochondria (Toro et al., 2009). On the other hand, when the Erdmann group ectopically targeted Pex3 to the mitochondria within Pex3-null yeast strains (Rucktaschel et al., 2010), peroxisomes were successfully regenerated from the mitochondria. Therefore, although an emerging pathway in peroxisomal cell biology, mitochondrial vesicles should be explored as potential contributors to the biogenesis of peroxisomes, particularly in mammalian cells. There may be significant variability in the relative contribution of ER and mitochondrial derived vesicles to peroxisomal biogenesis, which

would likely be linked to the functional diversity of the organelles in different tissues and organisms.

It is also possible that mitochondrial derived vesicles could carry cargo to functionally distinct peroxisomes, rather than playing a role in their biogenesis. There has been evidence that peroxisomes can be functionally differentiated within single cells, having distinct densities, import competencies, and protein composition (Schrader et al., 1994; Fahimi et al., 1996; Volkl et al., 1999; Islinger et al., 2012; Costa et al., 2013). However, it is not known how this might be achieved. Given the functional coupling of the mitochondria and the peroxisomes—particularly in mammalian cells—a direct vesicular transport route may allow the rapid shuttling of metabolites or proteins with very tight spatial and temporal regulation. A vesicular transport route could selectively target "active" peroxisomes and deliver metabolites or enzymes selectively to these organelles (**Figure 1**). Vesicles may also provide protection from potentially toxic, hydrophobic, or more complex mitochondrial cargoes. These could include heme, lipids like mitochondrial-generated PE or cardiolipin (Wriessnegger et al., 2007), or metabolic intermediates. It has been largely concluded that catalase is imported into peroxisomes in the heme-loaded form, indicating that heme would be an unlikely cargo for MDV transport. However, earlier work by Lazarow and DeDuve used radiolabelled pulse-chase experiments in rat liver elegantly demonstrated that heme loading of catalase occurred only after import (Lazarow and de Duve, 1973). Again, it cannot yet be excluded that some heme could be transported into peroxisomes from the mitochondria in vesicular carriers.

Finally, it is also possible that the mitochondrial protein import and folding machinery is more efficient than peroxisomes, leading some common enzymes to be shipped to peroxisomes only after their rapid assembly in the mitochondria. MAPL, with it's two transmembrane domains may fall into this category. Dually targeted proteins could potentially utilize both transport routes, depending on the conditions.

There are a number of challenges remaining to identify the molecular machinery that regulates this vesicular transport route, to understand which types of cargoes are segregated into the vesicles, and how these vesicles select and fuse with a subset of peroxisomes. Answers will likely come from large scale screening efforts and the development of cell-free assay systems. Such a screen of 4000 viable deletions in yeast provided compelling evidence for a direct link between the mitochondria and peroxisomes. This genome-wide scan of factors affecting peroxisomal biogenesis in yeast identified only 4 ER proteins, but 41 mitochondrial proteins whose loss affected peroxisomal numbers, shape, or function (Saleem et al., 2010). This can be compared to the loss of 46 nuclear-targeted proteins that similarly affected the peroxisomes. Surprisingly, none of the 4 ER proteins are known to function in vesicle formation, instead were mapped to fatty acid synthesis, farnysylation, lipid modifications and signal transduction (Saleem et al., 2010). Clearly many ER proteins, including many of those required for ER transport are essential, and therefore would not be seen in this screen of non-essential genes. On the other hand, the mitochondrial genes spanned multiple functions, from mitochondrial translation to respiration and mtDNA distribution. The robust effects on peroxisomal function upon the loss of so many mitochondrial genes should reinforce our efforts to consider the dynamic interplay between peroxisomes and mitochondria in all organisms. Also notable in this screen was the fact that the loss of 9 vacuolar proteins also led to a reduction in peroxisomal numbers and content (Saleem et al., 2010). These proteins included fusion factors like the SNAREs Vam3 and Nyv1, and the Rab GTPase, Ypt7. Future work will be required to understand the functions of these genes in peroxisomal behavior.

PEROXISOME AND MITOCHONDRIAL DYNAMICS: THE IMPLICATIONS OF A SHARED MECHANISM

One of the most striking parallels between the mitochondria and peroxisomes is the conservation of the fission machinery in diverse organisms from yeast to plants. Deletion of the dynamin-related protein Drp1 [also called Dlp1 (dynamin-like protein) and Dnm1 (in yeast)] led to elongated peroxisomes and mitochondria (Koch et al., 2003; Kuravi et al., 2006; Kobayashi et al., 2007; Motley et al., 2008). The recruitment of this cytosolic GTPase requires its single-membrane spanning receptors Mff and Fis1, which are dually imported into both organelles (Koch et al., 2005; Delille and Schrader, 2008; Motley et al., 2008). Other mitochondrial fission factors, including GDAP1 are also imported into peroxisomes in a Pex19-dependent manner (Huber et al., 2013). Interestingly, MAPL activates mitochondrial fission through the SUMOylation of Drp1 (Braschi et al., 2009). As described above, MAPL is a cargo that is transported to the peroxisomes in mitochondrial derived vesicles (MDVs) (Neuspiel et al., 2008; Braschi et al., 2010). Since MAPL was delivered only to a subpopulation of peroxisomes in HeLa cells, it suggests that MAPL-mediated activation of peroxisomal fission would be specific to either early peroxisomes, or functionally specialized organelles. Our lab continues to work on this pathway to elucidate the impact of MAPL on peroxisomal function and dynamics. Peroxisomes employ other factors, including the family of Pex11 proteins, which promote peroxisomal elongation (Koch et al., 2004; Kobayashi et al., 2007), indicating that there are also organelle specific factors regulating their division.

Having established that mitochondrial and peroxisomal fission utilize common machinery, what does this mean for the cell? On one hand, this fact further supports the idea that the mitochondria are a contributor to the ancestry of the peroxisome. But of more immediate relevance, it suggests that the mechanisms and signaling pathways that activate the fission machinery of the two organelles are coupled. In other words, when mitochondria fragment, peroxisomes should also fragment. For example, is Drp1 also stably recruited to peroxisomes during the apoptotic program, and would this contribute to the mechanisms of cell death (Frank et al., 2001; Wasiak et al., 2007)? In contrast, the inhibition of Drp1 by PKA phosphorylation during autophagy could trigger peroxisomal elongation (Gomes et al., 2011; Rambold et al., 2011). Would this affect the breakdown of fatty acids during starvation to promote gluconeogensis? Are longer peroxisomes also functionally more efficient, or resistant to degradation by pexophagy? These are important questions that will hopefully be answered soon.

Recent studies in mitochondrial fission have also highlighted a critical role for the endoplasmic reticulum in defining the site

of Drp1 scission sites (Friedman et al., 2011; Murley et al., 2013). Does Drp1 recruitment somehow mark a site to tether the peroxisomes to the ER? Is the ER functionally required to mark sites of peroxisomal division? These are questions that are likely the topic of current investigation in many labs. There has been some advance in our understanding of how peroxisomal division in yeast may be regulated by signaling pathways, through the peroxisome-specific fission factor Pex11. Yeast Pex11 was shown to be phosphorylated in both *Pichia pastoris* and *Saccharomyces cerevisiae* in the presence of oleate (Knoblach and Rachubinski, 2010; Joshi et al., 2012). In *Saccharomyces* the phosphorylation was mediated by a cyclin-dependent kinase Pho85p, potentially linking peroxisomal fragmentation, and segregation during the cell cycle (Joshi et al., 2012). In *Pichia*, Pex11 phosphorylation facilitated an interaction with Fis1 in steady state (Knoblach and Rachubinski, 2010). This provides further evidence that peroxisomal dynamics are tightly regulated through signaling cascades in multiple organisms. Much more work remains to be done to fully understand the functional implications of peroxisomal length, and whether/when fission may be controlled by signaling pathways.

Another emerging aspect of peroxisomal and mitochondrial dynamics is the contribution of organelle plasticity to quality control. It is clear that functionally aberrant, or damaged mitochondria or peroxisomes must be removed in order to ensure the survival of the cell. For peroxisomes, the primary mechanism is through pexophagy, where the autophagic machinery engulfs "old" peroxisomes for degradation in the autophagosomes (Nordgren et al., 2013). This implies that there may be conserved mechanisms that target Drp1 selectively to fission sites where dysfunctional organelles will be removed. However, a role for Drp1 in pexophagy has not yet been established.

Peroxisomes also contain a number of proteases to degrade unfolded or misassembled complexes, including the LonP2 enzyme which is paralagous to the mitochondrial LonP, and degrades oxidized proteins in a similar manner (Kikuchi et al., 2004). Finally, retrotranslocation pathways called RADAR for Receptor Accumulation and Degradation in the Absence of Recycling, functions in a ubiquitin-dependant manner similar to the ERAD pathway of removal from the ER (Leon et al., 2006; Leon and Subramani, 2007). Whether errors in these pathways are a primary cause of disease is something that is becoming a very important area for future research.

A newly identified mechanism for mitochondrial quality control is also the use of vesicular carriers that selectively remove oxidized proteins and lipids from otherwise intact organelles (Soubannier et al., 2012a,b). Interestingly, peroxisomes within fungi like *Neurospora crassa* are known to segregate assembled complexes of Hex-1 protein oligomers that pinch off into specialized organelles called woronin bodies. These structures target and physically block the leakage of hyphal contents within broken fungal branches (Tenney et al., 2000; Tey et al., 2005). Mechanistically the Hex-1 oligomers have been shown to interact with peroxisomal protein import components within a subclass of peroxisomes, which stimulates import to fuel the generation of Hex-1 crystals (Liu et al., 2011). This process effectively "differentiated" this subclass of peroxisomes to function in the generation of Hex-1 crystals rather than their other functions in redox control or beta-oxidation. This segregation was effectively reconstituted in a yeast model ectopically expressing the Hex-1 protein. The generation of the Hex-1 containing woronin bodies in this system was dependent on Dnm1/Vps1 for their division (Wurtz et al., 2008). In addition, peroxisomes in the yeast *Hansenula polymorpha were shown to segregate mutant catalase aggregates through a fission-dependent process, which were targeted to the autophagosome* (Manivannan et al., 2013). This indicates that peroxisomes also have a capacity to segregate cargo for their selective removal. Whether this processes involves the generation of small vesicles (~100 nm with coat proteins, cargo enrichment mechanisms, etc.), or is done exclusively through the segregation and Drp1-dependent fission of larger, non-vesicular structures (or both) needs to be further explored. In any case, the segregation of cargo is a specific process in cell biology that requires complex mechanisms and regulation.

A decade ago the field of mitochondrial dynamics was largely considered phenomenological. However, time has proven the fundamental importance of mitochondrial shape and position in the regulation of mitochondrial function (Nunnari and Suomalainen, 2012). A similar future awaits the field of peroxisomal dynamics. At least in mammalian systems, the functional consequences of precise peroxisomal positioning and contacts within the cell, and the question of regulated division and elongation during various cellular transitions is primed for new discovery.

THE HABITS OF A MATURE PEROXISOME

Functional peroxisomes have mechanisms for selective protein turnover (Nordgren et al., 2013), but the organelle itself is thought to remain stable within the cell for 1–4 days (Price et al., 1962; Poole et al., 1969). During this time proteins are imported, and they perform a number of major functions including the beta oxidation of very long chain fatty acids, the breakdown of peroxide, and the synthesis of specific compounds like bile acids, ether phospholipids like plasmalogen, etc (Wanders, 2013). Some of these functions, like the generation of plasmalogen, involve biochemical pathways present in anaerobic bacteria, further supporting a prokaryotic lineage for peroxisomal enzymes (Goldfine, 2010). However, the habits of peroxisomes in fungi, plants, and animals can vary widely, where entire biochemical pathways have been lost and/or expanded across the species, from fungi to plants and animals (Islinger et al., 2010). The generation of plasmalogens is one example, and the synthesis of bile is also specific to animals. For each of these pathways, the substrates and products of reactions performed within peroxisomes are acquired from, or targeted to, other cellular organelles. Historically, metabolite transport was assumed to occur by free diffusion, without requiring any specific contact sites. However, emerging cell biological studies continue to highlight the importance of direct organelle contacts between peroxisomes and the ER, mitochondria, and lipid droplets (Schrader et al., 2013). The task ahead is to determine the molecular mechanisms and regulation of these contacts, and determine whether these contacts really play an essential role in the funneling of metabolites. If so, it is conceivable that functionally distinct peroxisomes may favor contacts with just one partner organelle (i.e., mitochondria, ER, or lipid droplets),

leading to a type of peroxisomal differentiation within single cells (**Figure 1**). Given the technical limitations in visualizing peroixisomal metabolism within living cells, we cannot yet distinguish these possibilities. As cell biologists, the concept of free diffusion is very unappealing given the kinetic disadvantages compared to regulated, targeted interorganelle transport and direct contact (Howe, 2005). Without entering into the biochemical details of peroxisomal metabolism which are elegantly described elsewhere (Wanders, 2013), we describe three examples of the functional contacts that are currently under investigation in various cell models.

Plasmalogen is an ether phospholipid generated from enzymes in both the peroxisomes and the ER (Braverman and Moser, 2012). Once it is synthesized, plasmalogen is localized with the ER, but more significantly within golgi membranes, mitochondria and the nucleus. However, the bulk of plasmalogen is secreted from cells, and used in a variety of processes including the generation of myelin (in brain), surfactant (in lung), and in the development of the lens in the eye (Gorgas et al., 2006). Many of the severe phenotypes in patients with peroxisomal deficiencies are due, in large part, to a loss in plasmalogen biosynthesis. The first three enzymes of this pathway are localized in the peroxisomes, and the last three enzymes reside in the ER. Plasmalogen synthesis begins with a fatty acid, which is likely stored in the lipid droplet. A peroxisomal surface enzyme called FAR1 converts the fatty acid into a fatty alcohol, which enters the peroxisome (Honsho et al., 2010). Given the close contact of the peroxisomes with both the lipid droplets and ER, it has been suggested that "kiss and run" events help to facilitate the transfer of substrates and products between these organelles (Schrader et al., 2013). Indeed, lipids are transported from the ER into the mitochondria through well-established contact sites called MAM, for microsomal associated microdomains (English and Voeltz, 2013). Similar sites appear to exist between the ER and mature peroxisomes (Raychaudhuri and Prinz, 2008), however, the molecular basis for these contacts is unknown.

Peroxisomes are also essential in the production of bile acid salts, which are secreted from the liver to emulsify dietary fats travelling through the gut (Ferdinandusse et al., 2009; Lefebvre et al., 2009). The two fatty acids Di- and Trihydroxycholestanoic Acid (DHCA and THCA) are produced from cholesterol in the ER, and are then transported to the peroxisomes, likely through PMP70/ABCD3 (Morita and Imanaka, 2012). Following a few rounds of beta-oxidation in peroxisomes, the resulting acetyl-CoA esters are converted to the taurine and glycine conjugates for export back into the cytosol. These bile acids are then released from the hepatocytes into the bile caniliculi, which can be stored in the gall bladder and secreted into the gut. Therefore, as in the synthesis of plasmalogens, the generation of bile acid involves the transport of metabolites between the ER, the peroxisomes, and the plasma membrane. Whether or not the peroxisomes are in contact with the plasma membrane for direct flux of the bile salts across the membrane has not been explored.

The most conserved function for peroxisomes is the beta-oxidation of very long chain fatty acids. In fungi like yeast, peroxisomes are responsible for the beta-oxidation of all fatty acids, therefore there is no obvious requirement for any mitochondrial/peroxisomal contacts in these organisms. However, yeast grown in the presence of oleic acid were shown to trigger significant direct contacts between peroxisomes, mitochondria and the lipid droplet, hinting toward the direct transfer of fatty acids through these contact sites (Pu et al., 2011). In higher eukaryotes, peroxisomes catabolize very long chain fatty acids, and transport the medium chain products and acyl-CoA moieties into the mitochondria for further oxidation (Wanders, 2013). Therefore, beta-oxidation in mammals likely involves direct contacts between the peroxisomes and mitochondria, although links to the lipid droplets are likely also implicated. Whether or not any lipids or substrates could be transported in vesicular carriers between these organelles is also unknown.

In all of these instances, as well as numerous biochemical pathways we haven't described here, there is a constant need for the peroxisomes to be in direct contact with various intracellular organelles. The primary partners are the ER and the mitochondria, although there is evidence for contacts with lipid droplets as well. Future work will continue to explore the functional importance and molecular specificity of these contacts.

PEROXISOMES AND MITOCHONDRIA AS UNIQUE SIGNALING PLATFORMS

As a final comment on the functional coupling between the peroxisomes and the mitochondria, we end with their important roles in intracellular signaling pathways. A well-established core function of both the mitochondria and peroxisomes is their ability to scavenge damaging reactive oxygen species or peroxides (Starkov, 2008; Bonekamp et al., 2009). ROS scavenging is important to minimize cellular damage, but the contribution of ROS to signaling pathways is of equal importance (Tschopp, 2011; Murphy, 2012; Sena and Chandel, 2012). Recent studies utilizing a peroxisomal-targeted redox probe in both mammalian and yeast cells demonstrated significant variation in peroxisomal redox state depending on the environmental conditions (Ivashchenko et al., 2011). Overall the peroxisomes and mitochondria exhibited much lower levels of oxidation than expected, given the focus on these organelles as hot beds of reactive species. The strict control over the levels of ROS in these organelles reaffirms their competence in neutralizing damage to protect the cell. As seen earlier in a number of peroxisomal mutant fibroblasts (Baumgart et al., 2001; Dirkx et al., 2005), disruption of peroxisome redox status adversely affected the mitochondrial redox state, further highlighting the functional links between the two (Ivashchenko et al., 2011). In these experiments, individual peroxisomes with very high oxidative status were eliminated through pexophagy, consistent with the concept of selective autophagy (Nordgren et al., 2013). Whether the redox status of the mitochondria and peroxisomes feeds back into changes within the ER remains unexplored.

Although the mitochondria and peroxisomes are able to minimize the accumulation of reactive species, this does not exclude a role for a highly localized and/or situation-specific use of oxidation mechanisms in signaling. Our own work investigating the molecular mechanisms that drive stress-induced mitochondrial fusion has shown that elevations in oxidized glutathione (GSSG) lead to the oligomerization and "priming" of the mitochondrial

fusion GTPases Mfn1 and Mfn2 (Shutt et al., 2012). This has led us to consider a global role for increased local oxidation as a means to initiate protein modifications that may lead to their activation. A second example of this is the more established redox sensor KEAP1, which normally targets the Nrf2 transcription factor for ubiquitination by a Skp/Cul3 ubiquitin ligase complex (Itoh et al., 2010). Upon increasing levels of GSSG, new disulfide bonds are formed within KEAP1, rendering it unable to bind Nrf2, which is then targeted to the nucleus where it transcribes a host of stress response genes. KEAP1 has been localized to the mitochondrial surface, through its interaction with the mitochondrial outer membrane protein PGAM5 (Lo and Hannink, 2008), suggesting that local redox transitions at the mitochondria could effectively control Nrf2 transcriptional responses.

Perhaps the most surprising links between the mitochondria and signaling pathways came a number of years ago with the identification of the mitochondrial anti-viral signaling protein MAVS. MAVS was identified from 4 independent groups simultaneously as an essential protein for the viral-induced transcription of Nf-kB (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Only one of the 4 groups examined this protein by microscopy and realized that it is a mitochondrial membrane anchored protein (Seth et al., 2005). MAVS contains a carboxy-terminal transmembrane domain and a cytosolic CARD domain. Upon infection, the cytosolic double-stranded viral DNA forms a complex with RIG-I, which binds the CARD domain of MAVS at the mitochondrial surface. From there, a complex series of protein interactions and oligomerization steps leads to the formation of extremely large, prion-like MAVS filaments (Hou et al., 2011; Berke et al., 2012). These filaments are even "contagious" as they can seed the formation of MAVS filaments in ectopic situations.

MAVS has also been seen to signal even earlier from the peroxisomes, again linking these two organelles as unique signaling platforms (Dixit et al., 2010). So why do these things occur on the mitochondrial or peroxisomal surface? Initially the mitochondrial localization of MAVs suggested some role in delaying apoptosis until the infected cell could secrete cytokines to alert the neighboring cells. However, this has been challenging to prove, and the localization upon non-apoptotic peroxisomes suggests something different. For example, one of the core observations during infections is the spike of ROS that occurs, and has been shown to play a critical role in the host response (Soucy-Faulkner et al., 2010). There is evidence that mitochondria, and likely peroxisomes, contribute to these ROS spikes (Sena and Chandel, 2012). As ROS levels increase on the surface of these organelles, it opens the possibility that transient disulfide switching may mechanistically activate the MAVS complexes (Xiong et al., 2011). The evolution of conserved, redox-sensitive cysteine residues within MAVS or associated proteins could help explain why these complexes target the mitochondria and peroxisomes. More recently, MAVS was shown to recruit the inflammasome to the mitochondrial surface, a process specific for certain classes of activators (Subramanian et al., 2013). So far the MAVs regulated complexes appear to be specific to innate rather than adaptive immunity. A common theme in immune activation is the requirement for ROS spikes upon infection (Tschopp, 2011). Therefore, we suspect that the reason the mitochondria and peroxisomes are commonly used as signaling platforms is due to the high local concentrations of ROS (and subsequently oxidized glutathione) that can trigger conformational changes through disulfide switching mechanisms. Future work will continue to explore these and other hypothesis.

CONCLUDING REMARKS

In this review we have highlighted a series of observations that illustrate the very tight functional, spatial, and regulatory links between the peroxisomes and the mitochondria. Evolutionary analysis coupled with the emergence of a vesicular transport route between the mitochondria and peroxisomes propels us to consider a role for mitochondria in peroxisomal biogenesis. Since ER-derived pre-peroxisomes are fusogenic (Boukh-Viner et al., 2005; van der Zand et al., 2012), and the mitochondrial cargo MAPL was seen to fuse with only a subpopulation of peroxisomes (Neuspiel et al., 2008; Braschi et al., 2010), we hypothesize that MDVs may contribute to early peroxisomal formation. Mature peroxisomes are also tightly integrated within complex biochemical cascades, funneling their substrates and products to the mitochondria, ER and sometimes lipid droplets (Schrader et al., 2013). Therefore, an alternative to a role in biogenesis is that MDVs could also selectively deliver metabolites to functional subclasses of peroxisomes within a cell. An analysis of the extensive metabolite flux required to flow between these organelles helps to fuel our speculation about functional specialization among peroxisomes. Clearly there is a great deal of work to do in order to distinguish these possibilities. Finally we proposed a general hypothesis where local oxidation may be used to activate cellular signaling pathways, which may explain why the mitochondria and peroxisomes work together as unique signaling platforms.

The critical importance of peroxisomes in physiology is chronically underappreciated within the wider scientific community. Along with their established links to the ER, we hope that increasing awareness of the obligate coupling of the peroxisomes to the mitochondria will encourage researchers to more carefully consider the contribution of peroxisomal dysfunction to disease progression. For example, a great deal of attention is currently being paid to the role of mitochondria in neurodegeneration, cancer and immunology, yet the impact of mitochondrial dysfunction on peroxisomes is virtually unexplored in these disease pathologies. There is a great deal of work to be done before we will fully understand the role of peroxisomal dysfunction in human disease. A first step will require a better characterization of the molecular mechanisms that regulate the behavior and biochemistry of peroxisomes as a dynamic and tightly integrated organelle.

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Import of proteins into the peroxisomal matrix

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Peroxisomes constitute a dynamic compartment in all nucleated cells. They fulfill diverse metabolic tasks in response to environmental changes and cellular demands. This adaptation is implemented by modulation of the enzyme content of the organelles, which is accomplished by dynamically operating peroxisomal protein transport machineries. Soluble import receptors recognize their newly synthesized cargo proteins in the cytosol and ferry them to the peroxisomal membrane. Subsequently, the cargo is translocated into the matrix, where the receptor is ubiquitinated and exported back to the cytosol for further rounds of matrix protein import. This review discusses the recent progress in our understanding of the peroxisomal matrix protein import and its regulation by ubiquitination events as well as the current view on the translocation mechanism of folded proteins into peroxisomes. This article is part of a Special Issue entitled: Origin and spatiotemporal dynamics of the peroxisomal endomembrane system.

Keywords: peroxisome, protein import, ubiquitination, biogenesis, translocation, targeting

INTRODUCTION

Peroxisomes are organelles that can be found in all nucleated cells. The number and morphology of peroxisomes varies significantly among different cell types, tissues or species. Peroxisomes are typically spherical organelles with a diameter from 0.1 to 1 μm that are surrounded by a single phospholipid bilayer membrane.

The peroxisomal luminal proteins are tightly packed in electron-dense, sometimes even crystalline matrix. The enzyme content varies depending on the cellular demands with the ability to adjust to the metabolic requirements of the cell. Accordingly, peroxisomes are considered to be multi-purpose organelles that contribute to the adaptation of cells to different environmental conditions. The peroxisomal matrix can harbor at least 50 different enzymes that are involved in diverse biochemical processes, such as beta-oxidation of fatty acids and the detoxification of hydrogen peroxide, which are considered as a main functions of peroxisomes (Schlüter et al., 2010). The beta-oxidation of fatty acids exclusively takes place in peroxisomes in yeast and plants, while in the case of mammalian cells, only the very long chain fatty acids (VLCF) are oxidized in peroxisomes, whereas shorter chain fatty acids are oxidized in mitochondria. Additionally, it has been established that peroxisomes are required for the synthesis of plasmalogens and bile acids in mammals (Wanders and Waterham, 2006) and that they contribute to certain biochemical steps of photorespiration of plants (Hu et al., 2012) as well as the final steps of penicillin biosynthesis in some filamentous fungi (Meijer et al., 2010). More recently explored functions of peroxisomes include an partial involvement in Vitamin K biosynthesis in plants (Babujee et al., 2010; Widhalm et al., 2012), calcium homeostasis in mammals (Lasorsa et al., 2008) as well as pheromone production in nematodes and insects (Joo et al., 2010; Spiegel et al., 2011). Moreover, peroxisomes contribute to the iron uptake and therefore virulence of pathogenic *Aspergillus* species, by containing enzymes required for the biosynthesis of siderophores (Gründlinger et al., 2013).

Typical peroxisomes together with specialized peroxisomes like glyoxysomes, glycosomes and Woronin bodies constitute the organelle family of "microbodies," whose members are all evolutionary related, sometimes even interconvertible compartments. The glycosomes of the protist order Kinetoplastida harbor glycolysis enzymes, whereas the glyoxysomes of the germinating plant seeds house enzymes of the glyoxylate cycle, and the Woronin bodies of filamentous fungi are tightly packed with a crystalline protein and function to seal septal pores in response to wounding (Pieuchot and Jedd, 2012).

The pivotal role of the correct topogenesis of peroxisomal proteins is also pointed out by the fact that dysfunction of human peroxisomes is associated with a spectrum of severe peroxisomal disorders, like e.g., Zellweger syndrome or X-linked adrenoleukodystrophy (Baes and Van Veldhoven, 2012; Nagotu et al., 2012; Poll-the and Gärtner, 2012; Waterham and Ebberink, 2012). These different disorders have in common that they are characterized by an abnormal peroxisome assembly and impaired peroxisomal function, in many cases resulting in multisystemic disorders that lead to death in early infancy. Furthermore, recent data demonstrate a link of peroxisome function and antiviral innate immunity as that they can promote a rapid response to

viral infection via peroxisomal antiviral signaling proteins (Dixit et al., 2010). In addition, it has been demonstrated that functional peroxisomes counteract the progressive brain damage and cognitive decline found in Alzheimer's disease (Kou et al., 2011; Lizard et al., 2012). Finally, the reactive oxygene metabolism connects peroxisomes to the molecular process of aging (Giordano and Terlecky, 2012; Manivannan et al., 2012).

All of the mentioned physiologic tasks strictly depend on a proper compartmentalization of the corresponding enzymes, which itself relies on a proper peroxisomal biogenesis.

The biogenesis of peroxisomes conceptually consists of the (1) formation and proliferation of the peroxisomal membrane, (2) peroxisome movement and inheritance as well as (3) the topogenesis of peroxisomal membrane and (4) matrix proteins (Platta and Erdmann, 2007; Fagarasanu et al., 2010; Islinger et al., 2012; Liu et al., 2012; Theodoulou et al., 2013). These tasks are essentially carried out by peroxisomal biogenesis factors, the peroxins. From the 34 peroxins described so far, at least 19 are known to be directly involved in different stages of peroxisomal matrix protein import (**Table 1**). In this review, we will focus on the recent developments concerning the topogenesis of peroxisomal matrix proteins.

MATRIX PROTEIN IMPORT

Because peroxisomes do not contain genetic material, all of their protein content is encoded in the nucleus, synthesized on free polyribosomes in the cytosol and targeted to the organelle in a post-translational manner [reviewed in Leon et al. (2006a)]. Recent data suggest that already the mRNA of most of the analyzed peroxisomal proteins is targeted to the proximity area of the peroxisome prior to the translation step (Zipor et al., 2009, 2011). The capacity to import proteins in a fully folded or even oligomeric and co-factor bound state is an extraordinary feature of peroxisomes and differentiates peroxisomes from other classical organelles like mitochondria or chloroplasts [reviewed in Leon et al. (2006a)]. While this concept had been established quite early, the question whether folded cargo proteins are preferably imported in an oligomeric or monomeric state has been picked up by two recent studies. While one study finds that mammalian Pex5p strictly imports tetrameric catalase (Otera and Fujiki, 2011), another study suggests that the catalase oligomer may be disassembled during the import process (Freitas et al., 2011).

In general, peroxisomes share the capability to translocate folded proteins with the Twin-Arg-Translocation (Tat) pathway of bacteria and thylakoid membranes (Albiniak et al., 2012; Palmer and Berks, 2012). However, while the entire machinery required for the translocation of the substrate protein is membrane-bound in the case of the Tat-system, some of the constituents of the peroxisomal translocation apparatus are soluble (Schnell and Hebert, 2003). The peroxisomal import receptors cycle between the cytosol and the peroxisomal membrane (Dammai and Subramani, 2001; Nair et al., 2004). According to the dynamics of the import receptors, the protein import process into peroxisomes can be divided into five stages such as (1) cargo recognition in the cytosol, (2) docking of the receptor/cargo- complex at the peroxisomal membrane, (3) cargo translocation over the membrane, (4) release of the cargo into the peroxisomal matrix, and (5) receptor recycling (**Figure 1A**).

CARGO RECOGNITION IN THE CYTOSOL

Newly synthesized peroxisomal matrix proteins are transported to their destination by means of a targeting sequence. The majority of the matrix proteins harbors a peroxisomal targeting signal type 1 (**PTS1**) at the carboxy-terminus, which is defined by the amino acids SKL and variants of the motif fitting the consensus (S/A/C)-(K/R/H)-(L/A). Because also additional adjacent residues have an impact on the cargo-recognition, the definition of the PTS1 can be extended to a dodecamer (Brocard and Hartig, 2006; Chowdhary et al., 2012). The conserved receptor for the PTS1-signal is **Pex5p**, which recognizes the PTS1-sequence via a tetratricopeptide repeats (TPRs) containing domain within its carboxy-terminal half. Crystal structures of the cargo-loaded and unloaded PTS1-receptor revealed that cargo binding induces major conformational changes within the receptor, which might generate a docking-competent state of the receptor (Stanley et al., 2006; Shiozawa et al., 2009; Fodor et al., 2012).

The peroxisomal targeting signal type 2 (**PTS2**) sequence is the second known peroxisomal targeting determinant. It is usually located within the first 20 amino acids of the cargo protein and has been defined as a nona-peptide by the amino acid signal (RK)-(LVIQ)-XX-(LVIHQ)-(LSGAK)-X-(HQ)-(LAF) (Petriv et al., 2004; Lazarow, 2006). In plants, approximately one third of peroxisomal matrix proteins harbor a PTS2-signal (Lingner et al., 2011; Chowdhary et al., 2012), whereas in *Saccharomyces cerevisiae* only three proteins are known to use this targeting sequence (Grunau et al., 2009; Jung et al., 2010). The PTS2-pathway is completely absent in *Caenorhabditis elegans* (Motley et al., 2000), *Drosophila melanogaster* (Faust et al., 2012) and the protist *Phaeodactylum tricornutum* (Gonzalez et al., 2011), which therefore import all matrix proteins via the PTS1-pathway.

The PTS2-cargo is recognized by **Pex7p**, which contains several tryptophan-aspartic acid (WD) repeats that mediate the binding. However, unlike the PTS1-receptor, Pex7p is necessary, but not sufficient to carry out all steps of the import process because it requires auxiliary proteins. These PTS2-co-receptors are the redundant **Pex18p** and **Pex21p** in *S. cerevisiae*, the orthologous **Pex20p** in most other yeasts and fungi as well as **Pex5L**, the longer of two splice isoforms of Pex5p, in mammals and plants (Schliebs and Kunau, 2006). Interestingly, *Podospora anserina* Pex20p has been reported to carry out a Pex7p-dependent function in matrix protein import as well as a Pex7p-independent function in meiocyte formation (Peraza-Reyes et al., 2011).

The targeting of a subset of peroxisomal matrix proteins does not essentially rely on one of the two classical targeting signals (**Figure 1B**). Some of these proteins can be co-imported via an association with canonical PTS-cargo proteins. This "piggy-back import" has been demonstrated for the enoyl-CoA isomerases Eci1p and Dci1p from *S. cerevisiae* (Yang et al., 2001) and the five acyl-CoA oxidase isoforms from *Yarrowia lipolytica* (Titorenko et al., 2002) and more recently also for mammalian Cu/Zn superoxide dismutase (Islinger et al., 2009). Other proteins, like

Table 1 | Peroxins involved in peroxisomal matrix protein import.

Peroxin	Enzyme/activity	Mw (KDa)	Function
Pex1p (PAS1)	AAA-type ATPase	117.3	Binds Pex6p and is involved in the dislocation of the PTS-receptors
Pex2p (CRT1, PAS5)	Ubiquitin-protein ligase (E3), RING-domain	30.8	Forms together with Pex10p and Pex12p the RING-complex and is involved in the ubiquitination of the PTS-receptors
Pex4p (PAS2, UBC10)	Ubiquitin-conjugating enzyme (E2)	21.1	Monoubiquitination of the PTS-receptors
Pex5p (PAS10)	PTS1 receptor, TPR-domain	69.3	Receptor for the PTS1-signal; required for the PTS1-dependent matrix protein import
Pex6p (PAS8)	AAA-type ATPase	115.6	Binds Pex1p and is involved in the dislocation of the PTS-receptors
Pex7p (PAS7, PEB1)	PTS2 receptor, WD40 domain	42.3	Receptor for the PTS2-signal; required for the PTS2-dependent matrix protein import; forms complex with PTS2-co-receptors (Pex18p/Pex20p/Pex21p)
Pex8p (PAS6)	Coordinator of protein import machinery	68.2	Peripheral intraperoxisomal membrane protein; bridges docking- and RING-complex; possibly involved in cargo disassembly
Pex10p (PAS4)	Ubiquitin-protein ligase (E3), RING-domain	39.1	Forms together with Pex2p and Pex12p the RING-complex and is involved in the ubiquitination of the PTS-receptors
Pex12p (PAS11)	Ubiquitin-protein ligase (E3), RING-domain	46.0	Forms together with Pex2p and Pex10p the RING-complex and is involved in the ubiquitination of the PTS-receptors
Pex13p (PAS20)	Core component of docking machinery, SH3-domain	42.7	Integral membrane protein required for the docking of receptor/cargo complexes; forms docking complex with Pex14p and Pex17p
Pex14p	Core component of docking machinery, PXXP-domain	43.7	Membrane protein required for the docking of receptor/cargo complexes; forms docking complex with Pex13p and Pex17p
Pex15p (PAS21)	Membrane anchor of the AAA-peroxins	43.7	Phosphorylated tail-anchored PMP that is involve in the recruitment of yeast Pex6p to the peroxisomal membrane
Pex17p (PAS9)	Docking complex	23.2	Membrane-associated protein that forms the docking complex with Pex14p and Pex17p
Pex18p	PTS2-co-receptor	32.0	Interacts with PTS2-receptor Pex7p; partially redundant with Pex21p (in *S. cerevisiae*)
Pex20p	PTS2-co-receptor	37.8	Involved in PTS2-dependent protein import, mostly as co-receptor of Pex7p (in most fungi, like e.g., *P. pastoris*)
Pex21p	PTS2 protein import	33.0	Interacts with PTS2-receptor Pex7p; partially redundant with Pex21p (in *S. cerevisiae*)
Pex22p (YAF5)	Peroxisomal protein import	19.9	Membrane protein required the recruitment of Pex4p to the peroxisomal membrane
Pex26p	Membrane anchor of the AAA-peroxins	33.9	Tail-anchored PMP that is involved in the recruitment of mammalian Pex6p to peroxisomal membrane
Pex33p (Pex14/17p)	Docking complex	52.5	Part of the docking complex in filamentous fungi, e.g., *N. crassa*

The names in brackets refer to the previous original designations in yeast. Except where indicated, the molecular mass refers to the S. cerevisiae protein.

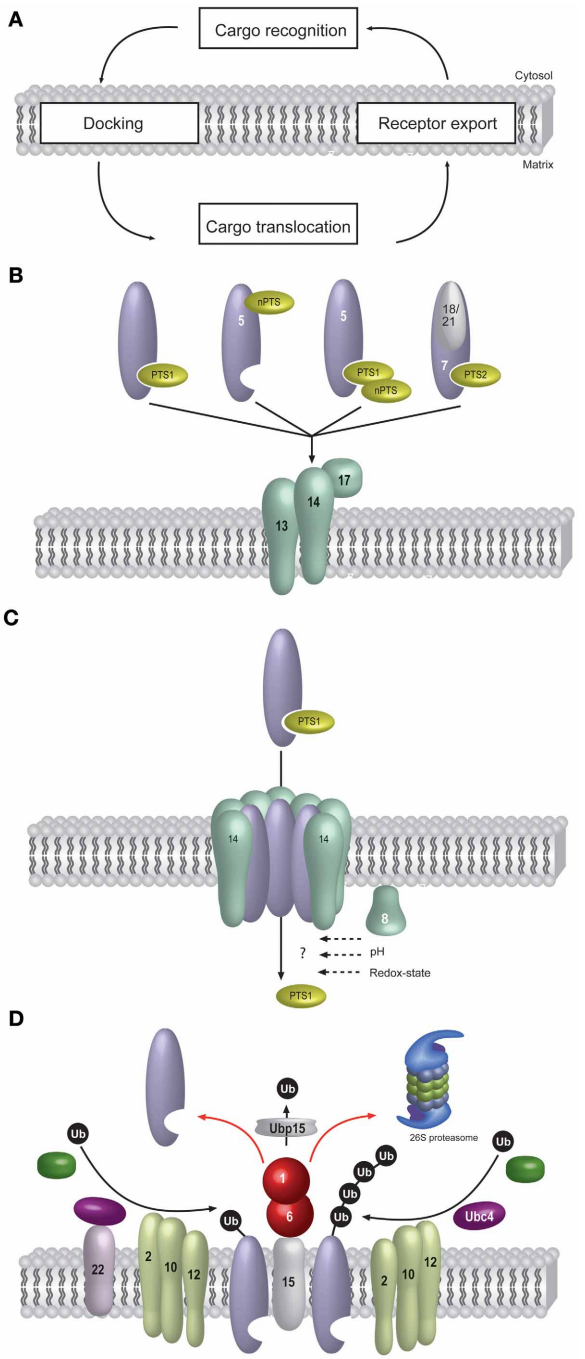

FIGURE 1 | Matrix protein import into peroxisomes. Most principles of peroxisomal protein import are evolutionary conserved. The schematic representation shown here refers to the situation in *Saccharomyces cerevisiae*. Peroxisomal matrix protein import takes place posttranslationally and requires an elaborate protein import machinery, consisting of peroxisome biogenesis factors, so called peroxins. **(A)** Another feature of peroxisomal import is the requirement for cycling receptors. The import process can conceptually be divided into cargo-recognition by the receptors in the cytosol, docking of the receptor/cargo-complex at the peroxisomal membrane, cargo translocation and finally export of the receptor back to the cytosol. **(B)** Cargo-recognition and docking: Proteins harboring a

(Continued)

FIGURE 1 | Continued
peroxisomal targeting signal of type 1 (PTS1) or type II (PTS2) are recognized in the cytosol by specific import receptors, Pex5p and Pex7p, respectively. Alternatively, some cargo proteins do not harbor a PTS or do not essential depend on it. Some of these non-PTS proteins (nPTS) bind to the N-terminus of Pex5p or to canonical PTS1-proteins. The cargo-loaded receptors are directed to a docking complex at the peroxisomal membrane. For this, the PTS2-receptor Pex7p requires auxiliary proteins, in baker's yeast these are the redundant Pex18p or Pex21p. The receptor-cargo-complexes bind to the docking-complex (Pex13p, Pex14p and Pex17p) at the peroxisomal membrane. The following steps are better known for the PTS1- than the PTS2-pathway. **(C)** Cargo-translocation: It is assumed that the association of Pex14p and cargo-loaded Pex5p leads to the formation of a transient pore, which functions as a protein-conducting channel. The cargo is translocated into the peroxisomal lumen in an unknown manner. In intraperoxisomal Pex8p, a pH-shift or the redox-state might be involved in receptor-cargo dissociation. **(D)** At the end of the import cascade, the receptor is recycled from the peroxisomal membrane back to the cytosol for another round of import. For this, Pex5p is monoubiquitinated by the Pex22p-anchored ubiquitin-conjugating enzyme Pex4p and the ubiquitin-protein ligase Pex12p, which forms the RING-finger complex together with the other ubiquitin-protein ligases Pex2p and Pex10p. Pex5p-polyubiquitination is performed by the ubiquitin-conjugating enzyme Ubc4p in conjunction with the ubiquitin-protein ligases Pex2p and Pex10p. For Pex5p, it has been demonstrated that the ubiquitin-signal leads to an ATP-dependent dislocation of Pex5p from the peroxisomal membrane. This process is performed by the AAA-type ATPases Pex1p and Pex6p, which are anchored to the peroxisomal membrane via Pex15p. During or shortly after receptor export to the cytosol, the ubiquitin is removed by the ubiquitin-hydrolase Ubp15p.

acyl-CoA oxidase from *S. cerevisiae* or alcohol oxidase from *H. polymorpha* (Klein et al., 2002; Gunkel et al., 2004), interact directly with Pex5p in a PTS1-independent fashion via the binding to the N-terminal region of Pex5p in a process, which is called "**non-PTS import**" (Van Der Klei and Veenhuis, 2006).

A new chapter of peroxisomal targeting signals has recently been opened by the finding that glycolytic enzymes of the analyzed fungi and mammalian species contain a cryptic PTS (Freitag et al., 2012). With exception of the well-established glycolytic enzymes found in glycosomes of Kinetoplastids (Gualdrón-López et al., 2012) and the report on the peroxisome-dependent glucose metabolism of the fungus *Cryptococcus neoformans* (Idnurm et al., 2007), these enzyme were thought to be strictly cytosolic in all species. However, they contain a cryptic peroxisomal targeting signal, which can be generated or eliminated in a species-specific manner by ribosomal read-through or alternative splicing (Freitag et al., 2012). This differential targeting to two locations may indicate a dynamic regulation of glycolysis by sequestering the key enzymes away from the cytosol.

ASSOCIATION OF THE CARGO-LOADED RECEPTOR WITH THE MEMBRANE VIA THE DOCKING COMPLEX

Several lines of evidence indicate that only the cargo-bound receptors are efficiently directed to the peroxisome (Gouveia et al., 2003; Grunau et al., 2009). The cargo-bound receptors associate with the peroxisomal membrane via a docking complex (**Figure 1B**), which consists in all known species of the core components Pex13p and Pex14p (Kiel et al., 2006). The absence of

either Pex13p or Pex14p significantly affects the import pathway of cargos targeted to the peroxisome (Azevedo and Schliebs, 2006; Williams and Distel, 2006). **Pex13p** is an integral membrane protein, which binds to Pex14p via its SH3-domain and also via an intraperoxisomal binding site (Pires et al., 2003; Schell-Steven et al., 2005). The N-terminal part of Pex13p also binds to the PTS2-receptor, while the SH3-domain of the yeast protein contains a binding site for the PTS1 receptor Pex5p (Williams and Distel, 2006). **Pex14p** contains a proline-rich segment for binding of the SH3-domain of Pex13p. Pex14p has been described as an carbonate-resistant integral membrane protein but in some species, it behaves like a peripheral protein (Azevedo and Schliebs, 2006). As Pex13p also Pex14p binds both PTS-receptors at different sites (Niederhoff et al., 2005). Nuclear magnetic resonance (NMR) and determination of the crystal structures (Neufeld et al., 2009; Su et al., 2009) revealed that the Pex5p/Pex14p interface comprises two hydrophobic cavities of Pex14p, which bind characteristic WXXXF/Y motifs of PTS1 receptor Pex5p. In general, Pex14p is considered to be the initial binding partner for the cargo-bound PTS1-receptor. However, because the available data addressing this question are limited and because of the observation that Pex13p can also associate with cargo-bound PTS-receptors (Grunau et al., 2009; Natsuyama et al., 2013), the individual contribution of Pex13p and Pex14p to the initial docking event remains to be further investigated.

In many species, the docking complex contains further peroxins in addition to Pex13p and Pex14p. Yeast **Pex17p** is a peripheral membrane protein of unknown function, which associates with peroxisomes via Pex14p but does not interact with Pex5p. A deficiency in Pex17p affects import of PTS1 as well as PTS2 proteins by an unknown mechanism (Azevedo and Schliebs, 2006). A homolog of Pex17p in higher eukaryotes has not yet been identified (Kiel et al., 2006). However, in filamentous fungi, a chimeric protein that consists of a Pex14p-like amino-terminal domain and a Pex17p-like carboxyl-terminal domain has been described (Managadze et al., 2010; Opaliński et al., 2010; Peraza-Reyes et al., 2011). This chimeric protein is called Pex14/17p in *P. anserina* and *Penicilium crysogenum*, while it is named Pex33p in *Neurospora crassa*.

Trypanosoma brucei contains two very different isoforms of Pex13p. While PEX13.1 resembles the conserved Pex13p, the PEX13.2 lacks the SH3 domain and contains a PTS1-signal at its carboxyl-terminus (Brennand et al., 2012; Verplaetse et al., 2012). It will be of interest to elucidate the special contribution of PEX13.2 to the protein import process, especially as it is part of the docking complex and essential for glycosome biogenesis.

A recent study of a Zellweger spectrum patient cell line describes the dimerization of human Pex13p (Krause et al., 2013). This dimerization occurs independently of the interaction to Pex14p and is required for PTS1-protein import (Krause et al., 2013). Also Pex14p can undergo dimerization, even though the functional impact is not understood yet (Su et al., 2010).

The association with the docking complex marks the entry of Pex5p to the protein-protein interaction network at the peroxisome (Hazra et al., 2002; Agne et al., 2003; Oeljeklaus et al., 2012). However, the collected data strongly indicate that components of the docking complex are far more than static receptor anchors and that they display a certain dynamic contribution to the peroxisomal protein import cascade.

TRANSLOCATION OVER THE TRANSIENT IMPORT PORE AND CARGO-RELEASE INTO THE MATRIX

The actual mode of matrix protein import is still elusive and how the cargo proteins traverse the peroxisomal membrane without affecting the permeability barrier remains hypothetical.

Transient import pore

Over the years, several models have been put forward of how folded and oligomeric proteins may traverse the peroxisomal membrane. The translocon has not yet been visualized and these models range from a channel consisting of multi-membrane spanning proteins and a transiently opened import pore to a pinocytosis-related model that completely lacks a translocon [as discussed in Girzalsky et al. (2009)].

The results collected in recent years strongly favor the idea of a transiently opened import pore (**Figure 1C**). It has been suggested that the major constituents of this dynamic pore may be membrane proteins Pex14p and Pex13p (Grou et al., 2009a) or Pex14p and the PTS1-receptor Pex5p (Erdmann and Schliebs, 2005). Indeed, one of the surprising features of Pex5p is that it can bind to lipids and change its topology at the peroxisomal membrane where it is partially carbonate resistant, adjusts to a partial protease-protected state and thereby behaves like an integral membrane protein (Gouveia et al., 2000, 2002; Platta et al., 2005; Kerssen et al., 2006). Furthermore, Pex5p together with Pex14p constitute the minimal unit for the translocation of the intraperoxisomal Pex8p across the membrane in *P. pastoris* (Ma et al., 2009). The functional interplay is further pointed out by the finding that at least in CHO cell lines, Pex5p can stabilize Pex14p (Natsuyama et al., 2013). *Leishmania donovani* Pex14p forms a homo-oligomeric complex, which undergoes major conformational changes upon Pex5p-binding (Cyr et al., 2008). Importantly, the Pex5p-Pex14p sub-complex of *S. cerevisiae* harbors pore forming activity in electrophysiological studies (Meinecke et al., 2010). This complex, which almost exclusively consists of Pex5p and Pex14p can be reconstituted into membranes and displays channel activity when incubated with a cargo-loaded soluble Pex5p (Meinecke et al., 2010). The size of this pore formed by the transiently gated ion conducting channel is variable up to 9 nm and therefore appears to accomplish the standards for the passage of folded proteins into the peroxisomal matrix. However, the exact composition of the pore as well as the driving force and the mechanism of cargo translocation remain elusive.

Cargo release

Data on how the cargo is released into the peroxisomal lumen are still scarce (**Figure 1C**). The first study to tackle this question directly suggests that the intraperoxisomal peripheral membrane protein **Pex8p** of *H. polymorpha* might be in involved in this process, because it can dissociate receptor-cargo complexes *in vitro* (Wang et al., 2003). In addition, the same study suggested that

a change in pH might induce a disassembly of Pex5p-oligomers into the monomeric form and thereby also induces the dissociation of the cargo from this complex (Wang et al., 2003). A recent study in *P. pastoris* suggests that Pex8p is involved in a redox-state dependent disassembly of Pex5p oligomers and cargo (Ma et al., 2013). However, it is not easy to generalize these results because the intraperoxisomal pH may vary significantly depending on the experimental condition (Visser et al., 2007) and because Pex8p is a less conserved yeast protein that seems to be absent in most other species (Kiel et al., 2006). *S. cerevisiae* Pex8p has been described to function as a structural link of the docking complex to the export machinery (Agne et al., 2003; Platta et al., 2013), while this task is accomplished by Pex3p in *P. pastoris* (Hazra et al., 2002). One hypothetical explanation could be that the proposed role of Pex8p in cargo release may be transferred to the conserved parts of the docking complex in other species. In this respect, it is interesting to note that a recent publication demonstrates that the amino-terminus of mammalian Pex14p plays a role in the release of Pex5p-bound PTS1-cargo from the translocation machinery into the peroxisomal matrix (Freitas et al., 2011).

The signal sequence of a subset of the imported proteins is proteolytically removed after the import in peroxisomes of mammals and plants (Kurochkin et al., 2007; Schuhmann et al., 2008; Okumoto et al., 2011a). In the case of mammalian beta-oxidation enzymes, the intraperoxisomal protease Tysnd1 is responsible both for the removal of the leader peptide from PTS2 proteins as well as for the processing of PTS1 proteins and controls thereby the proper activity of these enzymes in beta-oxidation (Okumoto et al., 2011a). Indeed, recent work demonstrates that a deficiency in Tysnd1 results in a mild Zellweger syndrome spectrum-resembling phenotype in mice (Mizuno et al., 2013).

UBIQUITINATION AND DISLOCATION OF THE RECEPTOR BY THE EXPORTOMER

Subsequent to cargo release, the receptor is exported to the cytosol by a molecular machinery called the peroxisomal exportomer (Platta et al., 2013). This machinery comprises sub-complexes consisting of mechano-enzymes that provide the pulling-force to extract the receptor from the membrane as well as sub-complexes that are required for the generation of the export-signal, which is the ubiquitination of the receptor (**Figure 1D**).

Recycling pathway of the PTS1-receptor

Most work has been dedicated to the ubiquitination of the PTS1-receptor Pex5p. Under wild-type conditions, the major modification of Pex5p is the attachment of a single ubiquitin moiety on a conserved cysteine residue (Kragt et al., 2005; Carvalho et al., 2007; Williams et al., 2007; Okumoto et al., 2011b). This monoubiquitination represents a prerequisite for the export of Pex5p back to the cytosol, which represents a molecular requirement that seems to be conserved from yeast to man (Carvalho et al., 2007; Platta et al., 2007, 2008; Okumoto et al., 2011b). The ubiquitin-conjugating enzyme (E2) **Pex4p** together with its membrane anchor **Pex22p** are required for this modification in *S. cerevisiae* (Platta et al., 2007; Williams et al., 2007, 2012). Mammalian cells lack clear Pex4p- and Pex22p-orthologs and here the functional-related isoforms UbcH5a, UbcH5b and UbcH5c catalyze the cysteine-dependent monoubiquitination (Grou et al., 2008). Proper monoubiquitination of Pex5p depends on an intact RING-peroxin complex (Kragt et al., 2005; Williams et al., 2008; Platta et al., 2009). Work in *S. cerevisiae* has demonstrated that **Pex2p** and **Pex12p** (Platta et al., 2009) as well as **Pex10p** (Williams et al., 2008; Platta et al., 2009) display ubiquitin-protein ligase (E3) activity. Recently, it could be confirmed that also each of the *A. thaliana* RING-peroxins has E3 activity (Kaur et al., 2013). The deletion of one of the RING-peroxin genes causes the instability of the entire complex (Hazra et al., 2002; Agne et al., 2003) and therefore inhibition of monoubiquitination of Pex5p (Williams et al., 2008; Platta et al., 2009). However, *in vitro* ubiquitination studies with recombinant proteins as well as additional work with RING-peroxin truncations lacking the catalytic RING-domain suggest that Pex12p is the E3 ligase directly responsible for monoubiquitination of Pex5p (Platta et al., 2009). At least *in vitro*, the activity of Pex12p can be synergistically enhanced in presence of the RING-domain of Pex10p (El Magraoui et al., 2012).

Following its monoubiquitination, the PTS1-receptor is exported back to cytosol in an AAA (ATPases associated with diverse cellular activities)-complex dependent manner. The peroxisomal AAA-type ATPases **Pex1p** and **Pex6p** play a non-redundant role in this process and are supposed to act as dislocases that extract the Pex5p from the membrane (Miyata and Fujiki, 2005; Platta et al., 2005). They are anchored to the peroxisomal membrane by the tail-anchored protein **Pex15p** in yeast as well as the orthologous Pex26p in mammals and APEM9 in plants (Birschmann et al., 2003; Matsumoto et al., 2003; Goto et al., 2011; Nashiro et al., 2011). The binding and hydrolysis of ATP by Pex1p and Pex6p is supposed to induce conformational changes that generate the force for the pull the receptor out of the membrane (Fujiki et al., 2012; Grimm et al., 2012). Although monoubiquitination of the PTS1-receptor plays a crucial role in its release, the exact molecular mechanism of substrate recognition and extraction from the membrane is still unclear. Interestingly, recent work from mammalian cells identified AWP1, which binds both Pex6p as well as ubiquitin (Miyata et al., 2012). This finding strongly suggest that AWP1 might function as specific linker, which enables the AAA-peroxins to transfer their pulling force to the monoubiquitinated receptor, thereby driving its export.

Degradation pathway of the PTS1-receptor

The cysteine-dependent monoubiquitination of Pex5p primes the PTS1-receptor for its recycling under normal conditions. However, in cases where the recycling pathway is hampered, Pex5p is polyubiquitinated on lysine residues and finally degraded in the 26S proteasome (Platta et al., 2004, 2007; Kiel et al., 2005; Williams et al., 2007) (**Figure 1D**). The polyubiquitination of *S. cerevisiae* Pex5p mainly depends on the ubiquitin-conjugating enzyme (E2) **Ubc4p** and the partially redundant Ubc5p and Ubc1p (Platta et al., 2004; Kiel et al., 2005; Kragt et al., 2005). The RING-peroxins Pex10p (Williams et al.,

2008) as well as Pex2p (Platta et al., 2009) have been suggested to function as corresponding ubiquitin-protein ligases (E3). It is interesting to note that a recent *in vitro* study demonstrates that Pex10p(RING) can enhance the ubiquitination activity of the Ubc4p-Pex2p(RING) unit (El Magraoui et al., 2012), indicating that possibly these two RING-peroxins act together in the catalysis of the Ubc4p-dependent ubiquitination. The proteasomal degradation of the polyubiquitinated *S. cerevisiae* Pex5p is supposed to represent a quality control system for aberrant PTS1-receptor molecules (Platta et al., 2013).

Deubiquitination

Pex5p found in the cytosol is not further ubiquitinated *in vivo*, indicating that the ubiquitin moiety is removed upon or shortly after membrane release of the receptor (**Figure 1D**). In general, ubiquitin hydrolases perform the cleavage of ubiquitin from substrates (Amerik and Hochstrasser, 2004). The corresponding deubiquitinating enzyme in *S. cerevisiae* for the removal of the ubiquitin moiety from the modified PTS1-receptor is **Ubp15p** (Debelyy et al., 2011), while the non-orthologous **USP9X** has been found to deubiquitinate the monoubiquitinated Pex5p in mammals (Grou et al., 2012). However, both studies suggest that also other deubiquitinating enzymes may act in a redundant manner on Pex5p. Furthermore, at least a minor fraction of the monoubiquitinated Pex5p can be deubiquitinated in a non-enzymatic manner by cleavage of the thioester bond between Pex5p and ubiquitin by a nucleophilic attack of glutathione (Grou et al., 2009b).

Functional role of ubiquitination in the PTS2-import pathway

The PTS2-co-receptor Pex18p of *S. cerevisiae* was the first peroxin that has been found to be ubiquitinated (Purdue and Lazarow, 2001). Recent studies analyzed the modified forms of PTS2-co-receptors in more detail. This resulted in the discovery that Pex18p as well as the orthologous *P. pastoris* Pex20p are ubiquitinated in a similar manner to the PTS1-receptor Pex5p. Both Pex18p and Pex20p are polyubiquitinated on lysine residues and monoubiquitinated on a cysteine. The monoubiquitination is essential for matrix protein import, while the polyubiquitination regulates the stability of the PTS2-co-receptors (Leon et al., 2006b; Hensel et al., 2011; Liu and Subramani, 2013). Pex4p, the E2 ubiquitin-conjugation enzyme and the RING peroxins (Pex2p, Pex10p, Pex12p) proved to be required for mono- and polyubiquitination of *P. pastoris* Pex20p (Liu and Subramani, 2013). It is interesting to note that Pex18p displays a constitutive turn-over even in wild-type cells, while the PTS2-receptor Pex7p is stable (Hensel et al., 2011). So far, no evidence for ubiquitination or degradation of Pex7p has been found in yeast. However, a recent report demonstrates that endogenous *Arabidopsis* Pex7p is degraded by the proteasome when the dominant-negative GFP-Pex7p is expressed and that this mechanism depends on the interaction of Pex7p with the Rab GTPase RabE1c (Cui et al., 2013). However, it remains to be elucidated whether the observed disposal of Pex7p is also present under normal conditions and if this pathway is conserved among species.

INTERCONNECTION OF RECEPTOR EXPORT AND CARGO IMPORT

The peroxisomal export machinery components and the mechanism by which they facilitate the cycling of peroxisomal receptors are evolutionary and functionally related to endoplasmic reticulum associated degradation (ERAD) (Schlüter et al., 2006; Gabaldón, 2010; Schliebs et al., 2010). ERAD signifies a mechanism by which accumulated misfolded and polyubiquitinated proteins are extracted from the ER membrane or lumen for their subsequent degradation by the proteasome (Hampton and Sommer, 2012). Both the peroxisomal protein import machinery as well as the ERAD machineries utilize ubiquitination to mark proteins for their ATP-dependent release from the membrane by AAA-type ATPases.

The collected evidence over the last years defined the peroxisomal exportomer as the energy-consuming entity for peroxisomal protein import. In general, this might indicate that the step of cargo translocation into the matrix itself is an ATP-dependent step. However, a study with a rat liver *in vitro* system suggests that cargo release may occur independently of ATP and therefore presumably before the ubiquitination step (Alencastre et al., 2009). A CHO cell *in vitro* system instead supports the model that cargo translocation relies on the hydrolysis of ATP (Miyata et al., 2009). Along this line, it has been suggested that the ERAD-like ATP-driven export of the ubiquitinated receptor might be mechanistically linked to cargo translocation, which is highlighted by the so called export-driven-import model (Schliebs et al., 2010). In support of this model, the presence of a functional receptor–export complex is a pre-requisite for the ATP-dependent import of matrix proteins into peroxisomes (Schliebs et al., 2010). Current work on the ubiquitination of the PTS2-co-receptor Pex18p in *S. cerevisiae* provided direct evidence for the export-driven-import model. The cysteine-dependent monoubiquitination of Pex18p, which is required for the export of the co-receptor from the peroxisomal membrane into the cytosol, was observed to be a prerequisite for translocation of cargo-loaded Pex7p across the peroxisomal membrane (Hensel et al., 2011). In this context, it is also interesting to note that the binding capacity for functional PTS-receptors at the peroxisomal membrane seems to be restricted. The attenuation of receptor export by functional impairment of the exportomer results in an accumulation of *S. cerevisiae* receptors at the membrane (Platta et al., 2004; Kiel et al., 2005) and therefore prevents the docking of incoming receptor-cargo complexes arriving from the cytosol. This assumption is in line with recent data from work in *A. thaliana* (Ratzel et al., 2011). The physiological defects of mutated and only insufficiently active Pex6p were partially restored when combined with a weakly expressed allele of the docking protein Pex13p (Ratzel et al., 2011). This strongly argues for a model in which PTS-receptor import and export rates have to be balanced to allow for a functional matrix protein import cascade.

In summary, the ATP-consuming peroxisomal receptor export machinery is thought to function as import motor for matrix proteins, either indirectly via balanced receptor import/export rates or directly via a linkage of cargo translocation with receptor ubiquitination and export.

CONCLUDING REMARKS

The import of peroxisomal matrix proteins differs significantly from other organelles like mitochondria or chloroplasts as peroxisomes can accommodate folded and oligomeric proteins that are targeted to the membrane via cycling receptors.

Much progress has been made in the understanding of the molecular requirements for recognition of the PTS1-signal of peroxisomal matrix proteins by the import receptor Pex5p, for which even several atomic structures are now available. In contrast our knowledge of the structure of Pex7p and mechanism of cargo recognition is still limited. It also still needs to be addressed whether cargo recognition by the peroxisomal import receptors and peroxisomal targeting of the cargo-loaded receptor occurs more or less haphazardly in the cytosol or is also well regulated as indicated by the above mentioned studies on mRNA targeting.

It is now well accepted that the PTS1 receptor Pex5p cycles between a soluble cytosolic state and a membrane-bound state and recent evidence indicated that the receptor at the membrane forms an integral part of the temporally formed import pore. Clearly, it will be of importance to elucidate the assembly of this pore as well as the modulation of its dynamics and gating by regulatory proteins or posttranslational modifications. Apart from this, it will be a significant advance to clarify the existence of an independent PTS2-selective import pore. The functional role of the translocon-associated proteins, e.g., the Pex17p-like and Pex13p-like peroxins or Pex8p is not really understood and needs to be experimentally addressed.

The collected data on the concerted action of sub-complexes of the peroxisomal protein import machinery has merged into the unified model of the receptor export machinery, the exportomer.

In light of its important contribution to the energy requirement of peroxisomal protein import and the proposed export-driven import model, the molecular dynamics of the exportomer during cargo translocation and export of the ubiquitinated receptor need to be investigated. Along this line, one of the most intriguing questions concerns the fact that the PTS1-receptor Pex5p as well as the PTS2-coreceptors Pex18p and Pex20p are ubiquitinated on a cysteine via a thioester bond and not by a more common isopeptide bond to a lysine. The functional relevance of this uncommon kind of ubiquitination remains to be disclosed.

Certainly, many questions regarding the molecular mechanism of matrix protein import into peroxisomes remain to be answered. This is also true for a possible cross-regulation of matrix protein import with other cellular processes, e.g., cellular redox-balance, nutrient and energy status as well as peroxisome maturation, division and degradation.

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Dual targeting of peroxisomal proteins

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Cellular compartmentalization into organelles serves to separate biological processes within the environment of a single cell. While some metabolic reactions are specific to a single organelle, others occur in more than one cellular compartment. Specific targeting of proteins to compartments inside of eukaryotic cells is mediated by defined sequence motifs. To achieve multiple targeting to different compartments cells use a variety of strategies. Here, we focus on mechanisms leading to dual targeting of peroxisomal proteins. In many instances, isoforms of peroxisomal proteins with distinct intracellular localization are encoded by separate genes. But also single genes can give rise to differentially localized proteins. Different isoforms can be generated by use of alternative transcriptional start sites, by differential splicing or ribosomal read-through of stop codons. In all these cases different peptide variants are produced, of which only one carries a peroxisomal targeting signal. Alternatively, peroxisomal proteins contain additional signals that compete for intracellular targeting. Dual localization of proteins residing in both the cytoplasm and in peroxisomes may also result from use of inefficient targeting signals. The recent observation that some *bona fide* cytoplasmic enzymes were also found in peroxisomes indicates that dual targeting of proteins to both the cytoplasm and the peroxisome might be more widespread. Although current knowledge of proteins exhibiting only partial peroxisomal targeting is far from being complete, we speculate that the metabolic capacity of peroxisomes might be larger than previously assumed.

Keywords: peroxisomes, protein import, alternative splicing, ribosomal read-through, glycolysis

INTRODUCTION

Peroxisomes are near-ubiquitous eukaryotic organelles that have been first described as microbodies in murine kidney-cells (Rhodin, 1954). Later, these organelles were shown to contain enzymes involved in the turnover of hydrogen peroxide (H_2O_2), which gave rise to the term peroxisomes (deDuve and Bauduin, 1966). One of the major sources of H_2O_2 is β-oxidation of fatty acids that occurs in peroxisomes throughout the eukaryotic kingdoms (Poirier et al., 2006). Beside these common tasks of fatty acid degradation and peroxide detoxification, peroxisomes exhibit a wide variety of other metabolic functions (Nyathi and Baker, 2006; van der Klei et al., 2006; Wanders and Waterham, 2006). A high degree of functional and metabolic specialization in different organisms even led to specific naming of peroxisomal subtypes. In plants and fungi, glyoxysomes harbor enzymes of the glyoxylate cycle, which is required for the anabolic use of acetyl-CoA as carbon source (Breidenbach and Beevers, 1967; Zimmermann and Neupert, 1980; Kionka and Kunau, 1985). In trypanosomes, the majority of glycolytic enzymes reside in specialized peroxisomes called glycosomes (Opperdoes and Borst, 1977). Filamentous ascomycetes contain "Woronin bodies" that play a mechanical role and seal septal pores (Jedd and Chua, 2000). In spite of their obvious functional and metabolic diversity all types of peroxisomes share a highly conserved import system for their matrix proteins (Gabaldon, 2010). Import is mediated by peroxisomal targeting sequences (PTS), that reside either at the C-terminus (PTS1) or at the N-terminus (PTS2) of proteins (Rucktäschel et al., 2011). C-terminal PTS1 motifs consist of about 12 amino acids that contain at the very end a characteristic tripeptide derived from the prototype sequence SKL (Gould et al., 1987, 1989; Brocard and Hartig, 2006). The commonly used consensus motif for C-terminal tripeptides is (S/A/C)-(K/R/H)-(L/M), while some studies suggest a more degenerated consensus (Lametschwandtner et al., 1998; Reumann et al., 2007). A few proteins contain internal motifs acting as PTS (Peterson et al., 1997; Klein et al., 2002; Gunkel et al., 2004; Oshima et al., 2008; Galland et al., 2010). PTS1 containing proteins are recognized by the cytoplasmic receptor Pex5 and are imported into peroxisomes in their fully folded, oligomeric and even cofactor bound form (Brocard et al., 1994; Glover et al., 1994; McNew and Goodman, 1994). A minority of proteins contains an N-terminal PTS2-motif, which is recognized by the soluble receptor Pex7 (Swinkels et al., 1991; Marzioch et al., 1994; Rucktäschel et al., 2011). PTS2-motifs exhibit the consensus sequence (R/K)(L/V/I)-X_5-(H/Q)(L/A) (Petriv et al., 2004). Interestingly, some species completely lack the PTS2 import pathway (Motley et al., 2000; Gonzalez et al., 2011). Some proteins have been described that lack any detectable PTS-motifs but are nevertheless found in peroxisomes. A quite unusual way to achieve peroxisomal import of proteins without PTS is "piggy-backing", since proteins can also be imported as oligomers (Glover et al., 1994; McNew and Goodman, 1994; Yang et al., 2001). It has been demonstrated

that peroxisomal import of the copper containing superoxide dismutase (SOD) is mediated via interaction with a chaperone that harbors a PTS1 (Islinger et al., 2009).

For several peroxisomal proteins dually targeted isoforms have been described. These isoforms execute the same or a similar function in at least one other place (for an overview see **Table 1**). In general, cells can use various mechanisms to achieve dual or multiple targeting of proteins. One of the best systems studied are mitochondrial proteins some of which occur also in other cellular compartments (for review Yogev and Pines, 2011). Here, we address the diversity of mechanisms to mediate dual targeting of peroxisomal proteins and distinguish five different strategies (see **Figure 1**): (1) gene duplication, (2) generation of alternative transcripts from single genes, (3) leaky scanning of start and stop codons, (4) competition between multiple targeting signals (5) partial peroxisomal localization of proteins, since the targeting signal is either modified or inefficient.

DUAL TARGETING OF PEROXISOMAL PROTEINS BY GENE DUPLICATION.

Enzymes that are part of shuttle systems to maintain homeostasis between organelles and the surrounding cytoplasm usually display dual targeting. E.g. redox homeostasis is reached by exchange of reduced small intermediates with their oxidized counterparts.

Table 1 | Overview on peroxisomal proteins with dual localization.

Enzyme	Organism	Mechanism for dual localization[a]	Localization	References
Malate dehydrogenase	*S. cerevisiae*	Gene duplication	Cyt, Mito, Pex	McAlister-Henn and Thompson, 1987; Minard and McAlister-Henn, 1991; Steffan and McAlister-Henn, 1992
NADPH-dependent isocitrate dehydrogenase	*S. cerevisiae*	Gene duplication	Cyt, Mito, Pex	Haselbeck and McAlister-Henn, 1991; Henke et al., 1998; Loftus et al., 1994; van Roermund et al., 1998
Citrate synthase	*S. cerevisiae*	Gene duplication	Mito, Pex	Kim et al., 1986; Lewin et al., 1990; Rosenkrantz et al., 1986
NADPH-dependent isocitrate dehydrogenase	*A. nidulans*	Alternative transcription initiation sites	Cyt, Mito, Pex	Szewczyk et al., 2001
Malate dehydrogenase	*Y. lipolytica*	Differential splicing	Cyt, Pex	Kabran et al., 2012
6-phosphogluconate dehydrogenase	*C. albicans*	Differential splicing	Cyt, Pex	Strijbis et al., 2012
Hydroxypyruvate reductase	Cucurbita sp. (cv. Kurokawa Amakuri Nankin)	Differential splicing	Cyt, Pex	Hayashi et al., 1996
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	*U. maydis*	Differential splicing	Cyt, Pex	Freitag et al., 2012
Phosphoglycerate kinase (PGK)	*A. nidulans*	Differential splicing	Cyt, Pex	Freitag et al., 2012
Phosphoglycerate kinase (PGK)	*U. maydis*	Ribosomal read-through	Cyt, Pex	Freitag et al., 2012
Triose phosphate isomerase (TPI)	*U. maydis*	Ribosomal read-through	Cyt, Pex	Freitag et al., 2012
Iron-containing superoxide dismutase	*L. polyedrum*	Alternative start codons	Plas/Mito, Pex	Bodyl and Mackiewicz, 2007
70-kDa heat shock protein	*Citrullus vulgaris*	Alternative start codons	Pex, Plas	Wimmer et al., 1997
3-Hydroxy-3-methylglutaryl coenzyme A lyase	*H. sapiens* (liver cells)	Multiple targeting signals	Mito, Pex	Ashmarina et al., 1999
Type II NAD(P)H dehydrogenase	*A. thaliana*	Multiple targeting signals	Mito, Pex	Carrie et al., 2008; Xu et al., 2013
Catalase A	*S. cerevisiae*	Multiple targeting signals	Mito, Pex	Petrova et al., 2004
NAD^+-dependent glycerol 3-phosphat dehydrogenase	*S. cerevisiae*	Protein modification (Phosphorylation)	Pex, Cyt/Nuc	Jung et al., 2010
Glucose-6-phosphat dehydrogenase	*A. thaliana*	Redox dependent heterodimerization	Pex, Plas	Meyer et al., 2011
Alanine-glyoxylate aminotransferase	*H. sapiens*	Protein folding	Cyt, Pex	Fodor et al., 2012
Epoxide hydrolase	*H. sapiens* (liver cells, kidney cells)	Level of expression, quarternary structure	Cyt, Pex	Arand et al., 1991; Enayetallah et al., 2006; Luo et al., 2008

[a] *Abbreviations: Cyt, cytosol; Mito, mitochondrion; Nuc, nucleus; Pex, peroxisome; Plas, plastid.*

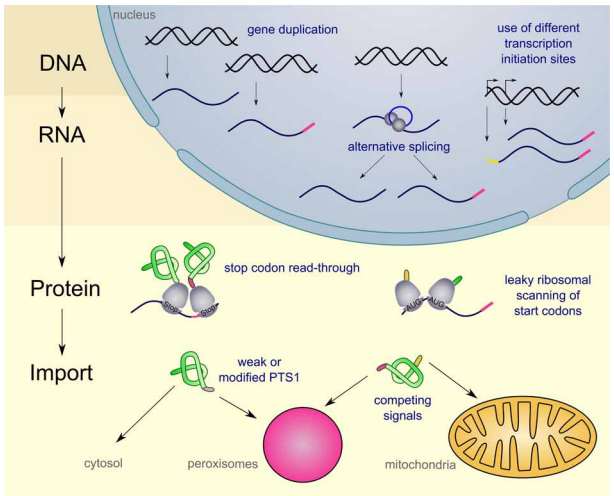

FIGURE 1 | Schematic overview on the diversity of mechanism leading to dual targeting of peroxisomal proteins. Peroxisomal targeting signals are indicated in pink, while mitochondrial signal sequences are indicated in yellow. For further explanations see text.

In mitochondria, recycling of NAD^+/NADH is achieved via a malate/aspartate shuttle system (Bakker et al., 2001). In peroxisomes, regeneration of NAD^+ during β-oxidation also depends on a related malate shuttle since the peroxisomal membrane is permeable for small molecules but impermeable for NADH (van Roermund et al., 1995; Antonenkov et al., 2004).

In *Saccharomyces cerevisiae* three genes encoding malate dehydrogenases have been identified. These code for distinct isoforms, which localize in the cytoplasm, mitochondria and peroxisomes, respectively (McAlister-Henn and Thompson, 1987; Minard and McAlister-Henn, 1991; Steffan and McAlister-Henn, 1992). The different isoenzymes not only carry different targeting signals, but also fulfill distinct functions and show specific regulation. All three malate dehydrogenases are involved in maintaining redox homeostasis. In addition, mitochondrial Mdh1 participates in the tricarboxylic acid cycle, cytosolic Mdh2 is required for gluconeogenesis and peroxisomal Mdh3 is an essential component of the glyoxylate cycle (McAlister-Henn and Thompson, 1987; Minard and McAlister-Henn, 1991; Steffan and McAlister-Henn, 1992)

$NADP^+$/NADPH homeostasis in peroxisomes and mitochondria is reached via an alternative shuttle that involves the exchange of isocitrate with alpha-ketoglutarate. This reaction is catalyzed by NADPH dependent isocitrate dehydrogenase (Idp). Similar to malate dehydrogenase, discrete genes (*idp1*, *idp2* and *idp3*) encode the mitochondrial, cytosolic and peroxisomal isoforms of Idp in *S. cerevisiae* (Haselbeck and McAlister-Henn, 1991; Loftus et al., 1994; Henke et al., 1998; van Roermund et al., 1998). Also, the mitochondrial (Cit1) and peroxisomal isoforms (Cit2) of citrate synthase are encoded by different genes in *S. cerevisiae* (Kim et al., 1986; Rosenkrantz et al., 1986; Lewin et al., 1990).

In yeast this type of multiple localization might be preferred since this species has undergone a large scale genome duplication during its evolution (Kellis et al., 2004). This allowed to attribute different cellular functions and localizations to these paralogous genes. In addition encoding isozymes by separate genes facilitates differential control and regulation not only on the transcriptional level, but also at the level of enzyme kinetics and allosteric control. It has been shown that in *S. cerevisiae*, which only contains a very limited number of introns, protein composition is nearly exclusively regulated by differential transcription (Goffeau et al., 1996).

In contrast, other eukaryotes make extensive use of post-transcriptional processes such as alternative splicing to adapt the proteome to environmental changes (Nilsen and Graveley, 2010).

DUAL LOCALIZATION RESULTING FROM DIFFERENT TRANSCRIPTS DERIVED FROM A SINGLE GENE

In contrast to budding yeast, where gene duplication is commonly used for dual targeting of isozymes, other species often use single genes to produce dually targeted proteins. In *Aspergillus nidulans* synthesis of cytoplasmic, mitochondrial and peroxisomal isoforms of NADP-dependent Idp results from alternative use of transcription initiation sites of the *idpA* gene (Szewczyk et al., 2001). The longer transcript encodes a protein which contains both an N-terminal mitochondrial targeting sequence (MTS) and a C-terminal PTS1. Downstream transcription initiation leads to a shorter *idpA* transcript coding for a protein without the MTS (Szewczyk et al., 2001). While the longer form is preferentially located in mitochondria, the shorter form lacking the MTS is targeted both to peroxisomes and the cytosol. The functional dominance of the N-terminal mitochondrial over the C-terminal peroxisomal targeting signal is most likely due to commitment to mitochondrial import occurring co-translationally before the C-terminus is synthesized (Danpure, 1997).

Differential splicing is another mechanism to generate compartment-specific isoforms from single genes (Yogev and Pines, 2011). In the yeast *Yarrowia lipolytica* the cytoplasmic and peroxisomal isoforms of malate dehydrogenase are generated from alternatively spliced transcripts that differ in their intron size by only four nucleotides. The resulting proteins are highly similar but only one of the Mdh isoenzymes carries a functional PTS1 (Kabran et al., 2012). A related mechanism has been reported for dual targeting of 6-phosphogluconate dehydrogenase (Gnd1) in *Candida albicans*. In this human pathogenic fungus, alternative splicing of *gnd1* transcripts leads to expression of a PTS2 containing isoform (Strijbis et al., 2012). Gnd1 is an essential enzyme of the oxidative branch of the pentose phosphate pathway. This pathway is used to generate NADPH and predominantly resides in the cytosol but has also been found in peroxisomes (Antonenkov, 1989; Corpas et al., 1998; Frederiks and Vreeling-Sindelarova, 2001; Boren et al., 2006; Reumann et al., 2007). Two other enzymes of this pathway, the glucose-6-phosphate dehydrogenase Zwf1 and the 6-phosphogluconolactonase Sol3, have been observed in peroxisomes in *C. albicans* (Strijbis et al., 2012). Differential splicing allows for regulation of dual targeting. In pumpkin leaves the ratio of peroxisomal and cytosolic isoforms of hydroxypyruvate reductase is achieved by light dependent differentially splicing (Hayashi et al., 1996; Mano et al., 1999, 2000).

An unexpected case of dual targeting by alternative splicing was recently described for fungal enzymes involved in glycolysis. This metabolic pathway is considered to be cytoplasmic

and glycolytic proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) often serve as cytoplasmic marker proteins in cell biology. A notable exception are trypanosomes, which have transferred the cytoplasmic glycolytic pathway into peroxisome-derived glycosomes (Opperdoes and Borst, 1977). This is considered as an adaptation to the unique lifestyle of these parasites in the bloodstream of vertebrates (Michels et al., 2006). In the basidiomycetous fungus *Ustilago maydis* a C-terminal extended peroxisomal isoform of GAPDH is expressed from an alternatively spliced transcript (Freitag et al., 2012). Inspection of other fungal species revealed that dual targeting of glycolytic enzymes is widespread. In the ascomycetous fungus *Aspergillus nidulans* the peroxisomal isoform of PGK but not of GAPDH is generated by differential splicing (Freitag et al., 2012).

DUAL LOCALIZATION VIA "LEAKY" START AND STOP CODONS

Further bioinformatic analysis of fungal genes coding for glycolytic enzymes revealed a novel molecular mechanism for dual targeting. Peroxisomal targeting of GAPDH, PGK and triose phosphate isomerase (TPI) is reached by ribosomal read-through of stop codons resulting in a fraction of C-terminally extended proteins ending with a PTS1 (Freitag et al., 2012). Stop codon read-through has been described for retroviral systems where it is used to enlarge protein diversity, but was also observed for cellular transcripts (Bertram et al., 2001; Jungreis et al., 2011). The efficiency of ribosomes to recognize stop codons is affected by sequence context and RNA secondary structure and might be subject of control (Bertram et al., 2001). Isoforms generated by ribosomal read-through correspond to a single transcript and escape detection by transcriptomics. Therefore, this novel mode of dual targeting is difficult to observe.

A related way to produce different isoforms from a single transcript is the use of alternative start codons. The iron-containing SOD of the dinoflagellate *Lingulodinium polyedrum* catalyzes dismutation of superoxide radicals to hydrogen peroxide and oxygen as the first line of defense against reactive oxygen species (ROS) (Bodyl and Mackiewicz, 2007; McCord and Fridovich, 1969). Translation initiation at the first start codon results in an SOD, which contains both an N-terminal targeting signal for plastids and mitochondria and a C-terminal PTS1. This form was suggested to reside in plastids and in mitochondria. Efficient peroxisomal targeting of SOD appears to depend on leaky ribosomal scanning and initiation at a downstream in-frame start codon resulting in an isoform lacking the N-terminal signal sequence (Bodyl and Mackiewicz, 2007). In watermelon cotyledons the mRNA molecules of a 70-kDa heat shock protein contain two in frame start codons. Translational initiation at the first start codon leads to a longer isoform, which carries a N-terminal presequence mediating plastid import, while the shorter isoform localizes to peroxisomes due to a PTS2 (Wimmer et al., 1997).

DUAL TARGETING OF PROTEINS WITH MULTIPLE TARGETING SIGNALS

Although it is commonly assumed that N-terminal signal sequences are dominant over PTS1 (Danpure, 1997), several examples are known where substantial peroxisomal targeting occurs even in the presence of an N-terminal MTS. 3-Hydroxy-3-methylglutaryl coenzyme A lyase (HL) catalyzes the conversion of β-hydroxy-β–methylglutaryl-CoA to acetoacetate, which is important during sterol biosynthesis in mitochondria. In human liver cells HL shows dual localization in mitochondria and peroxisomes. Peroxisomal HL still contains the N-terminal mitochondrial signal sequence, suggesting that dual localization of HL results from an intricate balance between mitochondrial and peroxisomal uptake (Ashmarina et al., 1999).

Another protein with competing signals is type II NAD(P)H dehydrogenase. This enzyme is typically located at the inner mitochondrial membrane but is also found in chloroplasts or peroxisomes (Xu et al., 2013). Three of the seven *Arabidopsis thaliana* genes encoding type II NAD(P)H dehydrogenases (ND) give rise to proteins which are dually targeted both to mitochondria and peroxisomes. These proteins carry an additional C-terminal signal for peroxisomal targeting. Intracellular distribution of the ND proteins with competing signals was shown to depend on the affinity of their signal sequences for their respective receptors/chaperones (Carrie et al., 2008).

If proteins contain competing targeting signals, localization studies with fluorescent proteins may result in ambiguous results. A number of *A. thaliana* acyl-activating enzymes localize either to peroxisomes or to other compartments depending on whether the fluorescent reporter protein was fused at the N- or the C-terminus (Hooks et al., 2012). Therefore it is still unclear whether these proteins occur outside of peroxisomes also in the natural situation.

Competition between the two targeting signals may also be affected by environmental factors. Catalase A (Cta1) of *S. cerevisiae* contains in addition to a non-canonical mitochondrial targeting signal, two peroxisomal targeting signals, an internal signal and a C-terminal PTS1 (Petrova et al., 2004). Both PTSs were shown to be sufficient to target Cta1 to peroxisomes (Kragler et al., 1993). The distribution of Cta1 between peroxisomes and mitochondria is influenced by growth conditions. In the presence of nutrients enhancing H_2O_2 formation, like oleic acid, catalase A is predominantly targeted to peroxisomes. In contrast, cultivation of yeast in raffinose leads to increased mitochondrial localization of Cta1. However, the molecular base for this differential targeting is still obscure (Petrova et al., 2004).

PROTEINS CARRYING REGULATED OR INEFFICIENT PTS

In all examples discussed above, multiple targeting signals are involved in dual localization residing either concomitantly in a single polypeptide or in different isoforms. In the case of proteins that occur both in peroxisomes and the cytoplasm alternative mechanisms may operate. Dual localization can also result from modified or weak PTS1 signals leading to inefficient import into peroxisomes. Partial peroxisomal localization is difficult to visualize with fluorescent marker proteins, since cytoplasmic fluorescence usually prevents detection of the peroxisomal localization. Therefore this type of dual targeting is likely to be missed in microscopic studies. For the similar case of partial mitochondrial targeting a lacZ-complementation assay has been successfully applied to verify dual targeting (Ben-Menachem et al., 2011). Photobleaching of the cytosolic fraction can also be used to visualize partial peroxisomal localization (Buch et al., 2009).

In the NAD^+-dependent glycerol 3-phosphate dehydrogenase (Gpd1) of *S. cerevisiae,* protein modification via phosphorylation is used to interfere with peroxisomal targeting (Jung et al., 2010). Gpd1 catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G3P) to cope with osmotic stress (Merkel et al., 1982; Chen et al., 1987). Gpd1 harbors an N-terminal PTS2, however, the subcellular distribution of Gpd1 depends on environmental factors. Upon osmotic cell stress, Gpd1 is relocated to both the cytosol and the nucleus. This altered localization is triggered by phosphorylation of two serine residues close to the PTS2, thus impairing peroxisomal import (Jung et al., 2010). In *A. thaliana*, peroxisomal import of glucose-6-phosphate dehydrogenase (G6PD1) is triggered by redox signaling and results in relocalization of G6PD1 from chloroplasts to peroxisomes. In this case, formation of a disulfide bridge allows recognition of an internal PTS (Meyer et al., 2011).

A weak/non-canonical PTS1 motif has recently been shown to be critical for proper folding of a PTS1 bearing protein due to prolonged duration of cytosolic localization prior to transfer into peroxisomes (Williams et al., 2012). Especially the import of proteins bearing non-canonical PTS1 motifs may depend on correct protein folding. Even minor misfolding may result in cytosolic localization as was demonstrated for the alanine-glyoxylate aminotransferase (AGT) of humans (Fodor et al., 2012). AGT is known to exhibit a variable distribution in mitochondria and/or peroxisomes in a variety of mammalian species (Danpure, 1997). Similarly, the non-canonical PTS1 motif of human epoxide hydrolase triggers peroxisomal import as a function of concentration and quaternary structure of the protein (Arand et al., 1991; Enayetallah et al., 2006; Luo et al., 2008).

Recent comprehensive studies of the peroxisomal proteome revealed additional proteins that have been previously annotated as cytosolic. Especially in plant peroxisomes, a variety of proteins with unconventional PTS1-motifs has been identified (Reumann et al., 2007; Reumann, 2011). Some of these proteins turned out to reside exclusively in peroxisomes, while others localize in the cytoplasm as determined by microscopy (Reumann et al., 2009). But this does not prove that these proteins are cytosolic since a minor fraction may reside in peroxisomes. At least for one of these proteins, a glutathione reductase, carrying a quite unusual PTS1 (-TNL), partial targeting to peroxisomes was demonstrated (Kataya and Reumann, 2010).

In fungi, proteome studies confirmed the partial peroxisomal localization of glycolytic enzymes and revealed a further candidate, fructose-bisphosphate aldolase (FBA) (Kiel et al., 2009; Managadze et al., 2010). Partial peroxisomal targeting of FBA is presumably mediated by a conserved C-terminal non-canonical PTS1-like motif (Kiel et al., 2009; Freitag et al., 2012). In *U. maydis*, this motif is able to trigger complete peroxisomal import if fused as a dodecamer to GFP, while a full-length GFP-FBA fusion protein results in cytoplasmic fluorescence. This suggests that partial peroxisomal import requires additional features of the protein that interfere with recognition of the unconventional PTS1. The combination of bioinformatic and experimental strategies revealed a heterogeneity of functional PTS1 motifs both in plants and fungi (Reumann, 2011; Freitag et al., 2012). Taken together these data indicate that partial peroxisomal targeting may occur more often than previously assumed. It has even been suggested that all cytosolic proteins may be found in any organelle at least in tiny amounts probably due to mistargeting. This hypothesis was proposed to explain the transfer of whole metabolic pathways from one compartment to another during evolution e.g. that of glycolysis in trypanosomes (Martin, 2010).

CONCLUDING REMARKS

The large variety of mechanisms leading to dual targeting of peroxisomal proteins (summarized in **Figure 1**) suggests that the metabolic capacity of peroxisomes might have been underestimated in the past. This idea is supported by the recent discovery of several hitherto unrecognized peroxisomal metabolic pathways. These include glycolysis in fungal peroxisomes, biotin synthesis in plants and fungi, as well as biosynthesis of secondary metabolites such as siderophores and antibiotics (Bartoszewska et al., 2011; Magliano et al., 2011; Tanabe et al., 2011; Freitag et al., 2012; Grundlinger et al., 2013). Especially for mammals, knowledge of the role of peroxisomes appears to be far from being complete (Schrader and Fahimi, 2008; Islinger et al., 2012). We envision that also in mammals the metabolic capacity of peroxisomes may be of greater variability with dual targeting playing a growing role.

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Permeability of the peroxisomal membrane: lessons from the glyoxylate cycle

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Glyoxylate serves as intermediate in various metabolic pathways, although high concentrations of this metabolite are toxic to the cell. In many organisms glyoxylate is fed into the glyoxylate cycle. Enzymes participating in this metabolism are located on both sides of the peroxisomal membrane. The permeability of this membrane for small metabolites paves the way for exchange of intermediates between proteins catalyzing consecutive reactions. A model, in which soluble enzymes accumulate in close proximity to both ends of pore-like structures forming a transmembrane metabolon could explain the rapid and targeted exchange of intermediates. The metabolites passing the membrane differ between the three model organisms *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Candida albicans*, which reflects the ease of evolutionary adaptation processes whenever specific transporter proteins are not involved. The atypical permeability properties of the peroxisomal membrane together with a flexible structural arrangement ensuring the swift and selective transport across the membrane might represent the molecular basis for the functional versatility of peroxisomes.

Keywords: glyoxylate, glyoxylate cycle, peroxisomes, pore forming protein, membrane permeability, metabolite transfer, metabolon, photorespiration

INTRODUCTION

Peroxisomes are defined as organelles encasing the metabolism of H_2O_2. This highly reactive molecule is generated by several oxidative reactions and degraded inside the organelle by abundant amounts of catalase. In addition, peroxisomes fulfill a number of important metabolic functions for eukaryotic cells requiring an active communication between the peroxisomal lumen and the cytosol or other organelles. Major functions include the β-oxidation of fatty acids, parts of the glyoxylate cycle and parts of the photorespiration (Hu et al., 2012; Waterham and Wanders, 2012). For β-oxidation fatty acids have to enter peroxisomes, and the resulting acetyl-CoA is distributed throughout the cells. Photorespiration in plants involves mitochondria, chloroplasts and peroxisomes and therefore requires extensive metabolite exchange. A key intermediate shared by the photorespiration and the glyoxylate cycle is glyoxylate, usually generated and metabolized inside peroxisomes. The glyoxylate cycle was originally considered a metabolic process localized to peroxisomes (Breidenbach and Beevers, 1967). This localization was rationalized as a means to increase the efficiency of the flux of intermediates. However, the finding that parts of the whole cycle are extra-peroxisomal in the yeast *Saccharomcyes cerevisiae* and in the plant *Arabidopsis thaliana* indicated, that a functional glyoxylate cycle requires the transfer of various metabolites across the peroxisomal membrane, too (Minard and McAlister-Henn, 1991; Courtois-Verniquet and Douce, 1993; Taylor et al., 1996; Kunze et al., 2002; Pracharoenwattana et al., 2007). The nature of the molecules crossing the peroxisomal membrane varies with the organism under study.

The single membrane separating the peroxisomal matrix from the surrounding was shown to be permeable only for small molecules such as tri- and dicarboxylates and amino acids (Antonenkov et al., 2009). Specific transporters have not yet been identified but features compatible with pore-like structures were demonstrated (Verleur and Wanders, 1993; Antonenkov and Hiltunen, 2006).

In this review we will address the questions how and where glyoxylate is generated and how metabolites of the two most prominent pathways partially localized to peroxisomes, the glyoxylate cycle and the photorespiration, are thought to cross the membrane on their way in and out of the peroxisomes.

PEROXISOMES AND GLYOXYLATE METABOLISM

Inside peroxisomes the accumulation of glyoxylate is prevented by conversion into glycine making use of a transaminase reaction or by condensation with acetyl-CoA into malate catalyzed by malate synthase (MLS), one of the key enzymes of the glyoxylate pathway (for reviews see Wanders and Waterham, 2006; Theodoulou and Eastmond, 2012). Glyoxylate can be produced from different precursor molecules (**Figure 1A**). Cleavage of isocitrate generates glyoxylate and succinate in the glyoxylate cycle (**Figure 1B**). The oxidation of glycolate to glyoxylate is catalyzed by glycolate oxidase being part of the photorespiratory process generating H_2O_2 within peroxisomes (**Figure 1C**). Another possible source of glyoxylate is the degradation of purines in those organisms metabolizing the intermediate uric acid (**Figure 1D**). Moreover, in mammals glyoxylate may represent a degradation product of hydroxyproline originally derived from collagen (Salido et al., 2012) or alternatively, may be the result of a conversion from

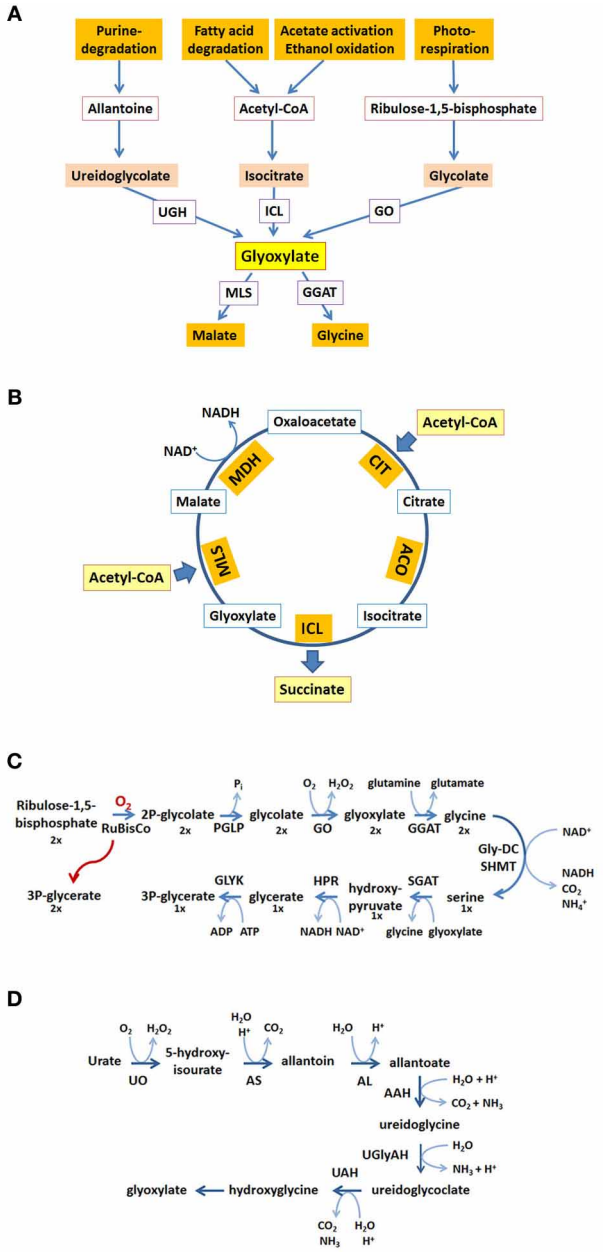

FIGURE 1 | Glyoxylate generation and consumption. (A) Glyoxylate is generated from different precursor molecules and converted into stable metabolites for further utilization. The proteins involved are ureidoglycolate hydrolase (UGH), isocitrate lyase (ICL), glycolate oxidase (GO), malate synthase (MLS), glutamine:glyoxylate aminotransferase (GGAT). **(B)** In the glyoxylate cycle two acetyl-CoA are condensed to succinate and 2 CoA (not shown). The proteins involved are citrate synthase (CIT), aconitase (ACO), isocitrate lyase (ICL), malate synthase (MLS), malate dehydrogenase (MDH). **(C)** The generation and consumption of glyoxylate in the photorespiratory process. The stoichiometry of the reaction sequence to obtain three molecules of 3-phospho-glycerate from two oxygenation products of ribulose-1,5-bisphosphate is indicated by the numbers below the molecules (1×, 2×). The proteins involved are ribulose-bisphosphate-carboxylase-oxygenase (RuBisCO), phosphoglycolate phosphatase (PGLP), glycolate oxidase (GO), glutamine:glyoxylate aminotransferase (GGAT), glycine-decarboxylase (GlyDC),

(Continued)

FIGURE 1 | Continued
serine-hydroxymethyltransferase (SHMT), serine:glyoxylate aminotransferase (SGAT), hydroxypyruvate reductase (HPR), glycerate kinase (GLYK). **(D)** The reactions in the purine degradation pathway leading to glyoxylate. The proteins involved are urate oxidase (UO), allantoin synthase (HIU-hydrolase + OHCU decarboxylase) (AS), allantoinase (alantoin amidohydrolase) (AL), allantoate amidohydrolase (AAH), ureidoglycine aminohydrolase (UGlyAH), ureidoglycolate amidohydrolase (UAH).

glycine catalyzed by D-amino acid oxidase generating H_2O_2 (Ohide et al., 2011).

The glyoxylate cycle allows the formation of 4-carbon units from 2-carbon units (**Figure 1B**). The resulting succinate serves to replenish the tricarboxylic acid (TCA) cycle representing the major collector and distributor of small carbon units. Alternatively, succinate or its follow-up product oxaloacetate serves as precursor for many biosynthetic processes. This biosynthetic pathway is absent in all animals except nematodes (Kondrashov et al., 2006). Acetyl-CoA fed into the glyoxylate cycle can be derived from different sources, such as β-oxidation of fatty acids, degradation of amino acids or in case of microbial organisms from external carbon sources such as ethanol or acetate. The glyoxylate cycle shares a series of three enzymatic activities with the TCA-cycle, namely malate dehydrogenase (MDH), citrate synthase (CIT), and aconitase (ACO) activity. The two unique activities, isocitrate lyase (ICL) and malate synthase (MLS) generate and consume the name-giving metabolite glyoxylate. The cleavage of isocitrate bypasses the decarboxylation reactions and the synthase reaction leads to the net-condensation of acetyl-CoA units. In many organisms these activities can be carried out by two or more isoenzymes with different localization signals and different expression patterns catalyzing the respective reactions.

Photorespiration is required in all photosynthetic organisms and serves as carbon recovery system (Maurino and Peterhansel, 2010). Oxygenation of ribulose-1,5-bisphosphate by ribulose-bisphosphate-carboxylase-oxygenase (RuBisCO) competes with CO_2 fixation upon high partial oxygen pressure leading to the formation of 2-phosphoglycolate and 3-phosphoglycerate in the chloroplast (**Figure 1C**). 3-phosphoglycerate is channeled into the Calvin cycle and 2-phosphoglycolate is recycled into 3-phosphoglycerate in a series of reactions involving peroxisomes and mitochondria. In peroxisomes glycolate is oxidized to glyoxylate generating H_2O_2, which is immediately decomposed by catalase. Glyoxylate is transaminated to glycine using either serine or glutamine as nitrogen source and further transported to the mitochondria. Here, two glycines are converted into one serine which is now returned into peroxisomes. The peroxisomal serine:glyoxylate aminotransferase and hydroxypyruvate reductase convert serine into glycerate which is transferred to chloroplasts, where it enters the Calvin cycle in the form of 3-phosphoglycerate. In summary, two glycolate molecules are consumed to produce glycerate and CO_2 and the generation of two H_2O_2 is confined to peroxisomes.

The degradation pathway of purines leads to uric acid, which in many organisms is further metabolized within peroxisomes to allantoine and allantoic acid (**Figure 1D**). In some animals

the allantoicase activity leads to the cleavage of allantoic acid into urea and glyoxylate (Hayashi et al., 2000). In plants, some fungi and bacteria allantoic acid is further processed within the endoplasmic reticulum into ureidoglycine and ureidoglycolate, both giving rise to glyoxylate upon degradation (Werner and Witte, 2011). The glyoxylate derived from purine degradation is supposedly converted into glycine or condensed with acetyl-CoA catalyzed by a MLS activity providing the versatile metabolite malate. In essence, the heterocyclic core structure is converted into a nitrogen containing product and glyoxylate. The latter is recycled into biosynthetic processes in all organisms, the nitrogen containing product is either excreted (animals) or recycled (plants).

ENZYMES ALLOCATED TO THE GLYOXYLATE CYCLE

Due to the fact that three of the five enzymatic activities contributing to the glyoxylate cycle are shared with the TCA cycle and additional enzymatic activities have been described an assignment of individual proteins to the glyoxylate cycle is not trivial. Some of the encoded proteins can be excluded due to an inappropriate expression pattern, but for some of the proteins only the phenotype of cells lacking the respective activity is a convincing argument for the function in the glyoxylate cycle. As the situation in the yeast *S. cerevisiae* has been analysed in detail previously (Kunze et al., 2006), we will only summarize the most important information and then concentrate on more recent results obtained in the thale cress *A. thaliana* as the plant model organism and in the human opportunistic fungus *Candida albicans*. The corresponding proteins are listed in **Table 1**.

In the genome of *S. cerevisiae* the key enzymes ICL and MLS are encoded by two genes each. Based on the high expression levels when cells grow on ethanol, acetate or fatty acids and the inability of cells lacking these proteins to grow on these carbon sources Icl1p and Mls1p have been attributed to the glyoxylate cycle (Fernandez et al., 1992; Hartig et al., 1992). The two others, Icl2p and Dal7p contribute to the mitochondrial propionate metabolism and the purine degradation, respectively (Hartig et al., 1992; Luttik et al., 2000). From the three genes coding for MDH, a mitochondrial (Mdh1p; McAlister-Henn and Thompson, 1987), a cytosolic (Mdh2p; Minard and McAlister-Henn, 1991), and a peroxisomal (Mdh3p; Steffan and McAlister-Henn, 1992) gene product are derived. Only the cytosolic and the mitochondrial variants are expressed when cells grow on C_2-carbon sources (ethanol/acetate) and the cytosolic enzyme was required for growth under these conditions (Minard and McAlister-Henn, 1991; McCammon, 1996). This experimental evidence indicates that the cytosolic Mdh2p participates in the glyoxylate cycle.

Similarly, among the three CIT proteins encoded within the yeast genome only Cit2p is considered to contribute to the glyoxylate cycle, because of its expression pattern on various carbon sources and because of the mitochondrial location of the other two enzymes Cit1p and Cit3p. Interestingly, Cit2p is essential for growth on ethanol or acetate only, when an alternative route probably involving one of the two mitochondrial activities is blocked (Van Roermund et al., 1995). Finally, one gene encoding ACO (ACO1; Gangloff et al., 1990) gives rise to the cytosolic and the mitochondrial activity (Regev-Rudzki et al., 2005) attributing Aco1p to the TCA- and the glyoxylate cycle, whereas Aco2p may be important for fermentation (Van den Berg et al., 1998).

Even more complex is the situation in *A. thaliana*. Although only one gene codes for ICL (Eastmond et al., 2000) and one for MLS (Cornah et al., 2004), eight genes encode NAD^+ dependent MDHs, five genes code for CITs and three genes code for ACOs (summarized in **Table 1**). The localization, expression pattern and phenotype of the corresponding T-insertion mutants may help to discern the respective function of each individual protein. Nevertheless, the attribution of a function in the glyoxylate cycle appears difficult. Three of the five CITs, CYS1-3 harbor peroxisomal targeting information whereas CYS4 and CYS5 include mitochondrial leader peptides (Pracharoenwattana et al., 2005). Similarly, among the MDH proteins two are peroxisomal, two mitochondrial, one is observed in chloroplasts and three in the cytosol (Pracharoenwattana et al., 2007) [The Arabidopsis Information Resource (TAIR), http://www.arabidopsis.org/, Huala et al., 2001].

The expression pattern of enzymes contributing to the glyoxylate cycle is very characteristic, namely high expression after imbibition during seed germination followed by strong reduction upon postgerminative growth. Isoenzymes that are not expressed in the phase of germination can be excluded from a contribution to the glyoxylate cycle, such as CYS1 (Pracharoenwattana et al., 2005). During this developmental phase both, the β-oxidation of fatty acids and the glyoxylate cycle are equally needed and therefore the expression profiles of the corresponding genes are similar (Eastmond and Graham, 2001; Rylott et al., 2001). A detailed and precise cluster analysis of expression profiles during seedling development in soybean remained inconclusive since the expression pattern of the two key enzymes ICL and MLS did not fall into the same cluster and expression differences of the isoforms of CIT and MDH were not distinguishable (Gonzalez and Vodkin, 2007).

An unambiguous attribution of a function in the glyoxylate cycle to a distinct protein could be based on the characteristics of cells deficient in the glyoxylate cycle function. *A. thaliana* seeds lacking ICL are able to germinate, but their seedling establishment is severely impaired in the absence of light or carbohydrates offered as alternative carbon source (Eastmond et al., 2000). In other words, gluconeogenesis is compromised but lipid respiration is still active in these mutants. *A. thaliana* seeds lacking MLS, the other unique enzyme of the glyoxylate cycle, display a similar defect in seedling establishment in the absence of light or carbohydrates. Regarding the compensation of the establishment defect by light the seeds respond differently. In the absence of MLS activity a lower light dose is required to promote seedling establishment than in the absence of ICL activity (Cornah et al., 2004). The latter phenotype led to the suggestion that in the absence of MLS glyoxylate, which is produced by ICL can enter gluconeogenesis by hijacking enzyme activities from the photorespiratory pathway.

Phenotypes of plants lacking various isoforms of either CYS or MDH are less revealing to discern a specific function. During seedling establishment both peroxisomal MDHs (pMDH1 and pMDH2) and peroxisomal CITs (CYS2 and CYS3) are expressed.

Seeds lacking either one of the peroxisomal activities present with disturbed fatty acid degradation (Pracharoenwattana et al., 2005, 2007). Thus, a contribution of these proteins to the glyoxylate cycle cannot be delineated from the block in seedling establishment obtained in these mutant plants, because the lack of fatty acid degradation elicits a similar block in seedling establishment. Conversely, no noticeable defect in establishment was observed for seedlings from plants lacking mitochondrial MDH excluding an essential role of these MDHs in either β-oxidation of fatty acids or the glyoxylate cycle (Tomaz et al., 2010). When the metabolic distribution of exogenously added acetate within seedlings is used as indicator for the functionality of the glyoxylate cycle, no differences between seedlings lacking the peroxisomal MDH and wild type seedlings could be observed (Pracharoenwattana et al., 2007). However, this assay shows differences in the re-routing of acetate between seedlings from wild-type plants and plants, which are blocked in the glyoxylate cycle due to lack of ICL or MLS, although the differences appear small in the absence of MLS (Cornah et al., 2004). Experiments to discern the individual roles of the three cytosolic MDH were not yet carried out. Similarly, among the three ACO proteins a function in the glyoxylate cycle has not been assigned to anyone of them (Peyret et al., 1995; Arnaud et al., 2007). Altogether, *in A. thaliana* the assignment of individual ACOs, CITs, and MDHs to the glyoxylate cycle remains open.

The situation in the yeast *C. albicans* is different. Each one of the enzymes ICL, MLS, and CIT is encoded by a single gene (Piekarska et al., 2008). In this organism the same enzyme Cit1p seems to contribute to the mitochondrial TCA-cycle and the glyoxylate cycle such as demonstrated for *Candida tropicalis* (Ueda et al., 1997). However, it cannot be excluded that a cytosolic form is derived from alternative translation as two variants are described (C4YLG7 and Q59ZZ5), but none of the described variants contains a peroxisomal targeting signal type 1 (PTS1; C-terminus KYIELVKNINKA). ACO activity is encoded by two genes, MDH activity by three genes (Jones et al., 2004). In each case one of the variants has a closer similarity to the respective glyoxylate cycle enzyme in *S. cerevisiae*, but experimental evidence for their role is missing.

SUBCELLULAR LOCALIZATION OF THE ENZYMES OF THE GLYOXYLATE CYCLE

In *S. cerevisiae* the enzymes contributing to the glyoxylate cycle are distributed between the peroxisomal matrix and the cytosol.

Table 1 | Enzymatic activities required for the glyoxylate cycle.

Enzyme	*Saccharomyces cerevisiae*			*Candida albicans*			*Arabidopsis thaliana*		
	Protein	**Gene**	**Targeting information**	**Protein**	**Gene**	**Targeting information**	**Protein**	**Gene**	**Targeting information**
Isocitrate lyase	**ICL1**	YER065C	Cytosolic	ICL1	CaO19.14134	Peroxisomal	ICL	At3g21720	Peroxisomal
	ICL2	YPR006C	Mitochondrial						
Malate synthase	**MLS1**	YNL117W	Peroxisomal	MLS1	CaO19.12296	Peroxisomal	MLS	At5g03860	Peroxisomal
	DAL7	YIR031C	PTS1						
Malate dehydrogenase	MDH1	YKL085W	Mitochondrial	MDH1	CaO19.12072	MITO	pMDH1	At2g22780	Peroxisomal
	MDH2	YOL126C	Cytosolic	MDH2	CaO19.7481		pMDH2/MDHG1	At5g09660	Peroxisomal
	MDH3	YDL078C	Peroxisomal	MDH3	CaO19.1278	PTS1	mDH3/MDHM1	At1g53240	Mitochondrial
							mDH4/MDHM2	At3g15020	Mitochondrial
							MDH5/MDHC1	At1g04410	
							MDH6/MDHC2	At5g43330	
							MDH7/MDHC3	AT5G56720	
							cMDH8/MDHP1	At3g47520	Chloroplast
Citrate synthase	CIT1	YNR001C	Mitochondrial	CIT1	CaO19.11871	Mitochondrial	CSY1	At3g58740	PTS2
	CIT2	YCR005C	Peroxisomal		Q59ZZ5		CSY2	At3g58750	Peroxisomal
	CIT3	YPR001W	Mitochondrial				CSY3	At2g42790	Peroxisomal
							CSY4	At2g44350	Mitochondrial
							CSY5	At3g60100	Mitochondrial
Aconitase	**ACO1**	YLR304C	Mitochondrial	ACO1	CaO19.13742	MITO	ACO1	At4g35830	Mitochondrial
	ACO2	YJL200C	Mitochondrial	ACO2	CaOrf19.6632	MITO	ACO2	At4g26970	Mitochondrial
							ACO3	At2g05710	Mitochondrial

Proteins, corresponding genes, accession number, and targeting information are shown for enzymes carrying out activities required for the glyoxylate cycle in S. cerevisiae, C. albicans, and A. thaliana. PTS and MITO indicates predicted targeting information (general prediction: http://wolfpsort.org/, PTS1-predictor: http://mendel.imp.ac.at/pts1/, Mito-predictor: http://ihg.gsf.de/ihg/mitoprot.html). Cytosolic, peroxisomal, mitochondrial, and chloroplast indicates experimentally verified localization. Bold underlined are proteins experimentally proven to contribute to the glyoxylate cycle.

Icl1p, Mdh2p, and Aco1p were described in the cytosol (Minard and McAlister-Henn, 1991; Taylor et al., 1996; Regev-Rudzki et al., 2005), and Cit2p was found in the peroxisomal fraction (Lewin et al., 1990). Mls1p is either targeted to peroxisomes when cells utilize oleic acid or distributed across the cytosol when cells utilize ethanol (McCammon et al., 1990; Kunze et al., 2002). Upon growth on ethanol or acetate as sole carbon source with all other glyoxylate cycle enzymes in the cytosol the extra-peroxisomal fraction of Cit2p might suffice to generate four-carbon units through the glyoxylate cycle.

In *A. thaliana* the key enzymes ICL and MLS are considered peroxisomal containing a C-terminal PTS1 [EGTSLVVAKSRM for ICL and IVAHYPINVSRL for MLS, Arabidopsis subcellular database (SUBA), (Heazlewood et al., 2007)]. In contrast, ACOs lack peroxisomal targeting information and were allocated to the cytosol and the mitochondria (Arnaud et al., 2007). The extra-peroxisomal location of ACO activity was confirmed in castor bean and potato (Courtois-Verniquet and Douce, 1993). Which of the other gene products, namely of MDH and of CIT, are contributing to the glyoxylate cycle is unclear. Among the MDHs it can be assumed that one or more of the three cytosolic isoforms participates in the glyoxylate cycle as mutant plants lacking either the peroxisomal (Pracharoenwattana et al., 2007) or the mitochondrial (Tomaz et al., 2010) isoenzymes show no signs of a glyoxylate cycle defect.

In *C. albicans* both key enzymes of the glyoxylate cycle, ICL and MLS were found in peroxisomes irrespective of the carbon source used (Piekarska et al., 2008), rendering this microorganism an alternative yeast model with key enzyme distribution similar to *A. thaliana*. However, the exclusive presence of one citrate synthase gene (CIT1) and the necessity of a peroxisomal and mitochondrial shuttle mechanism for acetyl-CoA for growth on oleic acid indicates that peroxisomal CIT is not available, but a cytosolic isoform might well be. The similarity of the MDH proteins to the homologs from *S. cerevisiae* suggests that the cytosolic Mdh2p contributes to the glyoxylate cycle as well. Finally, the cytosolic ACO Aco1p (Jones et al., 2004) might be supplemented by a minor peroxisomal subfraction that is caused by a weak PTS1 [HGSALNFIKSKY, http://mendel.imp.ac.at/pts1/, Neuberger et al., 2003].

Briefly summarized, the localization of proteins participating in the glyoxylate cycle on different sides of the peroxisomal membrane in all three model organisms requires the transport of intermediates across the lipid barrier.

TRANSFER OF METABOLITES

Usually, the concentration of free intermediates occurring in metabolic pathways is relatively low, because a local accumulation of participating enzymes, e.g., within an organelle, or a physical interaction between successive enzymes allows a channeling of intermediates. In extreme cases, large protein complexes such as the fatty acid synthase transfer small molecules from one active center to the next. If a membrane separates consecutive steps an efficient metabolic pathway requires a direct shuttling of intermediates either through a transporter protein or through a proteinaceous channel linking enzymes on both sides of the membrane. Alternatively, a comparably high net concentration of intermediates might facilitate diffusion controlled transfer, which appears unlikely for highly reactive compounds such as glyoxylate.

PERMEABILITY OF THE PEROXISOMAL MEMBRANE

To seal certain reactions in a compartment and to restrict the generation of reactive molecules cells seem to spare no effort to translocate the corresponding proteins and metabolic precursors into distinct compartments. The localization of various oxidases to peroxisomes is a typical example for an energy spending activity preventing contamination of the cytosol by H_2O_2. In turn, the membrane delimiting a compartment thought to protect the cellular interior from detrimental effects by small, highly reactive molecules could be expected to be impermeable for such substances. However, early studies provided evidence for a permeability of the peroxisomal membrane for small solutes such as urate or amino acids and for density gradient material such as sucrose (De Duve and Baudhuin, 1966; Van Veldhoven et al., 1987). For cofactors and larger substrates required for peroxisomal enzymes such as NAD or acetyl-CoA the peroxisomal membrane was shown to act as barrier similar to the inner mitochondrial membrane (Van Roermund et al., 1995). Shuttle systems were proposed to functionally connect the peroxisomal lumen with the cytosol exchanging substrates and keeping the cofactors in the reduced or in the oxidized state as required (Elgersma et al., 1995; Van Roermund et al., 1995; Antonenkov and Hiltunen, 2006; Visser et al., 2007).

A concept for the peroxisomal membrane permeability reconciling conflicting data was put forward by Hiltunen and coworkers (Antonenkov et al., 2004a,b). Pore-like structures permit the free exchange of small solutes (MW < 300D) across an otherwise impermeable membrane inhibiting the transfer of molecules such as acetyl-CoA, ATP, or NAD. Accordingly, pore-forming activities were reported in peroxisomes isolated from plants, mammalian tissue and yeast (Reumann et al., 1995; Antonenkov et al., 2005; Grunau et al., 2009), but the molecular nature of the channel proteins remained largely unknown. In contrast, specific transporters for adenine nucleotides and NAD were identified corroborating the impermeability of the peroxisomal membrane for such bulky molecules (Palmieri et al., 2001; Bernhardt et al., 2012; for reviews see Antonenkov and Hiltunen, 2012; Hu et al., 2012).

The localization of enzymatic activities of the glyoxylate cycle on different sides of the peroxisomal membrane requires an efficient transport of intermediates across the lipid barrier (Kunze et al., 2006). When acetyl-CoA is generated inside the peroxisomal matrix—usually via β-oxidation of fatty acids—it remains confined to peroxisomes probably because of its size (Van Roermund et al., 1995; Antonenkov and Hiltunen, 2006). CIT catalyzes the condensation of acetyl-CoA with oxaloacetate into citrate, which in turn is exported and serves as substrate for extra-peroxisomal ACO. The resulting isocitrate is imported into peroxisomes in those organisms in which the corresponding cleavage activity, ICL resides inside peroxisomes (*A. thaliana, C. albicans*). Thereby, succinate, the net product of the cycle is released within peroxisomes and requires an additional export mechanism. Conversely, glyoxylate is directly handed over to the second acetyl-CoA

consuming enzyme, MLS, which is a peroxisomal constituent in all organisms.

Some organisms such as *S. cerevisiae* do not harbor a peroxisomal ICL (Taylor et al., 1996), but instead generate the products of this reaction in the cytosol. The disadvantage that a small reactive molecule such as glyoxylate needs to be translocated across a membrane to reach MLS might be balanced by the advantage that succinate is already in the cytosol, which is one step closer to its usual final destination, mitochondria. The interesting observation that the relevant MDH activity for the glyoxylate cycle is exerted by cytosolic isoforms in the yeast *S. cerevisiae* and supposedly also in *A. thaliana* calls for an additional export of malate and the subsequent import of oxaloacetate to close the cycle (Minard and McAlister-Henn, 1991; Pracharoenwattana et al., 2007).

All in all a series of transport steps is required to complete a full round of the glyoxylate cycle. If ICL is extra-peroxisomal, e.g., in the yeast *S. cerevisiae*, citrate and malate are exported, and glyoxylate and oxaloacetate are imported (**Figure 2A**). If ICL resides inside peroxisomes citrate, malate and succinate are exported, whereas isocitrate and oxaloacetate are imported (**Figure 2B**), a situation occurring in the plant *A. thaliana*. A similar flux of intermediates occurs in *C. albicans*, although citrate is generated in the cytosol and does not occur in peroxisomes (**Figure 2C**).

The export of malate and import of oxaloacetate as part of the glyoxylate cycle mimics a redox-shuttle system consisting of a peroxisomal and a cytosolic MDH activity that mediates a net-export of reduction equivalents generated by the β-oxidation of fatty acids to the cytosol. Thus, peroxisomal malate generated by MLS might be fueled into the same path to the cytosol as malate generated by the re-oxidation of NADH (peroxisomal *Sc*Mdh3p or *At*pMDH1/pMDH2). Similarly, the transport of isocitrate might be part of a shuttle mechanism coupling the import of isocitrate and the export of α-oxo-glutarate with the reduction of $NADP^+$ inside peroxisomes. Such a hypothetical net-exchange is based on a cytosolic isocitrate dehydrogenase (*Sc*Idp2p) and a peroxisomal isoform (*Sc*Idp3p) converting isocitrate into α-oxo-glutarate and CO_2 and *vice versa*.

The export of citrate is not restricted to the glyoxylate cycle but also presents a mode of export for C_2-units that are generated as acetyl-CoA by fatty acid β-oxidation within peroxisomes. In this case the export of citrate is ultimately balanced by the import of oxaloacetate, the precursor for CIT. The export of citrate is considered the only export pathway for C_2-units in plants since lack of the peroxisomal CITs, CYS2 and CYS3, blocks β-oxidation of fatty acids in *A. thaliana* (Pracharoenwattana et al., 2005), whereas in the yeast *S. cerevisiae* an additional export system for acetyl-units exists. For the latter, a peroxisomal form of carnitine acetyl-transferase (Cat2p; Elgersma et al., 1995) generates acetyl-carnitine that can be translocated across the membrane and rebuilt into acetyl-CoA either in the cytosol or within mitochondria employing either one of three carnitine acetyltransferases, Cat2p, Yat1p, or Yat2p (Swiegers et al., 2001). In *S. cerevisiae* the β-oxidation of fatty acids requires only one mode of export and thus the lack of either the peroxisomal CIT Cit2p or the carnitine acetyltransferase Cat2p localized to the peroxisomes and the mitochondria is tolerated for growth on oleic acid as carbon source, but upon deletion of both genes (ΔCIT2ΔCAT2)

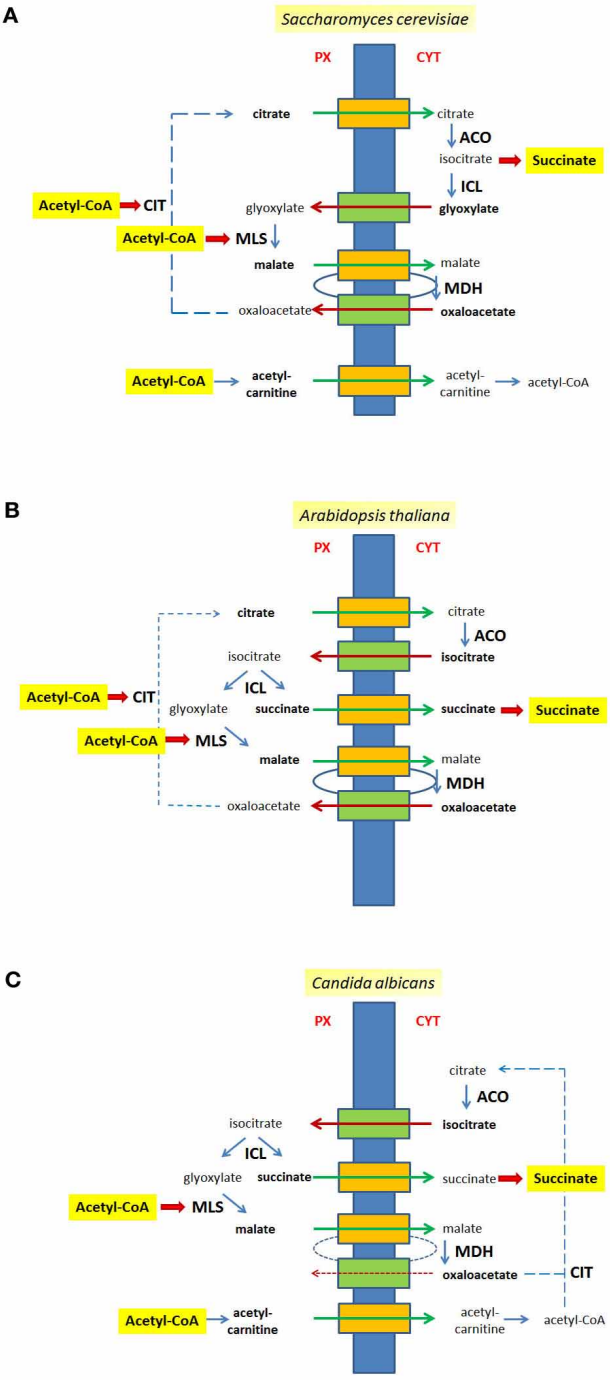

FIGURE 2 | Metabolites of the glyoxylate cycle crossing the peroxisomal membrane. The peroxisomal membrane facilitates the transfer of small metabolites. The transport of glyoxylate cycle intermediates and of C_2-units is shown for *S. cerevisiae* **(A)**, *A. thaliana* **(B)**, and *C. albicans* **(C)**. Hypothetical pore-forming proteins permitting the export of intermediates are colored orange, hypothetical pore-forming proteins permitting the import of intermediates are colored green. Broken lines are drawn to close the glyoxyate cycle. Intermediates that cross the peroxisomal membrane are indicated bold. *Px*, peroxisomal side of the membrane, *Cyt*, cytosolic side of the membrane.

cells were not able to utilize oleate (Van Roermund et al., 1999). Moreover, under these conditions the cytosolic re-conversion of acetyl-carnitine into acetyl-CoA is also essential, as mutant cells lacking CIT2 and either one of the other carnitine acetyltransferases (YAT1 or YAT2) were unable to consume oleic acid (Swiegers et al., 2001). In *C. albicans* a peroxisomal condensation of acetyl-CoA with oxaloacetate producing citrate is not possible due to the lack of a peroxisomal CIT. Therefore, the export of acetyl-units depends on the carnitine form and consequently, in the absence of the peroxisomal isoform of Cat2p cells were unable to grow on oleic acid (Strijbis et al., 2010).

The transfer of the various small intermediates is compatible either with specific transporter proteins or with atypical permeability properties of the membrane. The mitochondrial and chloroplast membranes contain numerous transporter proteins specific for small organic compounds, but corresponding proteins were not identified in peroxisomes. Few examples may illustrate this. In mitochondria of *S. cerevisiae* a carnitine acylcarnitine carrier protein Crc1p and a citrate-oxoglutarate carrier were identified, but no peroxisomal paralog was discovered yet (Van Roermund et al., 1999; Castegna et al., 2010). Similarly, a succinate-fumarate transporter was identified in the inner mitochondrial membranes of *S. cerevisiae* and *A. thaliana* [Sfc1p/Acr1p (Bojunga et al., 1998) and AtMSFC1 (Catoni et al., 2003), respectively], but no homologous protein was identified to mediate plant peroxisomal succinate export. Furthermore, a glycolate/glycerate transporter required for photorespiration (PLGG1) has been identified in the chloroplast membrane (Pick et al., 2013), but no homologous proteins were found in plant peroxisomes.

Despite overwhelming evidence for the free exchange of small solutes the molecular nature of pore-forming activities in plant and yeast peroxisomes remained unknown (Reumann et al., 1997, 1998; Grunau et al., 2009). Moreover, the yeast peroxisomal membrane pores were shown to conduct solutes of the glyoxylate cycle (Antonenkov et al., 2009; Grunau et al., 2009). So far, only in mammalian peroxisomes a protein, Pxmp2, was identified that exhibits channel-forming capacities (Rokka et al., 2009).

Provided that the transport of intermediates is essential for the metabolic activity, the lack of such pores should result in non-functional peroxisomes. Therefore, the corresponding genes should have turned up in various genetic screens searching for mutants with dysfunctional peroxisomes. However, more than one gene could encode redundant functions escaping the detection of these genes in screens. Likewise, such porins should be rather abundant constituents of the peroxisomal membrane and as such should have been identified in various proteomic approaches. The most abundant yeast peroxisomal membrane protein reported is Pex11p, but its localization at the outer surface renders a function in solute transport rather unlikely (Erdmann and Blobel, 1995; Van Roermund et al., 2000; Opalinski et al., 2011).

BIPARTITE ENZYME DISTRIBUTION OF GLYOXYLATE CYCLE ENZYMES: SUBCELLULAR DISTRIBUTION FOLLOWS GENERATION OF SUBSTRATES

The hypothesis that substrate availability was the driving force for changes in enzyme localization, has been proposed based on observations in the yeast *S. cerevisiae*. In this organism Mls1p localization differs between cells grown in medium containing ethanol as sole carbon source, when acetyl-CoA is generated primarily in the cytosol, and cells utilizing oleic acid generating acetyl-CoA within peroxisomes (Kunze et al., 2002, 2006).

In other organisms such as the plant *A. thaliana* ICL and MLS are peroxisomal. In this case an efficient coupling of the glyoxylate cycle to β-oxidation of fatty acids is essential, because both, energy and biomass production for germination and seedling outgrowth rests on the β-oxidation of fatty acid. The resulting acetyl-CoA is used to generate energy via the TCA-cycle and oxidative phosphorylation and to feed the glyoxylate cycle to cover the needs for biosynthetic processes. For the opportunistic fungus *C. albicans* carbohydrates and fatty acids are the prevalent carbon sources in its natural habitat, the mammalian gut. Thus, acetyl-CoA is expected to be obtained primarily inside mitochondria following glycolysis or inside peroxisomes via β-oxidation under such conditions. Even when these cells were grown in the presence of ethanol or acetate both key enzymes (ICL and MLS) are located in peroxisomes (Piekarska et al., 2008). Needless to mention, that neither *C. albicans* nor plants ever face ethanol or acetate as carbon source in their natural environment. This supports the hypothesis that the prevailing source of acetyl-CoA under natural conditions determines the localization of glyoxylate cycle key enzymes. For both model systems neither evolutionary pressure nor man-made selection forced the organism to change the intracellular location of parts of the glyoxylate cycle.

In contrast, strains of baker's yeast, *S. cerevisiae*, have been selected for efficient growth in the presence of ethanol. Consequently, this organism most efficiently utilizes acetyl-CoA generated from this carbon source in the cytosol, which is apparently supported by the potential to relocate the complete glyoxylate cycle to the cytosol.

The bipartite localization of enzymes provokes unusual intricacies for a straightforward metabolic pathway consisting of five enzymatic activities. Evolutionary optimization and pressure to increase efficiency demand a physiological advantage to compensate for this complexity. We suggest that the distribution of the glyoxylate cycle enzymes on different sides of the peroxisomal membrane might be due to the combination of (i) the unavoidable provision of its substrate, acetyl-CoA, inside peroxisomes by fatty acid β-oxidation (Kunze et al., 2006) and (ii) the incompatibility of some of its enzymes, namely ACO (Verniquet et al., 1991) and to a certain extent also ICL (Yanik and Donaldson, 2005), with the oxidative milieu within peroxisomes including high H_2O_2 concentrations. Thus, during evolution neither compartmentation of the complete pathway within peroxisomes nor the transfer of all enzymes into the cytosol appeared as feasible alternative.

However, the tight coupling of peroxisomal acetyl-CoA generation and its fueling into the glyxoylate cycle that appears optimal for growth of *A. thaliana* and *C. albicans* in their natural habitats might restrain the incorporation of extra-peroxisomal acetyl-CoA into the glyoxylate cycle. When such organisms utilize the less physiological carbon source acetate as carbon source they satisfy all their energetic and biosynthetic needs from acetyl-CoA that is primarily generated in the cytosol. In this case the peroxisomal membrane could act as barrier that separates

cytosolic acetyl-CoA from those peroxisomal enzymes of the glyoxylate cycle that utilize it, namely MLS in both organisms and CIT in *A. thaliana*. Under these conditions the mitochondrial energy production from cytosolic acetyl-CoA is not expected to be limited, but the biosynthetic capacity of the glyoxylate cycle is restricted by the transfer of acetyl-CoA into peroxisomes involving a specific import mechanism for this intermediate. Such a limitation is avoided in *S. cerevisiae*, where the glyoxylate cycle can be relocated to the cytosol.

Interestingly, *A. thaliana* and *C. albicans* can grow under conditions when acetate is utilized as sole carbon source (Hooks et al., 2004; Zhou and Lorenz, 2008), but they are dependent on specific peroxisomal functions. In *A. thaliana* a genetic screen for mutants that are unable to utilize exogenous acetate identified two genes encoding peroxisomal proteins (Hooks et al., 2004). The peroxisomal transporter protein *COMATOSE* known to transport fatty acids (Hooks et al., 2007) and the intraperoxisomal short chain acyl-CoA/acetyl-CoA synthase (At3g16910, AAE7) (Turner et al., 2005; Shockey and Browse, 2011) are required for the integration of acetate into organic compounds (Allen et al., 2011). Interestingly, neither *C. albicans* nor *S. cerevisiae* cells require functional peroxisomes for the utilization of acetate, as cells harboring a deletion of a PEX-gene can grow on medium solely containing this carbon source (Piekarska et al., 2008). However, upon selective interruption of fatty acid β-oxidation by the ablation of the enzyme exerting the second step (Fox2p), *C. albicans* cells cannot utilize acetate any more, but *S. cerevisae* cells can do so (Hiltunen et al., 1992; Piekarska et al., 2008). This phentotype of *C. albicans* cells might be due to their inability to feed cytosolic acetyl-CoA into the glyoxylate cycle, because MLS is enclosed by the peroxisomal membrane. This notion is supported by the observation, that the ability to utilize acetate efficiently can be restored in these cells (ΔFOX2), when the compartmentation of peroxisomal enzymes is prevented by the deletion of the PTS1-receptor PEX5 (ΔFOX2ΔPEX5) (Piekarska et al., 2008). Thus, in organisms optimized for an efficient consumption of fatty acids the redirection of cytosolic acetyl-CoA units obtained from other sources such as acetate into peroxisomes is a critical step for its fueling into the glyoxylate cycle.

METABOLIC FLUXES IN RELATED METABOLIC PATHWAYS

Photorespiration represents another example for a metabolic process in which glyoxylate is formed and intermediates traverse the peroxisomal membrane. Considering only the transport reactions between chloroplasts and peroxisomes two molecules of glycolate are transported into peroxisomes and one molecule glycerate is exported. The exchange with the mitochondria includes the export of two molecules of glycines and the import of one molecule serine (**Figure 3**). Already 40 years ago peroxisomes, mitochondria, and chloroplasts were often seen in close vicinity in the electron microscope (Frederick and Newcomb, 1969). A direct apposition of the peroxisomal membrane and the outer membranes of chloroplasts (Schumann et al., 2007) supports the hypothesis of direct exchange of metabolites thereby avoiding diffusion of intermediates into the cytosol. In leaves of *A. thaliana*, in which due to a mutation in a peroxisomal membrane protein the close apposition of chloroplasts and peroxisomes is lost, glyoxylate accumulates (Schumann et al., 2007).

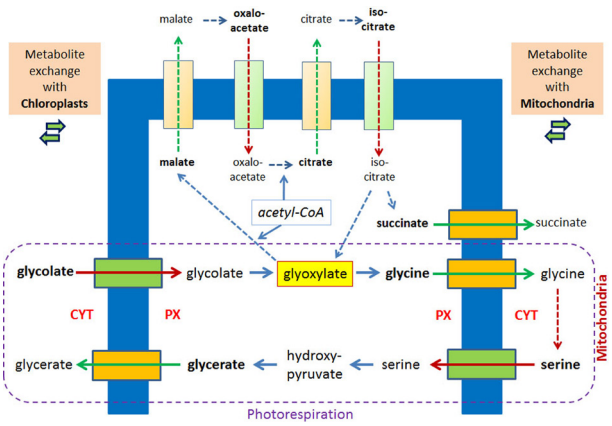

FIGURE 3 | Metabolite transport processes for the glyoxylate cycle and the photorespiratory process are considered to be similar. In plant peroxisomes glyoxylate is a key metabolite of the glyoxylate cycle and the photorespiration, the latter involving mitochondria and chloroplasts. Hypothetical pore-forming proteins permitting the export are colored orange, hypothetical pore-forming proteins permitting the import are colored green. The broken lines with arrow heads indicate reactions of the glyoxylate cycle and the conversion of two molecules of glycine into serine inside mitochondria without further details. Reactions of the photorespiration process are encircled.

Under normal conditions the glyoxylate cycle and the photorespiration do not overlap although they occur in the same organelle and share glyoxylate as key intermediate. This separation is due to the developmental program, which shows highest glyoxylate key enzyme activities during seed germination [2–4 days postimbibition (Eastmond and Graham, 2001)], whereas the enzymes of the photorespiration are induced by light (Bertoni and Becker, 1993) upon cotyledon development. Upon this development the change of glyoxylate cycle containing peroxisomes ("glyoxysomes," see Pracharoenwattana and Smith, 2008) into photorespiratory active peroxisomes is considered prototypical for the exchange of enzymatic equipment of such organelles (Titus and Becker, 1985; Behrends et al., 1990) and is even accompanied by the highly surprising removal of ICL from "glyoxsomes" (Lingard et al., 2009). However, employing artificial experimental conditions by exposing outgrowing seedlings to light these developmental programs become blended, and so does the enzymatic equipment. This is demonstrated by the comparison of plants in which the glyoxylate cycle is interrupted either by ICL deficiency or MLS deficiency. The observed growth defect of these plants in the dark can be completely overcome by exposing seedlings lacking MLS to light, but seedlings lacking ICL can only partially recover (Cornah et al., 2004). The suggestion that the addition of photorespiratory equipment allows the re-direction of glyoxylate from MLS to serine/glutamine-glyoxylate aminotransferase is supported by the re-organization of the incorporation pattern of radioactively labeled acetyl-CoA into different water soluble intermediates. Thereby, wild type plants and plants lacking MLS display a more similar phenotype than plants lacking ICL.

MODEL FOR THE FLUX OF INTERMEDIATES ACROSS A MEMBRANE

The localization of enzymes catalyzing successive metabolic reactions on opposite sides of the peroxisomal membrane and the permeability of this membrane for small molecules raises the question how fast small metabolites can cross the barrier membrane. Such a transfer of intermediates might be accomplished either (i) by an unspecific pore or (ii) by a series of specific transporter or facilitator proteins or (iii) by the direct coupling of specific enzymes on both sides of an unspecific pore forming a transmembrane metabolon. A simple diffusion controlled mechanism involving only a pore-like structure appears unlikely, because after reaching the other side of the membrane small metabolites could easily diffuse and the concentration might become (quite) low. This may happen, permitting the adjustment of concentrations between the cytosol and the peroxisomal matrix. However, such a model appears less likely for instable, highly reactive or even toxic intermediates such as glyoxylate. Alternatively, specific transporters or facilitators might close the gap between successive enzymatic reactions and guarantee specificity of transport, however these proteins have not been found so far. An interesting alternative might be a transmembrane metabolon comprised of transiently accumulating metabolic enzymes such as the glyoxylate cycle enzymes on both sides of an unspecific pore. We propose that in this extended form of the classical metabolon (Srere, 1987) pore-like channels participate in the assembly of supramolecular complexes, thereby linking proteins on both sides of the membrane enabling the swift transfer or exchange of metabolites. Such structural arrangement could enhance efficiency and ensure sufficient flux of intermediates across the peroxisomal membrane. Importantly, the transiently formed transmembrane metabolon would generate only local concentration peaks of intermediates at the entry sides of the pores. In our model metabolically active proteins transiently acquire a pore leading to a rapid transfer of the corresponding metabolites across a short distance. In this respect the observation seems interesting that polyethylene glycol (PEG), which stabilizes peroxisomal membrane integrity during the isolation procedure (Antonenkov et al., 2004a) is also known as stabilizing agent for supramolecular protein complexes such as metabolons (Beeckmans and Kanarek, 1981; Barnes and Weitzman, 1986). Such a model would explain how a variety of substrates could cross the membrane without specific transporter molecules. The selectivity would be maintained via the transient association of proteins on both sides of the pore-like channels providing control and efficiency.

According to our model in *S. cerevisiae* the loosely associated protein complex outside peroxisomes would consist of ACO, ICL, and MDH, on the inside of MLS and CIT, and the channel(s) would allow transferring citrate and malate from the matrix to the cytosol and/or glyoxylate and oxaloacetate from the cytosol to the peroxisomal matrix. When the peroxisomal Mdh3p has no access to the complex formed it is excluded from participation in the glyoxylate cycle. In plants, five metabolites have to cross the membrane. ACO and MDH acting outside peroxisomes require the export of citrate and malate and the import of isocitrate and oxaloacetate for further processing. The product succinate needs to be exported as well to make it available for the cellular metabolism. Since according to our model ICL would loosely associate with CIT and MLS it is reasonable to suggest that the simultaneous export of succinate using the same channel unit represents an additional drive for the cleavage reaction.

Furthermore, the peroxisomal membrane would *per se* not represent a principal barrier for glyoxylate cycle intermediates or any other small metabolite, but controls the velocity of the continuous flux. In addition, this model could also explain how shuttles may function. A local concentration of enzymes on both sides of a membrane-spanning pore could facilitate the shuttling of reduction equivalents in [isocitrate/α-oxo-glutarate (Van Roermund et al., 1998)] or out [malate/oxaloacetate (Van Roermund et al., 1995), lactate/pyruvate (Baumgart et al., 1996), G3P/DHAP (Gee et al., 1974)] of the peroxisomal matrix (for review see Antonenkov and Hiltunen, 2006). The exchange of the reduced and oxidized intermediates would occur due to a local association of the corresponding enzymes, Mdh2p/Mdh3p or Idp2p/Idp3p, on each side of a channel.

CONCLUSIONS AND PERSPECTIVES

The peroxisomal matrix is surrounded by a single membrane that allows the enclosure of a variety of highly reactive and even toxic compounds. However, *in vitro* the membrane appears permeable to small molecules and neither a chemical nor an electrochemical gradient has been detected, which represents a major difference to the mitochondrial inner membrane or the thylakoid membrane. The biophysical properties of the peroxisomal membrane demands pore-like structures in addition to the few transmembrane proteins with transporter function for larger molecules. The frequent exchange of intermediates between peroxisomes and the intracellular environment requires directed transport processes with high selectivity, which appears incompatible with pore-like exchange modules. However, a model in which soluble enzymes accumulate in proximity to both ends of such pore-like structures forming a transmembrane metabolon would explain rapid and selective exchange based on a local increase in intermediate concentration. The observation that the enzymes of the glyoxylate cycle are distributed across both sides of the peroxisomal membrane would be prototypical for such transport processes, because an efficient transfer of small organic metabolites across the peroxisomal membrane is essential for this pathway. The high variability in the nature of small organic molecules transported across the peroxisomal membranes between *S. cerevisiae*, *C. albicans*, and *A. thaliana* may reflect the ease of adaptation processes whenever specific transporter proteins are not involved. Similar considerations are applicable to the photorespiration, although the direct exchange of metabolites with other organelles renders the latter apparently more complex. A transport system of such high versatility generates an organelle of high plasticity and allows rapid adjustments to environmental changes and to developmental programs generally considered an important feature of peroxisomes.

Altogether, the peroxisomal membrane markedly differs from membranes of other metabolically active organelles, which might represent an intermediate step in the development of an organelle with novel properties. Thus, it can serve as model system to investigate and to understand transport processes across membranes with reduced complexity.

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Peroxisome biogenesis in mammalian cells

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To investigate peroxisome assembly and human peroxisome biogenesis disorders (PBDs) such as Zellweger syndrome, thirteen different complementation groups (CGs) of Chinese hamster ovary (CHO) cell mutants defective in peroxisome biogenesis have been isolated and established as a model research system. Successful gene-cloning studies by a forward genetic approach utilized a rapid functional complementation assay of CHO cell mutants led to isolation of human peroxin (*PEX*) genes. Search for pathogenic genes responsible for PBDs of all 14 CGs is now completed together with the homology search by screening the human expressed sequence tag database using yeast *PEX* genes. Peroxins are divided into three groups: (1) peroxins including Pex3p, Pex16p, and Pex19p, are responsible for peroxisome membrane biogenesis via classes I and II pathways; (2) peroxins that function in matrix protein import; (3) those such as three forms of Pex11p, Pex11pα, Pex11pβ, and Pex11pγ, are involved in peroxisome proliferation where DLP1, Mff, and Fis1 coordinately function. In membrane assembly, Pex19p forms complexes in the cytosol with newly synthesized PMPs including Pex16p and transports them to the receptor Pex3p, whereby peroxisomal membrane is formed (Class I pathway). Pex19p likewise forms a complex with newly made Pex3p and translocates it to the Pex3p receptor, Pex16p (Class II pathway). In matrix protein import, newly synthesized proteins harboring peroxisome targeting signal type 1 or 2 are recognized by Pex5p or Pex7p in the cytoplasm and are imported to peroxisomes via translocation machinery. In regard to peroxisome-cytoplasmic shuttling of Pex5p, Pex5p initially targets to an 800-kDa docking complex consisting of Pex14p and Pex13p and then translocates to a 500-kDa RING translocation complex. At the terminal step, Pex1p and Pex6p of the AAA family mediate the export of Pex5p, where Cys-ubiquitination of Pex5p is essential for the Pex5p exit.

Keywords: CHO cell mutants, genetic phenotype-complementation, import machinery, membrane assembly, pathogenic genes, peroxins, peroxisome targeting signals, Zellweger syndrome

INTRODUCTION

Molecular mechanisms of peroxisome biogenesis, including peroxisomal import of newly synthesized matrix and membrane proteins, have been one of the major foci in the peroxisome research. Studies at the molecular level on both peroxisome assembly and peroxisome biogenesis disorders (PBDs) rapidly progressed in the last three decades. Studies on cloning of genes, particularly including those of a very low-level expression, have benefited from so-called functional cloning of genes, mostly cDNAs in mammalian cases, by phenotype complementation assay using cell mutants defective of biological pathways. The identification and characterization of numerous essential genes, termed *PEX*s encoding peroxisome biogenesis factors termed peroxins, by means of the genetic phenotype-complementation of peroxisome assembly-defective cell mutants, named *pex* mutants impaired in *PEX* genes. Such mutants from Chinese hamster ovary (CHO) cells (**Table 1**; see below) (Fujiki, 1997, 2000), several yeast species including *Saccharomyces cerevisiae* (Erdmann et al., 1989), *Pichia pastoris* (Gould et al., 1992; Liu et al., 1992), *Hansenula polymorpha* (Cregg et al., 1990), and *Yarrowia lipolytica* (Nuttley et al., 1993) (also see reviews Van Der Klei and Veenhuis, 1996; Kunau, 1998; Tabak et al., 1999; Subramani et al., 2000; Titorenko and Rachubinski, 2001; Lazarow, 2003), and plant *Arabidopsis thaliana* (Hayashi and Nishimura, 2006) have made invaluable contributions to the investigations of peroxisome biogenesis and protein trafficking in eukaryotes (Schatz and Dobberstein, 1996; Wickner and Schekman, 2005). We herein summarize mammalian model cell systems in studying biogenesis, physiology, and human disorders of peroxisomes.

Abbreviations: CG, complementation group; CHO, Chinese hamster ovary; DLP1, dynamin-like protein 1; EST, expressed sequence tag; PBD, peroxisome biogenesis disorder; PTS, peroxisomal targeting signal; ZS, Zellweger syndrome.

GENETIC APPROACHES TO STUDYING MAMMALIAN PEROXISOME BIOGENESIS

Basically two mutually complementary approaches have been taken for isolation of *PEX* genes encoding peroxins, i.e., the genetic phenotype-complementation of peroxisome biogenesis-defective mutants of mammalian somatic cells such as CHO cells and a combination of the human ortholog isolation by homology search on the human expressed sequence tag (EST) database using yeast *PEX* genes and cells derived from the patients with PBDs of 14 different genotypes, i.e., complementation groups (CGs)

(**Table 1**; see below) (Fujiki, 1997, 2000, 2003; Gould and Valle, 2000; Weller et al., 2003).

MAMMALIAN CELL MUTANTS DEFICIENT OF PEROXISOME

Genetic heterogeneity consisting of 14 CGs were identified in PBDs by cell-fusion CG analysis using fibroblast cell lines derived from PBD patients (Fujiki, 2000; Ghaedi et al., 2000a; Gould and Valle, 2000; Matsumoto et al., 2001), where CGs 4 and 7 were revealed to be the same CGs as CGs 6 and 5, respectively (**Table 1**). A new CG, CG15, of ZS was also identified (Shimozawa et al., 2004), hence indicative of totally 13 genotypes of PBDs. The primary defect for PBDs was revealed to be the impaired biogenesis of peroxisomes (Fujiki, 2000; Gould and Valle, 2000). With respect to somatic animal cell mutants, 12 CGs of peroxisome-deficient CHO cell mutants were isolated, including a mutant ZP114 of a CG distinct from human CGs (**Figure 1**; **Table1**). A PBD patient of the 14th CG, CG16, was recently identified with pathogenic gene *PEX11β* (Ebberink et al., 2012). Together, genetic heterogeneity comprising 15 CGs are currently identified in mammals including humans and CHO cells.

PEROXISOME BIOGENESIS GENES

Genetic phenotype-complementation screening

*PEX*s were isolated by genetic phenotype complementation of peroxisome biogenesis-deficient mutants of mammalian somatic cells including CHO cells (**Figure 1A**) and of several yeast species including *S. cerevisiae*, *P. pastoris*, *H. polymorpha*, and *Y. lipolytica* (Distel et al., 1996; Subramani et al., 2000; Fujiki et al., 2006b). Two mutually distinct but complementary approaches have been taken to identify and clone mammalian *PEX* genes.

A direct cloning approach has been taken by means of genetic complementation with peroxin cDNA essential for assembly of

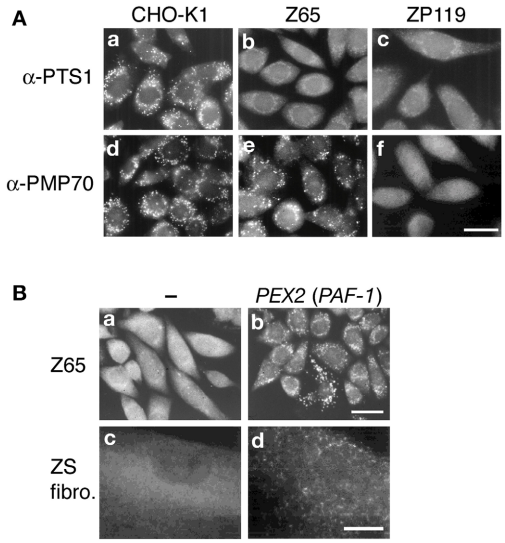

FIGURE 1 | Morphology of peroxisomes in CHO cell mutants defective in peroxisome biogenesis and cloning pathogenic genes of PBDs. (A) Cells are stained with antibodies to PTS1 **(a–c)** and PMP70 **(d–f)**. Cells are as indicated at the top. Scale bar, 20 μm. In contrast to the wild-type CHO-K1 cells, PTS1 proteins are discernible in the cytosol in *pex2* Z65 and *pex19* ZP119. Z65 contains PMP70-positive peroxisomal remnants, whilst ZP119 is absent from such peroxisome ghosts, indicative of the defect of membrane protein import. **(B)** Cloning of pathogenic gene of PBD. Peroxisome-restoring *PEX* gene were isolated by functional complementation assay using CHO mutant. Restoration of peroxisomes in Z65 **(a)** by transfection of rat liver cDNA library **(b)**. Transformed cells positive in catalase import contained *PAF-1* (*PEX2*). In fibroblasts from a patient with ZS of CG10 **(c)**, expression of *PAF-1* restored the impaired import of catalase **(d)**. Scale bar, 20 μm **(a,b)**; 30 μm **(c,d)**.

Table 1 | Complementation groups (CGs) and *PEX* genes of peroxisome deficiencies.

Gene	CG		Phenotype	CHO mutants	Peroxisome ghosts	Peroxin	
	US/EU	Japan				(kDa)	Characteristics
PEX1	1	E	ZS, NALD*, IRD*	Z24, ZP107	+	143	AAA family
PEX2	10	F	ZS, IRD*	Z65	+	35	PMP, RING
PEX3	12	G	ZS	ZPG208	−	42	PMP, PMP-DP
PEX5	2		ZS, NALD	ZP105*, ZP139	+	68	PTS1 receptor, TPR family
PEX6	4(6)	C	ZS, NALD*	ZP92	+	104	AAA family
PEX7	11	R	RCDP	ZPG207	+	36	PTS2 receptor, WD motif
PEX10	7(5)	B	ZS, NALD		+	37	PMP, RING
PEX11β	16		ZS		+	28	PMP
PEX12	3		ZS, NALD, IRD	ZP109	+	40	PMP, RING
PEX13	13	H	ZS, NALD*	ZP128	+	44	PMP, PTS1-DP, SH3
PEX14	15	K	ZS	ZP110	+	41	PMP, PTS1-DP, PTS2-DP
PEX16	9	D	ZS		−	39	PMP, PMP-DP
PEX19	14	J	ZS	ZP119	−	33	CAAX motif, PMP receptor
PEX26	8	A	ZS, NALD*, IRD*	ZP124, ZP167	+	34	PMP, Pex1p-Pex6p recruiter
				ZP114	+		

*, *Temperature-sensitive phenotype.*

ZS, Zellweger syndrome; IRD, infantile Refsum disease; NALD, neonatal adrenoleukodystrophy; RCDP, rhizomelic chondrodysplasia punctata; DP, docking protein; PMP, peroxisome membrane protein; TPR, tetratricopeptide repeat.

peroxisomes in CHO cells. Establishment of an effective method, termed P12 (12-(1′-pyrene)dodecanoic acid)/ultraviolet selection method, made it feasible to isolate revertant (transfectant) cells showing a morphologically and biochemically normal peroxisome-phenotype, whereby *PEX2* (formerly *PAF-1*) encoding the 35-kDa membrane peroxin Pex2p with RING zinc-finger motif was cloned for the first time (Tsukamoto et al., 1991) (**Figure 1B**). Expression of *PEX2* (called *Zellweger gene*) in fibroblasts from a ZS patient of CG10 (F) complemented the impaired peroxisome biogenesis (Shimozawa et al., 1992) (**Figure 1B**). Dysfunction of *PEX2* caused by a homozygous nonsense point mutation at R119ter was shown for the first time to be responsible for ZS, a prototype of the PBDs (Shimozawa et al., 1992). A more practical approach, i.e., a transient expression assay, was also developed for further isolation of *PEX* cDNAs including nine others, *PEX1*, *PEX3*, *PEX5*, *PEX6*, *PEX12*, *PEX13*, *PEX14*, *PEX19*, and *PEX26* (Fujiki, 2003; Fujiki et al., 2006b) (**Figure 2**). These *PEX*s were shown to be the pathogenic genes involved in PBDs of nine CGs (Weller et al., 2003; Fujiki et al., 2006b; Fujiki, 2011) (**Table 1**).

Expressed sequence tag homology search

As an alternative method, the homology search by screening the human EST database using yeast *PEX* genes successfully led to isolation of human ortholog genes responsible for PBDs: *PEX1*, *PEX3*, *PEX5*, *PEX6*, *PEX7*, *PEX10*, *PEX12*, *PEX13*, and *PEX16* (Weller et al., 2003; Fujiki et al., 2006b).

All of pathogenic genes responsible for PBDs of currently identified 13 CGs have been successfully cloned within about 10 years after the first isolation of the ZS gene, *PEX2*, by such extensive search using the mutually complementary methods.

BIOGENESIS OF PEROXISOMES

MEMBRANE BIOGENESIS

Three mammalian peroxins, Pex3p, Pex16p, and Pex19p, were isolated by the functional phenotype-complementation assay on CHO cell mutants (Matsuzono et al., 1999; Ghaedi et al., 2000b) and the EST database search using yeast *PEX* genes (Kammerer et al., 1997, 1998; Honsho et al., 1998; South and Gould, 1999) and were shown to be exclusively required for membrane

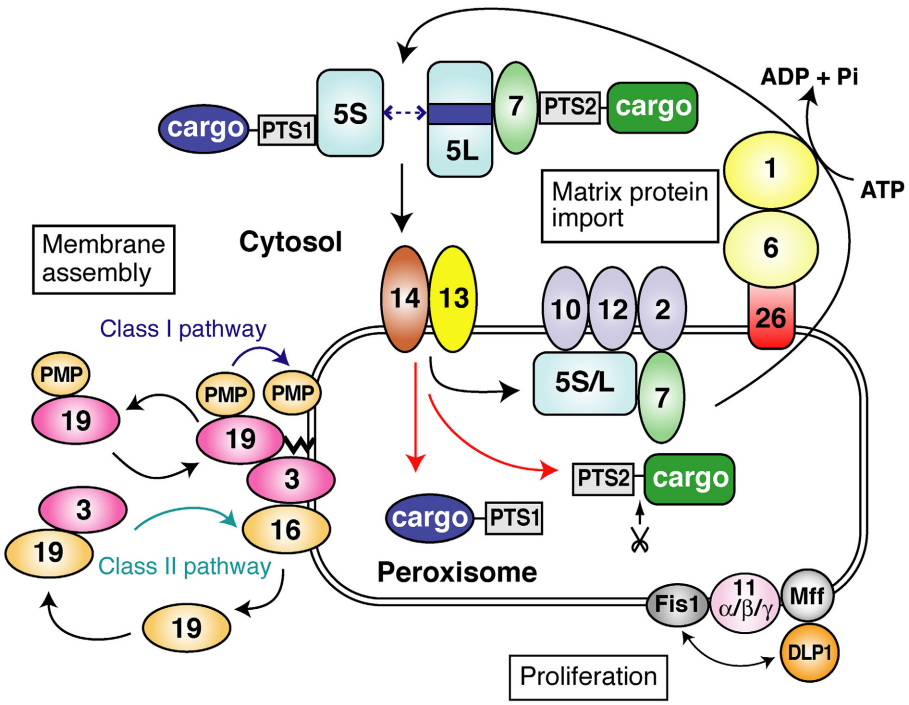

FIGURE 2 | A schematic view of peroxisome biogenesis in mammalian cells. The subcellular localization and molecular characteristics of peroxins are shown. Peroxins are classified into three groups: (1) peroxins that are required for matrix protein import; (2) those including Pex3p, Pex16p and Pex19p, responsible for peroxisome membrane assembly via classes I and II pathways (see in this figure); (3) those such as three forms of Pex11p, Pex11pα, Pex11pβ, and Pex11pγ, apparently involved in peroxisome proliferation where DLP1, Mff, and Fis1 coordinately function. PTS1 and PTS2 proteins are recognized by Pex5p and Pex7p, respectively, in the cytoplasm. Two isoforms, Pex5pS and Pex5pL, of Pex5p are identified in mammals. PTS1 proteins are transported by homo- and hetero-oligomers of Pex5pS and Pex5pL to peroxisomes, where Pex14p functions as a convergent, initial docking site of the "protein import machinery" translocon. Pex5pL directly interacts with the PTS2 receptor, Pex7p, carrying its cargo PTS2 protein in the cytosol and translocates the Pex7p–PTS2 protein complex to Pex14p. PTS1 and PTS2 proteins are then released at the inner surface and/or inside of peroxisomes, downstream Pex14p and upstream Pex13p. Pex5p and Pex7p subsequently translocate to other translocon components, named translocation complex comprising the RING peroxins, Pex2p, Pex10p, and Pex12p. Both Pex5p and Pex7p finally shuttle back to the cytosol. In regard to peroxisome-cytoplasmic shuttling of Pex5p, Pex5p initially targets to an 800-kDa docking complex containing Pex14p and then translocates to a 500-kDa translocation complex comprising RING peroxins. At the terminal step of the protein import reaction, Pex1p and Pex6p of the AAA family catalyze the export of Pex5p, where Cys-ubiquitination of Pex5p is prerequisite to the Pex5p exit.

assembly of peroxisomes. Mechanistic insights on membrane biogenesis are addressed here.

Peroxins essential for membrane assembly of peroxisomes

Of 13 peroxins of which mutations are responsible for PBDs, Pex3p, Pex16p, and Pex19p were identified as essential factors for PMP assembly in several species including humans (Baerends et al., 1996; Götte et al., 1998; Honsho et al., 1998; Matsuzono et al., 1999; South and Gould, 1999; Ghaedi et al., 2000a; Hettema et al., 2000; Sacksteder et al., 2000; South et al., 2000; Otzen et al., 2004) (**Figure 1**). Pex19p is a predominantly cytoplasmic protein that shows a broad PMP-binding specificity; Pex3p serves as the membrane-anchoring site for Pex19p-PMP complexes (Class I pathway); and Pex16p—a protein absent in most yeasts (Eitzen et al., 1997; South and Gould, 1999) functions as the receptor for Pex19p complexes with newly synthesized Pex3p (Matsuzaki and Fujiki, 2008) (Class II pathway) (**Figures 2**, **3**). The function of Pex16p is not conserved between different species. In addition, under debate remains whether Pex19p has a chaperone-like role in the cytosol or at the peroxisome membrane and/or functions as a cycling import receptor for newly synthesized PMPs (Fujiki et al., 2006a).

Gene defects of peroxins required for both membrane biogenesis and matrix protein import

Impairment of Pex3p, Pex16p, and Pex19p, causes the most severe PBD, ZS, of three CGs, CG12 (G), CG9 (D), and CG14 (J), respectively (Weller et al., 2003; Fujiki et al., 2006b, 2012; Fujiki, 2011) (**Table 1**).

Pex19p. *PEX19* encodes 33-kDa farnesylated protein harboring farnesylation CAAX box motif localized mostly in the cytosol and only partly anchored to peroxisomal membranes (Matsuzono et al., 1999). *PEX19* expression complemented impaired peroxisome assembly in fibroblasts from a patient with CG14 (J) PBD. This patient was a homozygote for inactivating mutation: a one-base insertion, A764, in a codon for Met^{255}, thereby resulting in a frameshift. Upon transfection of *PEX19* into a CHO *pex19* mutant ZP119 devoid of peroxisomal remnants called ghosts, most striking was formation of peroxisomal membranes, prior to the import of matrix proteins (Matsuzono et al., 1999; Sacksteder et al., 2000). This was the first demonstration of the membrane assembly process during peroxisome biogenesis, particularly differentiated from the soluble protein import.

Pex16p. Fibroblasts from a ZS patient of CG9 (D) are deficient in peroxisomal membrane remnants, as in *PEX19*-defective fibroblasts of CG14 (J). Expression of *PEX16* encoding 336-amino-acid peroxisomal membrane protein restored peroxisomal membrane biogenesis and matrix protein import in CG9 (D) fibroblasts (Honsho et al., 1998; South and Gould, 1999), of which mutation was a homozygous nonsense mutation R176ter (Honsho et al., 1998). More recently, other mutations are identified: exon 10 skip (Shimozawa et al., 2002) and five novel homozygous mutations (Ebberink et al., 2010).

Pex3p. Mammalian *PEX3* encodes 42-kDa integral membrane protein of peroxisomes (Ghaedi et al., 2000a,b). Upon expression

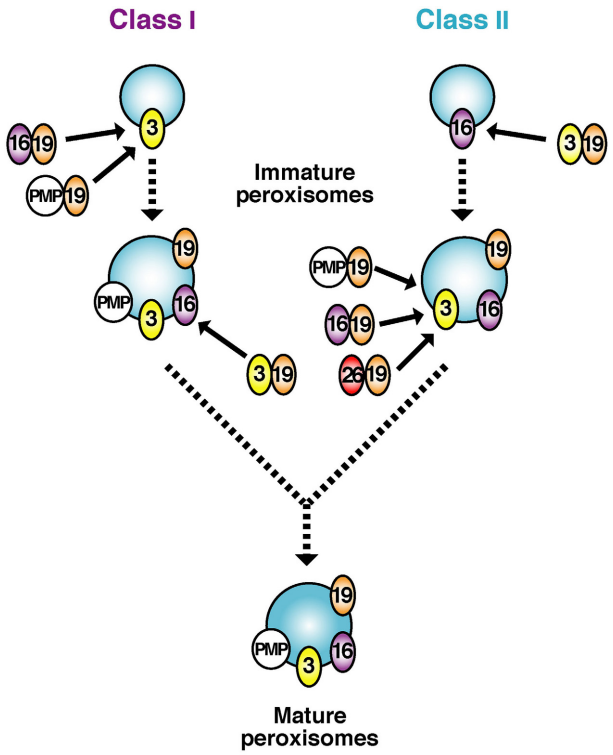

FIGURE 3 | A model for early stages of peroxisomal membrane biogenesis involving mutually dependent targeting of Pex3p and Pex16p, named classes I and II pathways. The initial membranes harboring Pex3p or Pex16p culminate in indistinguishable, matured peroxisomes. Pex19p forms complexes in the cytosol with newly synthesized PMPs including Pex16p and C-tailed anchored membrane proteins such as Pex26p and transports them to the membrane protein receptor Pex3p, whereby peroxisome membrane is assembled (Class I pathway). With respect to biogenesis of Pex3p, Pex19p likewise forms a complex with newly synthesized Pex3p and translocates it to the Pex3p receptor, Pex16p (Class II pathway). Of note, peroxisomes are assembled no matter which pathway initially proceeds.

of *PEX3* in a CHO *pex3* mutant (Ghaedi et al., 2000b) and fibroblasts from three ZS patients of CG12 (G) (Ghaedi et al., 2000a), peroxisomal membrane vesicles were assembled prior to the import of soluble proteins (Ghaedi et al., 2000a; Muntau et al., 2000; Shimozawa et al., 2000; South et al., 2000; Fujiki et al., 2006a; Fujiki, 2011), as in *pex19* and *pex16* patients-derived cells (see above), likewise implying the temporally differentiated translocation of matrix proteins into peroxisomal membrane vesicles. Two types of mutations, exon 11 deletion and a single-nucleotide insertion in the codon for Val^{182} in exon 7, in *PEX3* were identified in the ZS patients (Ghaedi et al., 2000a; Muntau et al., 2000; Shimozawa et al., 2000).

Taken together, Pex3p, Pex16p, and Pex19p are categorized as a peroxin exclusively required for the assembly of peroxisome membranes. They function as essential factors in the transport process of membrane proteins and membrane vesicle assembly in a concerted manner. Two distinct pathways were recently suggested for the import of PMPs: a Pex19p- and Pex3p-dependent

class I pathway for PMP-import complex, except for Pex3p (Fang et al., 2004; Matsuzono et al., 2006) and a Pex19p- and Pex16p-dependent class II pathway for Pex3p (Matsuzaki and Fujiki, 2008) (**Figures 2**, **3**). It is noteworthy that C-tailed anchor-type peroxin Pex26p, the recruiter of Pex1p-Pex6p complex, is transported in a Pex19p-dependent (Halbach et al., 2006), class I pathway (Yagita et al., 2013), which is distinct from the GET3-dependent topogenesis of yeast Pex15p, a functional ortholog of Pex26p (Schuldiner et al., 2008).

Involvement of ER in peroxisome biogenesis

In regard to involvement of the ER in peroxisome biogenesis, ER was postulated to provide the initial "seed" for recruiting other components required for peroxisome assembly (Kim et al., 2006; Ma et al., 2011; Tabak et al., 2013). Several groups suggested a different view of peroxisomal membrane biogenesis that peroxisomes are formed from ER upon induction of Pex3p (Hoepfner et al., 2005; Kragt et al., 2005; Kim et al., 2006), although the significance of such observations remains under debate. Recently, a study (Motley and Hettema, 2007) suggest that peroxisomes are generally formed by growth and division under normal conditions and that only under a condition where no peroxisome is present in a cell, they can be formed from the ER after the expression of the complementing *PEX* gene, whilst another study (Van Der Zand et al., 2010) proposes that all peroxisomal membrane proteins are transported via ER. Meanwhile, we demonstrated that Pex3p, the membrane receptor for Pex19p-complexes with PMPs including Pex16p, is directly targeted to peroxisomes in a Pex19p-Pex16p dependent class II pathway in mammalian cells such as CHO and human cell lines (Matsuzaki and Fujiki, 2008). Moreover, it is noteworthy that several peroxisomal membrane proteins might be transported to peroxisomes via ER (Lam et al., 2010; Agrawal et al., 2011; Yonekawa et al., 2011), likely implying a sort of semi-autonomous property of peroxisomes. At any event, the issue with respect to how peroxisome membrane is assembled is one of the important and of highly interesting problems to be tackled (Ma et al., 2011; Fujiki et al., 2012; Tabak et al., 2013).

MATRIX PROTEIN IMPORT

Ten peroxins including Pex1p, Pex2p, Pex5p, Pex6p, Pex7p, Pex10p, Pex12p, Pex13p, Pex14p, and Pex26p are involved in protein import into peroxisomal matrix (**Figure 2**) (Fujiki et al., 2006a).

Peroxisome-cytoplasmic shuttling of import receptors

PTS1 and PTS2 proteins are recognized by Pex5p and Pex7p, respectively, in the cytoplasm. In mammalian cells, PTS1 proteins are transported by homo- and hetero-oligomers of Pex5pS and Pex5pL to peroxisomes, where Pex14p functions as the initial site of an 800-kDa "docking complex." Pex5pL translocates the Pex7p–PTS2 protein complex to Pex14p (Otera et al., 2002; Miyata and Fujiki, 2005). After releasing the cargoes, Pex5p and Pex7p translocate to a 500-kDa "translocation complex" comprising the RING peroxins, Pex2p, Pex10p and Pex12p (Miyata and Fujiki, 2005). Both Pex5p and Pex7p finally translocate back to the cytosol (Dammai and Subramani, 2001; Gouveia et al., 2003; Nair et al., 2004; Miyata and Fujiki, 2005; Platta et al., 2005; Miyata et al., 2009). At the terminal step of the protein import reaction, AAA peroxins, Pex1p and Pex6p, recruited to Pex26p (Pex15p in yeast) on peroxisomes catalyze the ATP-dependent export of Pex5p (Miyata and Fujiki, 2005; Platta et al., 2005). Ubiquitination of Pex5p is prerequisite for the Pex5p exit (Carvalho et al., 2007; Williams et al., 2007; Okumoto et al., 2011).

Mono-ubiquitination of the conserved cysteine residue at position 11 in the N-terminal region of mammalian Pex5p plays an essential role in the recycling, especially in the export step from peroxisomes to the cytosol (Grou et al., 2009; Okumoto et al., 2011; Miyata et al., 2012), as in yeast (Platta et al., 2009). A cytosolic factors, AWP1/ZFAND6 involved in the recycling of Pex5p is recently identified in mammals (Miyata et al., 2012); USP9X and Ubp15 are suggested as a potential deubiquitinase in mammals (Grou et al., 2012) and yeast (Debelyy et al., 2011), respectively. A distinct redox state may affect the recycling of Pex5p requiring Cys-ubiquitination, thereby leading as a possible cause to the phenotype of deficiency in protein import in *PEX*-defective cells.

REDOX STATE OF NORMAL AND PEROXISOME-DEFICIENT CELLS

In peroxisomes possessing a fatty acid β-oxidation system in wild-type CHO cells, the redox state within the peroxisomes is more reductive than that in the cytosol, despite the fact that reactive oxygen species are generated within the peroxisomes (Yano et al., 2010). Moreover, to our surprise, the redox state in the cytosol of *pex* cell mutants is more reductive than that of the wild-type CHO cells (Yano et al., 2010). Such distinct redox state may affect the recycling of Pex5p requiring Cys-ubiquitination, thereby leading as a possible cause to the phenotype of deficiency in protein import in *PEX*-defective cells including cell lines from patients with PBDs. A potential way to cure the PBD patients may be a screening for agents that moderate the abnormal cytosolic redox state in the *pex* cell lines including the cells with nonredox-sensitive mutations in *PEX*s. It is noteworthy that in *P. pastoris* PTS1-cargo release from Pex5p is achieved by a redox-regulated oligomer to dimer transition of Pex5p and aided by Pex8p (Ma et al., 2013). Interestingly, intraperoxisomal redox status is strongly influenced by environmental growth conditions (Ivashchenko et al., 2011).

GENE DEFECTS OF PROTEINS FOR PEROXISOMAL MORPHOGENESIS

Three isoforms of Pex11p family, Pex11pα (Abe et al., 1998; Li et al., 2002a), Pex11pβ (Abe and Fujiki, 1998; Schrader et al., 1998; Li et al., 2002b), and Pex11pγ (Li et al., 2002a; Tanaka et al., 2003), are identified as factors involved in morphogenesis of peroxisomes in mammals (Kobayashi et al., 2007; Delille et al., 2010; Koch et al., 2010; Itoyama et al., 2013). In mammalian cells, dynamin-like protein 1 (DLP1) (Koch et al., 2003; Li and Gould, 2003; Tanaka et al., 2006; Waterham et al., 2007), fission 1 (Fis1) (Koch et al., 2005; Kobayashi et al., 2007), and mitochondrial fission factor (Mff) (Gandre-Babbe and Van Der Bliek, 2008; Otera et al., 2010; Koch and Brocard, 2012; Itoyama et al., 2013) are shown to be involved in the fission of peroxisomes.

In regard to peroxisomal dysmorphogenesis in humans, only two patients have been identified with a different defect in any

of the proteins involved in the proliferation and division of peroxisomes. The first reported patient was a severely affected female patient, who died 1 month after birth and postmortally was found to have a dominant-negative heterozygous mutation in the *DLP1* gene, which resulted in a severe fission defect of both peroxisomes and mitochondria (Waterham et al., 2007). More recently, the first patient with a defect of peroxisomal division due to a homozygous nonsense mutation in the *PEX11*β gene was reported as the 14th CG (CG16) of PBDs (Ebberink et al., 2012) (**Table 1**).

PERSPECTIVE

Mammalian cell mutants of 15 CGs defective of peroxisome biogenesis have been identified, including PBD patients' fibroblasts and CHO mutant cell lines (**Table 1**). Pathogenic genes are now elucidated for all of PBD CGs. Biochemical functions of peroxins involved in the import of matrix proteins are better elucidated, whilst molecular mechanisms underlying the membrane assembly are less understood. Defects in peroxisomal morphogenesis have also been recently reported. Investigations using the cloned peroxins and *pex* mutants including CHO mutants and those from PBD patients will shed light on the mechanisms involved in biogenesis and morphogenesis of peroxisomes and pathogenesis of PBDs.

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Peroxisome degradation in mammals: mechanisms of action, recent advances, and perspectives

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Peroxisomes are remarkably dynamic organelles that participate in a diverse array of cellular processes, including the metabolism of lipids and reactive oxygen species. In order to regulate peroxisome function in response to changing nutritional and environmental stimuli, new organelles need to be formed and superfluous and dysfunctional organelles have to be selectively removed. Disturbances in any of these processes have been associated with the etiology and progression of various congenital neurodegenerative and age-related human disorders. The aim of this review is to critically explore our current knowledge of how peroxisomes are degraded in mammalian cells and how defects in this process may contribute to human disease. Some of the key issues highlighted include the current concepts of peroxisome removal, the peroxisome quality control mechanisms, the initial triggers for peroxisome degradation, the factors for dysfunctional peroxisome recognition, and the regulation of peroxisome homeostasis. We also dissect the functional and mechanistic relationship between different forms of selective organelle degradation and consider how lysosomal dysfunction may lead to defects in peroxisome turnover. In addition, we draw lessons from studies on other organisms and extrapolate this knowledge to mammals. Finally, we discuss the potential pathological implications of dysfunctional peroxisome degradation for human health.

Keywords: peroxisomes, organelle quality control, reactive oxygen species, protein import, organelle turnover, autophagy, pexophagy, lysosomes

INTRODUCTION

Peroxisomes were first observed in electron microscopy studies by the Swedish doctoral student Johannes Rhodin in 1954 (Rhodin, 1954) and, approximately a decade later, for the first time isolated from rat liver and biochemically characterized by the Belgian Nobel Laureate Christian de Duve and his colleague Pierre Baudhuin (de Duve and Baudhuin, 1966). The name "peroxisome" derives from the early observation that the organelle is involved in processes that both generate and decompose hydrogen peroxide (H_2O_2). Over the last half century, our knowledge about this highly dynamic and plastic organelle has virtually exploded. For example, it is now known that mammalian peroxisomes are involved in multiple metabolic pathways, including the breakdown of various carboxylates via α- and β-oxidation, and the synthesis of bile acids, docosahexaenoic acid (DHA) and ether-phospholipids (Van Veldhoven, 2010). Importantly, many of the enzymes involved in these processes produce reactive oxygen or nitrogen species (ROS or RNS) as part of their normal catalytic cycle (Fransen et al., 2012). To combat the destructive effects of these molecules, peroxisomes also contain various antioxidant enzymes of which catalase is perhaps the best known (Antonenkov et al., 2010). The necessity of peroxisomes for normal development and physiology is illustrated by the existence of a group of genetic disorders associated with peroxisomal deficiencies. These diseases are generally subdivided into two groups: the peroxisome biogenesis disorders (PBDs) (Nagotu et al., 2012) and the single peroxisomal enzyme deficiencies (PEDs) (Wanders and Waterham, 2006). In recent years, peroxisome (dys)function has also been associated with a wide variety of age-related maladies, including cancer, type 2 diabetes, and neurodegeneration (Fransen et al., 2013).

PHYSIOLOGICAL IMPORTANCE OF PEROXISOME HOMEOSTASIS

Currently, it is generally accepted that the localization and activity of many proteins (e.g., kinases, phosphatases, transcription factors, etc.) are reversibly controlled by the cellular composition and concentration of specific lipids and (redox-derived) signaling mediators (Hekimi et al., 2011; Schug et al., 2012). As peroxisomes are actively involved in the metabolism of many of these compounds, it is not surprising that these organelles are increasingly recognized as potential signaling platforms in diverse biological processes such as inflammation (Zmijewski et al., 2009), apoptosis (Li et al., 2002; Hasegawa et al., 2010), innate immunity (Dixit et al., 2010; Horner et al., 2011), cellular aging (Beach et al., 2012; Giordano and Terlecky, 2012), diabetes (Elsner et al., 2011; Hwang et al., 2012), and cancer development (Reddy et al., 1980; Frederiks et al., 2010). This is perhaps best illustrated by the observation that peroxisomes play a central role in the cellular metabolism of H_2O_2, a key molecule in cellular redox signaling (Fransen et al., 2012). For example, peroxisomes seem to be responsible for as much as 35% of the

total H_2O_2 production in rat liver (Boveris et al., 1972), and fibroblasts derived from hypocatalasemic patients accumulate H_2O_2 and are oxidatively damaged (Wood et al., 2006). In addition, overexpression of acyl-CoA oxidase 1, a H_2O_2-producing enzyme of the peroxisomal fatty acid β-oxidation pathway, has been shown to activate the redox-sensitive transcription factor NF-κB in a substrate concentration-dependent manner (Li et al., 2000); and overexpression of catalase, a peroxisomal enzyme that decomposes H_2O_2, sensitizes cells (and animals) to certain types of stressors by dampening H_2O_2-mediated signaling pathways (Carter et al., 2004; Chen et al., 2004). Finally, as high ROS levels are also known to cause significant damage to cell structures (Nathan and Ding, 2010), excessive production of peroxisomal ROS may overwhelm the cellular antioxidant defenses and mediate cellular injury or even cell death (Elsner et al., 2011; our unpublished observations). In this context, it is also interesting to mention that, to carry out their functions, peroxisomes physically and functionally interact with other cell organelles (Horner et al., 2011; Beach et al., 2012; Kohlwein et al., 2013), and that disturbances in peroxisome function have been reported to trigger signaling events that ultimately activate mitochondrial and endoplasmic reticulum stress pathways (Koepke et al., 2007; Ivashchenko et al., 2011; Kovacs et al., 2012). In summary, these observations (among others) clearly illustrate that changes in peroxisomal metabolism have a tremendous impact on many cellular processes, and as such it is of vital importance for humans (and organisms in general) to adjust peroxisome function and abundance to cellular needs.

REGULATION OF PEROXISOME ABUNDANCE

Peroxisome abundance is strictly regulated by the rates of organelle formation, division and turnover. Peroxisomes can be formed either *de novo* from the ER or by growth and asymmetric division of pre-existing organelles (**Figure 1A**) (Fransen, 2012). The latter process is, to a great extent, regulated by the Pex11p family of proteins. Indeed, the expression levels of members of this protein family have been shown to correlate with the number of peroxisomes in a cell (Schrader et al., 1998; Thoms and Erdmann, 2005), and overexpression of human Pex11pβ promotes peroxisome proliferation independent of peroxisomal metabolic activity (Li and Gould, 2002). For more detailed information regarding these processes, we refer the reader to other recent reviews (Ma et al., 2011; Schrader et al., 2012).

As mentioned above, peroxisomes can rapidly modulate their number, size, and function in response to cellular needs. Nowhere else is this better illustrated than in the methylotrophic yeasts *Hansenula polymorpha* and *Pichia pastoris*, where peroxisome number and size are massively increased when the cells are grown in media containing methanol as the sole carbon source (van der Klei and Veenhuis, 2006). This finding may not be surprising given that these organelles harbor the key enzymes of methanol metabolism (van der Klei and Veenhuis, 2006). As the enhanced peroxisomal activity is no longer needed when the cells are recultivated in media containing ethanol or glucose as carbon source, these methanol-induced peroxisomes are rapidly degraded by a process called "pexophagy" (see below) (Manjithaya et al., 2010). A similar phenomenon, albeit less pronounced, can also be observed in rodents upon the administration (and subsequent removal) of a variety of xenobiotics, collectively known as peroxisome proliferators (Reddy et al., 1980; Yokota, 1993). Agents that are frequently used to induce peroxisome proliferation in this class of animals include hypolipidemic drugs (e.g., fibrates), industrial phthalate ester plasticizers, and several types of fatty acids (Cho et al., 2008). These compounds act by binding to the nuclear receptor Peroxisome Proliferator-Activated Receptor α (PPARα) (Issemann and Green, 1990), which heterodimerizes with the Retinoid X Receptor (RXR) to regulate gene expression through PPAR-responsive elements in target DNA (Chandra et al., 2008). Interestingly, human cells do not respond similarly to PPARα agonists (Lawrence et al., 2001). However, some drugs such as 4-phenylbutyrate and niclosamide can act as potent PPARα-independent peroxisome proliferators in these cells (Sexton et al., 2010).

It is well-known that peroxisome number is significantly reduced in fibroblasts from patients with PBDs or peroxisomal fatty acid β-oxidation deficiencies (Chang et al., 1999). Intriguingly, in fibroblasts from the latter class of patients, this decrease in number coincides with an increase in peroxisome diameter but has apparently no effect on the expression levels of peroxisomal membrane proteins (PMPs) (Chang et al., 1999; and references therein). Together with the observations that (1) the reduced abundance of peroxisomes in cells with peroxisomal β-oxidation deficiency correlates with a loss of the corresponding enzyme activity and not with peroxisomal import defects (Chang et al., 1999), and (2) overexpression of ACOT8, one of the peroxisomal acyl-CoA thioesterases that inhibit fatty acid oxidation by depleting acyl-CoA substrates, reduces peroxisome abundance in normal human fibroblasts (Chang et al., 1999), these data suggest that this dysmorphogenesis is caused by alterations in peroxisomal β-oxidation metabolite levels. This hypothesis is in line with the findings of a recent study (Itoyama et al., 2012) showing that treating cells with DHA, a major product of peroxisomal β-oxidation, restores peroxisome number in cells deficient in peroxisomal β-oxidation, but not in PBD cells. Importantly, this process is time-, dose-, and Pex11p-dependent, but PPARα-independent. As peroxisomes in control fibroblasts fail to proliferate in response to DHA treatment, these findings also underscore the complexity of the regulation of peroxisome abundance under normal conditions.

PEROXISOME QUALITY CONTROL MECHANISMS

To maintain their health, cells need to keep organelles in a functional state. Over the years, multiple quality control mechanisms have been described, including (1) organellar chaperones and proteases that, respectively, promote proper protein folding and proteolytic removal of terminally damaged proteins (Haynes and Ron, 2010; Walter and Ron, 2011), (2) retrotranslocation of misfolded proteins from the organelle to the cytosol for proteasomal degradation (Taylor and Rutter, 2011; Brodsky, 2012), and (3) autophagic degradation of dysfunctional organelles (Farré et al., 2009). A similar situation most likely exists for peroxisomes. In the following paragraphs, we discuss the components and mechanisms involved in peroxisomal proteostasis. For the mechanisms of how dysfunctional peroxisomes are degraded by the autophagic

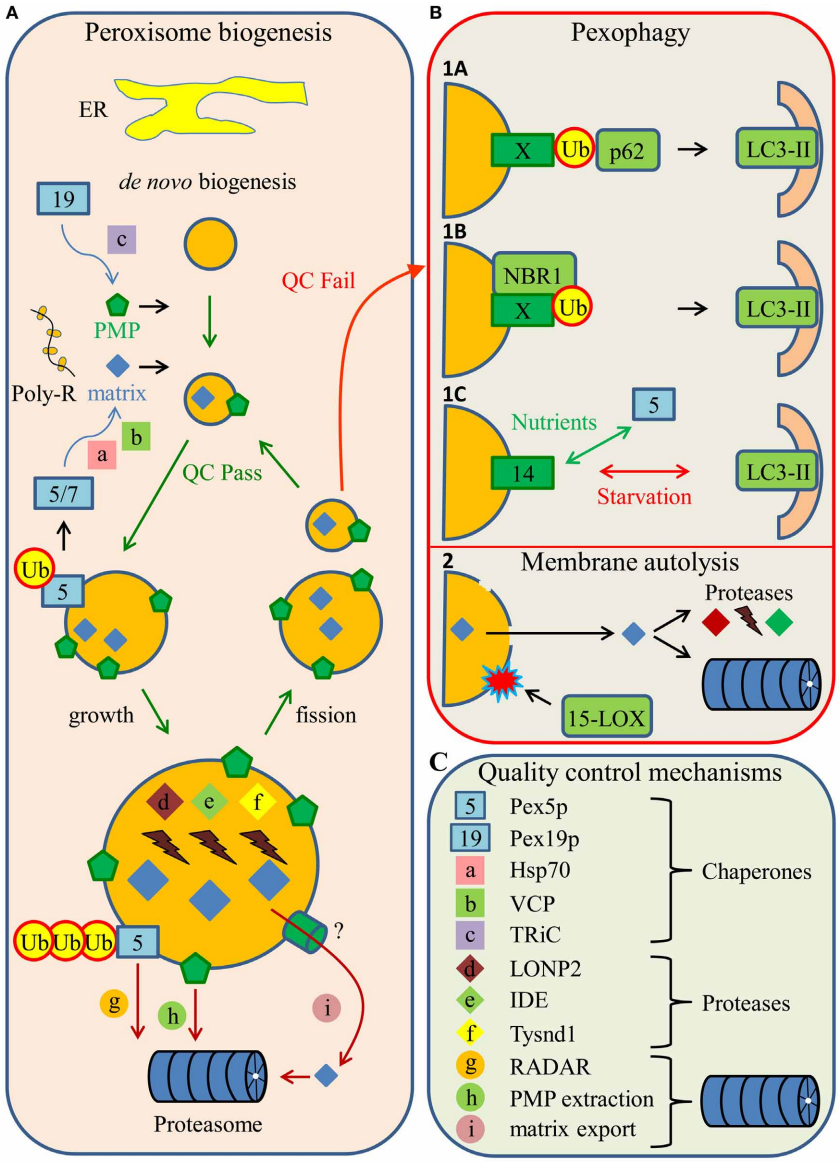

FIGURE 1 | Peroxisome biogenesis, quality control, and turnover in mammalian cells. (A) Peroxisomes can be formed *de novo* from the ER or by growth and asymmetric fission of pre-existing organelles. Peroxisomal matrix (matrix) and membrane (PMP) proteins are translated on free polyribosomes (Poly-R) in the cytosol, where they are recognized by their cognate import receptors Pex5p, Pex7p, or Pex19p (these and other peroxins are represented by numbers). Importantly, Pex5p and Pex19p possess chaperone-like activities. In addition, matrix protein folding is facilitated by the cytosolic chaperones Hsp70 and VCP, whereas PMP folding is assisted by the chaperonin TRiC (all non-peroxin-related quality control mechanisms are indicated by lower-case letters and listed in panel **C**). At the peroxisomal membrane, Pex5p is either mono- or poly-ubiquitinated (Ub). In the case of mono-ubiquitination, Pex5p is extracted from the membrane into the cytosol for a new round of matrix protein import. However, upon poly-ubiquitination, Pex5p is degraded by the proteasome in a process known as RADAR. Superfluous or dysfunctional PMPs are also targets for proteasomal degradation. The peroxisomal matrix harbors several proteases (e.g., LONP2, IDE, and Tysnd1) that function as regulators of intra-peroxisomal proteostasis. In addition, excessive peroxisomal matrix proteins may be exported to the cytosol where they are degraded by cytosolic proteases or the proteasome. During their life cycle, peroxisomes are constantly exposed to quality control (QC) mechanisms, and in case of failure, it is likely that the organelle is targeted for degradation. **(B)** Mammalian peroxisomes can be degraded by distinct pathways, pexophagy and 15-LOX mediated membrane autolysis. Three mechanisms have been proposed for how dysfunctional peroxisomes can be removed by the autophagic machinery. **(1A)** The first one involves the recognition of a ubiquitinated PMP (X) by an autophagic adaptor protein p62 which, in turn, bridges the peroxisome with the developing autophagosome via LC3-II. **(1B)** The second mechanism involves another adaptor protein, NBR1, which, similarly to p62, recognizes dysfunctional peroxisomes via ubiquitinated PMPs and links the organelles with the autophagic machinery through LC3-II. NBR1 is also capable of binding directly to the peroxisomal lipid bilayer. **(1C)** A third mechanism describes the competitive nature of the Pex14p binding partners Pex5p and LC3-II. Under nutrient-rich conditions, Pex5p is the preferred binding partner, whereas in nutrient-starved conditions, interaction with LC3-II is favored. Importantly, peroxisomes are only degraded upon re-supplementation of nutrients. **(2)** Finally, the peroxisomal membrane can undergo 15-lipoxygenase (15-LOX)-mediated autolysis, which subsequently leads to proteasome- or protease-dependent degradation of peroxisomal proteins. **(C)** Peroxisomal protein quality control mechanisms.

machinery, please see section Peroxisome Degradation of this chapter below.

The involvement of chaperones and proteases is central to many organellar quality control systems (Chen et al., 2011). Indeed, newly imported proteins often need to be proteolytically processed, properly folded, and assembled into functional units to acquire their activity. In this context, it is important to note that peroxisomes have the capacity to import fully folded and oligomeric matrix proteins (Lanyon-Hogg et al., 2010). This finding suggests that the quality control of proteins destined for the peroxisomal matrix may occur, at least partially, in the cytosol. Such control mechanisms may be mediated by cytosolic heat shock proteins (Hsps) or cytosolically located peroxins (proteins involved in peroxisome biogenesis) displaying chaperone-like activity. Potential candidates include members of the Hsp70 family of proteins (Walton et al., 1994; Harano et al., 2001), valosin-containing protein (Murakami et al., 2013), and Pex5p, the import receptor for peroxisomal matrix proteins containing a C-terminal peroxisomal targeting signal (PTS1) (**Figure 1**) (Freitas et al., 2011). Importantly, as the peroxisomal matrix protein translocation machinery can also accommodate the import of unfolded proteins (Brocard et al., 2003), one would expect peroxisomes to contain classical Hsps (e.g., members of the Hsp70 superfamily). Here it should be mentioned that Hsc70 molecules can be co-imported into peroxisomes by interacting with unfolded PTS1-bearing albumin (Brocard et al., 2003). In addition, one cannot exclude the possibility that the peroxisomal matrix harbors other proteins having a chaperone-like activity. One such protein may be the peroxisomal Lon protease (see below), of which the *Penicillium chrysogenum* orthologue has been shown to possess chaperone activity *in vitro* (Bartoszewska et al., 2012). Finally, peroxisome formation and maintenance also require the proper assembly of membrane proteins. In this context, it should be emphasized that Pex19p, the cycling import receptor for newly synthesized PMPs, also exhibits chaperone-like activity (**Figure 1**) (Jones et al., 2004). In addition, it has been reported that *in vitro* translated PMP22 forms a complex with TRiC (**Figure 1**) (Pause et al., 1997), a cytosolic chaperonin known to fold a large number of protein substrates (Spiess et al., 2006).

Several proteins in the peroxisomal matrix are post-translationally processed by specific proteases (Okumoto et al., 2011). In addition, as peroxisomes constantly produce ROS (Fransen et al., 2012), the presence of sophisticated intraperoxisomal quality control mechanisms is essential. Damaged, oxidized and misfolded proteins need to be degraded in order to maintain peroxisome proteostasis and function. To date, three proteases have been identified in mammalian peroxisomes, including insulin degrading enzyme (IDE) (Authier et al., 1994), peroxisomal Lon protease (LONP2) (Kikuchi et al., 2004), and trypsin domain-containing protein 1 (Tysnd1) (**Figure 1**) (Kurochkin et al., 2007). IDE has been shown to degrade the cleaved leader peptide of the peroxisomal enzyme thiolase as well as oxidized lysozyme, a model substrate for oxidized proteins (Authier et al., 1994; Morita et al., 2000). LONP2 is a multifunctional protein that has chaperone-like functions (see above) and displays proteolytic activity toward (superfluous) β-oxidation enzymes (Yokota et al., 2008; Okumoto et al., 2011). The *P. chrysogenum* orthologue of this protein can degrade oxidized proteins *in vitro*, and an inactivation of its function has been shown to be associated with the formation of protein aggregates in the peroxisomal matrix and enhanced oxidative stress (Bartoszewska et al., 2012). In this context, it is important to note that LONP1, the mitochondrial Lon protease, is the most important quality control protease in the mitochondrial matrix, where it selectively degrades damaged, unassembled and misfolded proteins (Venkatesh et al., 2012). Finally, Tysnd1 has been shown to be responsible for the specific processing of β-oxidation enzymes in the peroxisomal matrix (e.g., the removal of leader peptide of 3-ketoacyl-CoA thiolase A) (Kurochkin et al., 2007; Mizuno et al., 2013). Interestingly, a recent study has shown that the proteolytic activities of Tysnd1 and LONP2 cooperatively regulate peroxisomal fatty acid β-oxidation (Okumoto et al., 2011). Taken together, these findings clearly show that mammalian peroxisomes contain a highly sophisticated protease-dependent house-keeping system to ensure protein quality within the organellar matrix.

Some time ago, it was demonstrated that the turnover rates of some PMPs (e.g., Pex3p and Pex16p) are much faster than that of matrix proteins (Matsuzaki and Fujiki, 2008; Huybrechts et al., 2009), and that the half-life of these PMPs can be extended by inhibiting the ubiquitin-proteasome system (UPS) (Huybrechts et al., 2009). These observations indicate that the peroxisomal membrane continuously undergoes quality control mechanisms in order to remove unnecessary or dysfunctional membrane proteins (**Figure 1**). Unfortunately, the mechanisms underlying this process remain unclear. However, in this context, it is necessary to mention that—as part of a quality control mechanism—membrane-associated PTS (co-)receptors (e.g., Pex5p, Pex7p, and Pex20p) also can be degraded by the UPS, at least in several organisms (**Figure 1**) (Léon et al., 2006; Cui et al., 2013). Under normal conditions, Pex5p and Pex20p become monoubiquitinated at a conserved cysteine residue. This triggers the subsequent ATP-dependent dislocation of these receptors from the peroxisomal membrane back to the cytosol where they become available for a new import cycle (Grou et al., 2009). However, under conditions where export of these receptors is impaired, these peroxins are polyubiquitinated on one or more lysines in their N-terminal tails and extracted from the peroxisomal membrane for degradation by the UPS by a process called RADAR (for Receptor Accumulation and Degradation in the Absence of Recycling) (Léon et al., 2006). As RADAR closely resembles ERAD (for Endoplasmic Reticulum-Associated protein Degradation) (Gabaldón et al., 2006; Schlüter et al., 2006), a pathway in which defective proteins in the ER are exported back to the cytosol for proteasomal degradation (Brodsky, 2012), it is tempting to speculate that peroxisomal matrix proteins also may exit the organelle for cytosolic degradation (**Figure 1**). This hypothesis is in line with the recent observation that, in plants, the efficient degradation of peroxisomal matrix proteins involves Pex6p, an AAA (ATPase Associated with various cellular Activities)-ATPase that is part of the Pex5p export machinery (Burkhart et al., 2013). Finally, it has been reported that in plant cells the degradation of peroxisome-associated Pex7p is triggered by binding to RabE1c, a small Ras-related GTPase (Cui et al., 2013). Note that, as Pex5p

levels are drastically reduced in skin fibroblasts from PBD patients that are blocked in normal receptor recycling (e.g., cells lacking Pex1p or Pex6p activity) (Dodt and Gould, 1996), the RADAR quality control pathway is most likely also conserved in mammals (**Figure 1**). So far, there is no evidence for a UPS-mediated degradation mechanism of mammalian Pex7p.

PEROXISOME DEGRADATION

To maintain a healthy cellular peroxisome population, dysfunctional and superfluous organelles need to be selectively removed. Throughout the years, several half-life studies on peroxisomal proteins have been performed, and—if one assumes that peroxisomes are degraded as a whole—most data indicate a peroxisomal half-life of around 1.5–2 days (Price et al., 1962; Poole et al., 1969; Huybrechts et al., 2009). This turnover process may occur randomly (e.g., as part of bulk sequestration of the cytoplasm) or selectively. However, one must assume that a cell—in order to assure a functional peroxisome population—preferably and specifically degrades non-functional organelles. Below we discuss the concepts of peroxisome removal, the initial triggers for peroxisomal degradation, and the factors for dysfunctional peroxisome recognition.

CONCEPTS OF PEROXISOME REMOVAL

Until now, ample evidence has been provided that peroxisomes are mainly degraded by the autophagy-lysosome pathway, in a process known as pexophagy (see below). In addition, it has been suggested that these organelles can be targets for 15-lipoxygenase (15-LOX)-mediated autolysis. Both degradation pathways are discussed in the following paragraphs.

Autophagy is a highly conserved intracellular pathway that delivers cytoplasmic substrates to lysosomes for subsequent degradation (Choi et al., 2013). Under basal conditions, this process provides a mechanism for the removal of long-lived proteins and the turnover of superfluous and damaged organelles (Mizushima et al., 2011). However, this degradation pathway can also be upregulated in response to different stress conditions such as hypoxia, heat, and starvation. Yeast genetics has been crucial for the elucidation of the molecular machinery responsible for autophagy, and, to date, 36 AuTophaGy-related (*ATG*) genes have been identified (Motley et al., 2012). Of these, many are part of the core autophagy machinery essential for the formation of canonical autophagosomes (see below), whereas others function only in different selective autophagy pathways (Mizushima et al., 2011). For more details regarding the molecular mechanisms of autophagy, we refer the reader to other excellent reviews (Klionsky et al., 2011; Mizushima et al., 2011).

Until now, three major types of autophagy have been characterized in eukaryotic cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). During macroautophagy, parts of the cytoplasm are engulfed within double- or multi-membrane delimited structures known as autophagosomes, which subsequently fuse with lysosomes where cargo is released (Mizushima et al., 2011). In contrast, microautophagy involves the direct engulfment of cytoplasmic portions at the lysosome by invagination, protrusion or septation of the lysosomal membrane (Chen and Klionsky, 2011). Finally, CMA is dependent on chaperones which selectively target cytosolic proteins containing a pentapeptide motif (KFERQ) to the lysosomal surface, where the protein is unfolded and transported across the membrane (Kaushik and Cuervo, 2012). Since CMA only degrades cytosolic proteins (Kaushik and Cuervo, 2012) and selective organellar microautophagy has not been unambiguously proven to take place in mammals (Mijaljica et al., 2011), macroautophagy is widely believed to be the major, if not only, pathway for organelle degradation in mammalian cells.

In 1966, de Duve and Baudhuin were the first scientists to discuss the occasional appearance of peroxisomes within autophagosomes, but thought that lysosomal degradation by itself was insufficient to account for the high cellular turnover of catalase (de Duve and Baudhuin, 1966). Since then, several studies on cultured cells have shown that in the presence of 3-methyladenine (3-MA), a macroautophagic inhibitor, peroxisome degradation is strongly inhibited (Luiken et al., 1992; Kondo and Makita, 1997; Huybrechts et al., 2009). After the discovery of peroxisome proliferators (Reddy et al., 1980), a new method became available to study the degradation of superfluous peroxisomes in rodents. As already mentioned above (see section Regulation of Peroxisome Abundance), treatment of these animals with hypolipidemic drugs massively increases the number of peroxisomes, which—after removal of stimulus—rapidly returns to basal levels. However, the mechanism by which peroxisomes disappear remained enigmatic until 1993, when excess peroxisomes were detected within autophagosomes and lysosomes upon addition of the lysosomal protease inhibitor leupeptin (Yokota, 1993). More recently, these observations were confirmed and extended to be macroautophagy-dependent (Iwata et al., 2006). This conclusion was based on the observation that degradation of proliferated peroxisomes was impaired in autophagy-deficient ($Atg7^{-/-}$) mouse hepatocytes (Iwata et al., 2006). Nevertheless, as peroxisome abundance still slightly decreased upon withdrawal of the proliferation stimulus, peroxisomes can most likely also be degraded by other mechanisms.

Another mechanism proposed to play a role in peroxisome degradation is 15-LOX-dependent membrane autolysis (**Figure 1B**). Lipoxygenases are a family of monomeric non-heme, non-sulfur iron dioxygenases, which catalyze the conversion of poly-unsaturated fatty acids (PUFAs) into conjugated hydroperoxides (Maccarrone et al., 2001). The actions of 15-LOX are thought to be important for organellar degradation in reticulocytes, central fiber cells of the eye lens, and keratinocytes (van Leyen et al., 1998). In these cells, the expression of 15-LOX peaks just before organellar degradation occurs (van Leyen et al., 1998). The potential role of 15-LOX has been strengthened by the observation that organellar degradation in these cells occurs independently of autophagy (Matsui et al., 2006), although this is an issue under debate (Betin et al., 2013). About a decade ago, it was shown that—in rat liver—peroxisomal membranes were disrupted when cells were fixed in medium conserving 15-LOX activity (Yokota et al., 2001). This process was effectively blocked upon addition of the 15-LOX inhibitors esculetin and propyl gallate (Yokota et al., 2001). In addition, it is important to note that 15-LOX was shown to colocalize with some, albeit not all peroxisomes (Yokota et al., 2001). Taken together, these finding suggest

that peroxisomes, depending on the cell type and/or their membrane lipid composition, may be targets for 15-LOX-mediated autolysis.

Finally, one cannot rule out that in certain cell types and/or under specific environmental conditions, peroxisome degradation may occur through other mechanisms. In this context, it is interesting to note that (1) there is some experimental evidence that cell organelles may also be degraded by non-conventional Atg5/Atg7-independent autophagy pathways (Nishida et al., 2009; Juenemann and Reits, 2012), and (2) inhibition of cellular respiration and uncoupling of oxidative phosphorylation in HeLa cells resulted in the selective elimination of dysfunctional mitochondria by a novel mechanism involving the formation of "mitoptotic bodies," which are subsequently extruded from the cells (Lyamzaev et al., 2008).

RECOGNITION FACTORS AND ADAPTOR PROTEINS FOR PEROXISOME REMOVAL

Mounting evidence suggests that autophagy is a more selective process than originally anticipated. Most of the pioneering studies on pexophagy have been done using methylotrophic yeasts as the model organism. Working with these yeasts has several advantages, including the relative ease by which peroxisome number, volume and content can be modulated by shifts in growth medium, and the fact that it is rather straightforward to genetically modify these organisms (Manjithaya et al., 2010). Below, we therefore include data from different organisms in order to get a clearer picture of mammalian pexophagy. Until now, every selective autophagy pathway requires the involvement of specific cargo receptors (Till et al., 2012). These receptors, which act independently or together with specific adaptor proteins, recognize their substrates and connect them with one or more components of the core autophagic machinery to allow their specific sequestration (Johansen and Lamark, 2011). To date, at least five autophagic receptors have been identified in mammals: p62, NDP52, optineurin, NIX, and NBR1 (Behrends and Fulda, 2012). These receptors work alone or co-operatively in targeting their substrates for selective degradation (Johansen and Lamark, 2011). The modular composition of binding domains and motifs in these receptors ensures efficient tethering of cargo to the site of developing and engulfing autophagosomes (Behrends and Fulda, 2012). Common for most of these receptors is that they contain both an LC3-Interacting Region (LIR) and a Ubiquitin-Binding Domain (UBD) (Behrends and Fulda, 2012). LC3 and its homologues GABARAP and GATE-16 are ubiquitin-like proteins that are synthesized as precursors and—upon autophagy induction—processed and localized to the autophagosomal membranes (Mizushima et al., 2011). The LIR and UBD domains render the adaptors capable of bridging a ubiquitinated substrate (e.g., organelles, protein aggregates, and bacteria) with the autophagic machinery, thereby selectively triggering degradation of the cargo.

Until now, at least three pexophagy receptors have been identified, including Atg30 (for *P. pastoris* and related yeasts) (Farré et al., 2008), Atg36 (for *Saccharomyces cerevisiae* and similar yeasts) (Motley et al., 2012) and NBR1 and/or p62 (for mammalian cells) (Kim et al., 2008; Deosaran et al., 2013). These proteins bridge peroxisomes with developing autophagosomes by simultaneously binding to protein(s) at the peroxisomal membrane and the autophagic machinery via different structural motifs (Till et al., 2012). The *P. pastoris* peroxisome receptor Atg30 interacts with peroxisomes through two PMPs, Pex3p, and Pex14p, and with the autophagic machinery via Atg11 and Atg17 (Farré et al., 2008). *S. cerevisiae* Atg36, another Atg11-interacting protein, is also recruited to peroxisomes in a Pex3p-dependent manner (Motley et al., 2012). Interestingly, both Atg30 and Atg36 are regulated by phosphorylation (Farré et al., 2013), trigger pexophagy upon overexpression (Farré et al., 2008; Motley et al., 2012), and interact with Atg11 (Farré et al., 2008; Motley et al., 2012). Atg11 is thought to function as a common adaptor protein for most, if not all, selective autophagy pathways in yeasts (Manjithaya et al., 2010). Note that, despite their functional similarities, Atg30 and Atg36 do not display any sequence homology (van der Zand and Reggiori, 2012).

Less is known about the selective pexophagy receptors in mammals. However, some years ago, it was discovered that peroxisomes can be degraded in a p62-dependent manner upon overexpression of a PMP (in this case PMP34 and Pex3p) fused to a cytosol-facing ubiquitin moiety (**Figure 1B**) (Kim et al., 2008). This phenotype can be significantly increased by employing a mutated ubiquitin protein incapable of being polyubiquitinated, thus eliminating proteasome-dependent removal of the proteins from the peroxisomal membrane (Kim et al., 2008). In addition, a recent study showed that pexophagy was triggered upon overexpression of NBR1, another adaptor protein (**Figure 1B**) (Deosaran et al., 2013). However, similar overexpression of p62 did not yield the same results, indicating that NBR1 most likely functions as endogenous pexophagy adaptor in mammals (Deosaran et al., 2013). This might stem from the fact that, even though these proteins share LIR and UBD domains, NBR1—but not p62—contains a membrane interacting amphipathic α-helical JUBA domain, capable of binding to the peroxisomal lipid bilayer (Deosaran et al., 2013). Nevertheless, it cannot be excluded that these proteins co-operate during pexophagy. Finally, it should be mentioned that, unlike Atg30 and Atg36, both p62, and NBR1 have been implicated in the selective degradation of other cargoes (Johansen and Lamark, 2011). Taken together, these data clearly indicate that mammalian pexophagy is regulated by at least one, and perhaps even more, of the currently identified autophagy receptors. An intriguing question that has risen from these studies is how these receptors recognize peroxisomes as their targets.

PEROXISOMAL COMPONENTS NECESSARY FOR PEXOPHAGY

Currently, most data point to a role of Pex3p and/or Pex14p in the recruitment of pexophagy-specific receptor proteins. For example, while in *H. polymorpha* peroxisome degradation is triggered by the removal of Pex3p (Bellu et al., 2002; van Zutphen et al., 2011), studies in *P. pastoris* and *S. cerevisiae* have shown that this peroxin is essential to recruit Atg30 and Atg36, respectively, to the peroxisome prior to degradation (Farré et al., 2008; Motley et al., 2012). In addition, *P. pastoris* Atg30 has been reported to interact with Pex14p (Farré et al., 2008), and the N-terminal 64 amino acids of this peroxin are required for pexophagy in *H. polymorpha*

(Bellu et al., 2001; van Zutphen et al., 2008). Interestingly, by redirecting Pex3p to the mitochondrial outer membrane in yeast cells lacking Atg32, the mitochondria-specific autophagy receptor (Okamoto et al., 2009), it is possible to recruit Atg36 to this organelle and trigger mitophagy (Motley et al., 2012).

In mammals, there is some evidence that Pex14p may play a role in pexophagy by interacting with LC3-II during nutrient-starvation (**Figure 1B**) (Hara-Kuge and Fujiki, 2008). In addition, a recent study by Deosaran and colleagues suggests that (mono)-ubiquitination of endogenous PMPs can trigger pexophagy (Deosaran et al., 2013). Unfortunately, no such protein has yet been identified. One potential candidate is Pex5p, which needs to be mono-ubiquitinated at the peroxisomal membrane in order to be recycled back to the cytosol (Platta et al., 2013). Indeed it has recently been observed that by inhibiting Pex5p recruitment to peroxisomes via down-regulation of Pex14p, pexophagy is—at least partly—prevented upon overexpression of NBR1 (Deosaran et al., 2013). However, since (1) Pex14p is heavily implicated in yeast pexophagy (Till et al., 2012; see above), and (2) this peroxin interacts with membrane-bound LC3-II during starvation conditions (Hara-Kuge and Fujiki, 2008), one cannot assertively claim that the lack of peroxisome turnover was due to the absence of Pex5p, and not to Pex14p (or any other Pex14p-interacting factor), at the peroxisomal membrane.

TRIGGERS FOR PEROXISOME DEGRADATION

Although relatively much is known about the concepts and recognition factors of peroxisome degradation, less data exist regarding the triggers for this process. As mentioned before, both superfluous and dysfunctional organelles need to be removed in order to maintain cellular homeostasis. The turnover of superfluous peroxisomes can be induced by returning to growth conditions in which the necessity of peroxisomes is reduced (see section Regulation of Peroxisome Abundance). In addition, overexpression of the pexophagy receptors Atg30, Atg36, and NBR1 has been shown to trigger peroxisome degradation by binding simultaneously to peroxisomes and the autophagic machinery (see section Recognition Factors and Adaptor Proteins for Peroxisome Removal). Accumulating evidence indicates that the initial signal for peroxisome degradation resides at the peroxisomal membrane, and that changes in its composition may be the key for pexophagy induction. This gives rise to a burning question in the field: how is, at a given time point, a select set of peroxisomes recognized by the autophagic machinery whereas others are not? A potential answer to this question could reside in the existence of peroxisomal subpopulations, where some peroxisomes are protected from degradation. In this context, it is important to mention that, in yeasts, at least one peroxisome is protected from degradation under pexophagy-inducing conditions (Leao-Helder et al., 2003). In addition, even high overexpression of pexophagic receptors does not yield a total cellular lack of peroxisomes (Farré et al., 2008; Motley et al., 2012; Deosaran et al., 2013). Furthermore, it is likely that organelle morphology may also play a role. For example, it has been shown that—during starvation-induced autophagy—mitochondria elongate and are therefore protected from mitophagy (Gomes et al., 2011). Since (1) peroxisomal morphogenesis is a very dynamic process (Ribeiro et al., 2012), and (2) peroxisomes are commonly elongated during proliferation (Schrader et al., 2012), one could envisage a similar protective mechanism for these organelles. In this context, it is worthwhile mentioning that studies in *P. pastoris* have shown that the larger the peroxisome, the more cargo-specific Atg proteins are essential for its sequestration (Nazarko et al., 2009).

Other essential questions that need to be addressed include the identity and order of events that lead to the substrate recognition signal at the peroxisomal membrane. In this context, one can envisage that the signal for peroxisome degradation stems from the peroxisomal matrix. Indeed, as (1) peroxisomes are important regulators of both ROS and lipid metabolism (Van Veldhoven, 2010; Fransen et al., 2012), and (2) it has been shown that inhibition of autophagy with 3-MA increased the amount of peroxisomes with a disturbed redox state (Ivashchenko et al., 2011), it is tempting to speculate that a disrupted redox equilibrium can lead to oxidation-specific peroxisomal membrane modifications, such as lipid peroxidation. Here it is also important to mention that mitochondria are depolarized and subjected to mitophagy upon compartment-specific ROS-generation (Kim and Lemasters, 2011; Wang et al., 2012). However, since peroxisomes do not contain a membrane potential, a similar mechanism seems unlikely to occur for these organelles. Nevertheless, as (1) it is well-known that the PTEN Induced Putative Kinase 1 (PINK1)-dependent recruitment of the E3 ubiquitin ligase Parkin to the mitochondrial outer membrane can trigger mitophagy (Lazarou et al., 2012; Ashrafi and Schwarz, 2013), and (2) targeting ectopically expressed PINK1 to the peroxisomal membrane recruits Parkin to these organelles and triggers pexophagy (Lazarou et al., 2012), it is very likely that the peroxisome- and mitochondria-specific turnover mechanisms converge at an early step. This hypothesis is also in line with the observation that mitochondria-targeted Pex3p triggers mitophagy in *H. polymorpha* mutants lacking Atg32 (see section Peroxisomal Components Necessary for Pexophagy) (Motley et al., 2012).

It is widely known that mitochondria harbor complex fusion and fission machineries, which allow them to mix, segregate and eliminate damaged components from the functional networking population (Twig et al., 2008). In addition, there is abundant evidence that mitochondrial dynamics and mitophagy are closely related, and that a dysfunctional mitochondrion has to be separated from the mitochondrial network before it can be sequestered by an autophagosome (Ashrafi and Schwarz, 2013). However, as peroxisomes cannot fuse with one another (Huybrechts et al., 2009; Bonekamp et al., 2012), alternative mechanisms must assist in assuring a healthy organelle population. One such mechanism may be asymmetric fission. This would render peroxisomes capable of sequestering non-functional proteins into the mother organelle, which—after a limited number of fission events—is targeted for pexophagy (Huybrechts et al., 2009; Delille et al., 2010). In this context, it is important to mention that, in *H. polymorpha*, protein aggregates within the peroxisomal lumen can be eliminated by the concerted action of asymmetric fission and subsequent autophagic degradation of the aggregate-containing organelle (Manivannan et al., 2013).

PHYSIOLOGICAL ROLE OF PEXOPHAGY

As already mentioned above (see section Physiological Importance of Peroxisome Homeostasis), peroxisomes play a prominent role in a variety of cellular metabolic and signaling processes. As such, a tight regulation of peroxisome biogenesis, dynamics, and degradation is important for human health. Over the years, it has become increasingly clear that not only defects in peroxisome biogenesis, but also disturbances in peroxisome degradation can contribute to disease (see section Physiological Importance of Peroxisome Homeostasis). This is illustrated below by three specific examples.

First, there is accumulating evidence that defects in pexophagy can facilitate the cellular aging process. For example, it is already known for more than a decade that the number of peroxisomes profoundly increases during cellular aging, and that these organelles display a reduced capacity to import matrix proteins, especially catalase (Legakis et al., 2002). Since (1) these cells contain peroxisomes with a disturbed H_2O_2 metabolism (Legakis et al., 2002), (2) there is strong evidence that oxidative stress plays a key role in the etiology and progression of cellular senescence (Salmon et al., 2010), and (3) the latter process is causally linked to organismal aging (Baker et al., 2011), it is very likely that these dysfunctional peroxisomes directly contribute to the age-related phenotype (Koepke et al., 2008). As these age-related changes in peroxisome number, matrix protein import, and ROS production can be mimicked in a *H. polymorpha* strain lacking Atg1, a crucial member of the core autophagic machinery (Aksam et al., 2007), these phenotypes in all probability result from an age-related decline in lysosomal function or pexophagy-specific factors.

Next, it has also been postulated that pexophagy is essential to maintain functional peroxisomes during endotoxin-induced stress (Vasko et al., 2013). In this study, it was shown that exposure of human vascular endothelial cells or mice to lipopolysaccharides (LPS) selectively induced pexophagy, and that inhibition of this process (e.g., by treating the cells with chloroquine or by employing lysosome-defective Lyst-mice) resulted in the accumulation of functionally compromised peroxisomes, an altered cellular redox equilibrium, and aggravated renal damage.

Finally, as it is well-known that the decreased autophagic flux observed in various lysosomal storage diseases (LSDs) often leads to an accumulation of dysfunctional mitochondria and cytoplasmic protein aggregates (Platt et al., 2012), the same is most likely true for peroxisomes. LSDs are a family of genetic disorders that perturb lysosomal homeostasis by the accumulation of specific macromolecules or monomeric catabolic products inside organelles of the endosomal-autophagic-lysosomal system (Platt et al., 2012). Interestingly, some LSDs such as Niemann-Pick disease type 1 (Schedin et al., 1997) and Krabbe disease (Haq et al., 2006) have also been associated with peroxisome dysfunction. In addition, as (1) a-series gangliosides and their precursor are common secondary storage metabolites in many LSDs, and (2) these compounds also increase in PBDs, it is very likely that peroxisomal dysfunction underpins secondary ganglioside storage in LSDs (Platt et al., 2012). Taken together, these data indicate that a disturbance in pexophagy may have a significant negative impact on human health and function.

CONCLUSIONS AND PERSPECTIVES

From the cumulative evidence presented in this review, it is clear that macroautophagy plays a pivotal role in the removal of obsolete peroxisomes in mammalian cells. However, as (1) these organelles can also be degraded under conditions where the conventional macroautophagy pathway is inactivated (Iwata et al., 2006), and (2) macroautophagy does not seem to be responsible for organelle turnover during lens and erythroid differentiation (Matsui et al., 2006), it is very probable that other cell- and/or condition-specific peroxisome degradation pathways exist. Candidate pathways may include micropexophagy, Atg5/Atg7-independent macropexophagy, and 15-LOX-mediated degradation. Importantly, crucial *in vivo* evidence for the presence of these or other peroxisome turnover routes is currently lacking. Yet, the recent identification of NBR1 as potential pexophagy receptor may shed more light on this issue (Deosaran et al., 2013). However, given the functional similarities of mammalian autophagy receptors (Behrends and Fulda, 2012), it is likely that by inactivating NBR1 (e.g., in cells or in an animal model) other autophagic receptors may shoulder the role of this protein. In addition, as NBR1 has been shown to be involved in other ubiquitin-regulated autophagy pathways (Kirkin et al., 2009), the phenotype observed upon NBR1 inactivation will not be solely due to impaired peroxisome degradation.

Virtually all experimental data suggest that, at least in yeasts, peroxisome degradation is a highly selective process (Manjithaya et al., 2010; Till et al., 2012). Although not yet unambiguously proven, several arguments support the view that this is also true for mammals. For example, peroxisomes induced by PPARα-agonists are selectively removed upon withdrawal of the proliferation stimulus (Yokota, 1993). In addition, although Pex14p has been shown to interact with LC3-II during nutrient starvation, peroxisome degradation only occurred when the cells were re-cultured in a normal medium (Hara-Kuge and Fujiki, 2008). These data are in line with the finding that starvation-induced autophagy of cell organelles occurs in an ordered fashion (Kristensen et al., 2008).

The observation that, in yeasts, peroxisome biogenesis and degradation converge on Pex3p and Pex14p offers the intriguing possibility that these peroxins may act as peroxisome fate decision makers. Whether this is also the case in mammals remains to be established. In this context, it is important to mention that (1) mammalian Pex14p can directly bind to Pex5p and LC3-II, and (2) the affinity of Pex14p for these proteins depends on the culture conditions (with Pex5p and LC3-II being the preferred interaction partner under nutrient-rich and starvation conditions, respectively) (**Figure 1B**) (Hara-Kuge and Fujiki, 2008). Interestingly, Pex14p is also capable of forming distinct oligomeric complexes at the peroxisomal membrane (Itoh and Fujiki, 2006). As the functions of these complexes are not yet characterized, it is tempting to speculate that some may fulfill a specific role in peroxisome turnover.

One of the most challenging aspects within the field is the identification of potential triggers for peroxisome degradation. As (1) excessive ROS-generation in mitochondria has been shown to trigger mitophagy (Kim and Lemasters, 2011; Wang et al., 2012), and (2) peroxisomes produce large amounts of ROS as

part of their normal metabolism (Fransen et al., 2012), it is very likely that also disturbances in peroxisomal redox metabolism may provoke signaling/damaging events that lead to structural changes in the peroxisomal membrane and ultimately result in organelle degradation. One such modification may be lipid peroxidation. Alternatively, the initial trigger may be generated by changes in peroxisomal lipid metabolism, a condition likely to affect the organellar membrane composition. In this context, it is worthwhile mentioning that (1) DHA, a PUFA synthesized by peroxisomal β-oxidation (Van Veldhoven, 2010), can promote negative membrane curvature (Bruno et al., 2007), and (2) NBR1, the putative pexophagy receptor in mammalian cells, contains a lipid binding domain that inserts into the peroxisomal membrane bilayer in a curvature-dependent manner (Mardakheh et al., 2010; Deosaran et al., 2013). These findings and the observation that DHA can also mediate peroxisome elongation (Itoyama et al., 2012) suggest that peroxisomal β-oxidation, peroxisome morphology, and pexophagy are closely intertwined processes. Importantly, such a mechanism would closely resemble that of mitochondria in that dysfunctional spherical organelles are segregated from a tubular network prior to degradation (Ashrafi and Schwarz, 2013).

Finally, it is very likely that the implications of dysfunctional peroxisome degradation have been overlooked throughout the years. In this context, it is essential to take into account that an increase in peroxisome number (e.g., during cellular aging) is not necessarily due to an augmentation of peroxisome biogenesis, but can also result from a decrease in peroxisome turnover rates. Unfortunately, with the current lack of animal models that are selectively deficient in peroxisome degradation, it is virtually impossible to predict the severity of the phenotype and/or the course of the disease of patients suffering from this impairment. However, given the recent breakthroughs in this field, we are convinced that such disease models will soon be available, and answers to these important questions rapidly obtained.

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Peroxisome deficient invertebrate and vertebrate animal models

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Although peroxisomes are ubiquitous organelles in all animal species, their importance for the functioning of tissues and organs remains largely unresolved. Because peroxins are essential for the biogenesis of peroxisomes, an obvious approach to investigate their physiological role is to inactivate a *Pex* gene or to suppress its translation. This has been performed in mice but also in more primitive organisms including *D. melanogaster*, *C. elegans,* and *D. rerio*, and the major findings and abnormalities in these models will be highlighted. Although peroxisomes are generally not essential for embryonic development and organogenesis, a generalized inactivity of peroxisomes affects lifespan and posthatching/postnatal growth, proving that peroxisomal metabolism is necessary for the normal maturation of these organisms. Strikingly, despite the wide variety of model organisms, corresponding tissues are affected including the central nervous system and the testis. By inactivating peroxisomes in a cell type selective way in the brain of mice, it was also demonstrated that peroxisomes are necessary to prevent neurodegeneration. As these peroxisome deficient model organisms recapitulate pathologies of patients affected with peroxisomal diseases, their further analysis will contribute to the elucidation of still elusive pathogenic mechanisms.

Keywords: inflammation, male fertility, phytanic acid, plasmalogens, PUFA, very long chain fatty acids, Zellweger syndrome

INTRODUCTION

Absence of peroxisomes in man leads to a devastating disease, clinically known as the hepato-renal syndrome of Zellweger. Affected baby's are born alive, but are severely hypotonic, mentally retarded with brain malformation, liver and kidney problems, and die generally with the first weeks of life (Wanders and Waterham, 2005). Understanding the anomalies at the cellular and organ level and the malformation during development in such patients with a peroxisome biogenesis disorder, requires access to suitable experimental material. Unfortunately, for man the sources are rather limited (fibroblasts, lymphoblasts, amniotic villi), and not representative for specialized cells/tissues. In addition, no natural occurring or inducible animal model is known. Hence, as soon as appropriate molecular techniques were established, animal models were created, starting of with PEX5 [1](Baes et al., 1997) and PEX2 (Faust and Hatten, 1997) deficient mice in 1997, followed later by inactivation of peroxins in other laboratory "pet-animals" like worms, fruitfly, or zebrafish.

In addition to these animal models, peroxisome deficient mutants were created in different yeasts, starting of with baker's yeast (Erdmann et al., 1989), followed by *Hansenula polymorpha* (Cregg et al., 1990) and *Pichia pastoris* (Gould et al., 1992); in filamentous fungi, *Neurospora crassa* (Sichting et al., 2003; Managadze et al., 2007), *Magnaporthe grisea* (Ramos-Pamplona and Naqvi, 2006), *Aspergillus oryzae* (Escano et al., 2009), in plants like *Arabidopsis* (Kaplan et al., 2001; Schumann et al., 2003; Fan et al., 2005), in trypanosomes (Banerjee et al., 2005; Galland et al., 2007). Some of the latter models are described elsewhere in this book, whereas for a treatise on human disorders linked to peroxisomes we refer to (Wanders and Waterham, 2005; Waterham and Ebberink, 2012).

Before discussing in more detail the different animal models, a general description of the metabolic functions of peroxisomes is given, followed by a short note about their biogenesis.

PEROXISOMAL METABOLISM

From a human pathological point of view, the main peroxisomal pathways are β-oxidation, α-oxidation, and ether lipid synthesis, and to a lesser extent glyoxylate metabolism and xanthine metabolism. Whereas peroxisomal β-oxidation seems universally present in all animals, although sometimes serving other purposes, some of the other pathways might be missing in

[1]For the sake of consistency, the nomenclature guidelines as formulated for rodents (http://www.informatics.jax.org/mgihome/nomen/gene.shtml) are followed throughout this text, gene symbols being italicized, first letter capitalized, whereas the protein is referred to by the corresponding gene symbol in standard capitalized font. Guidelines related to nomenclature in other species can be found at following URLs: nematodes (http://www.wormbase.org/about/userguide/nomenclature#fda31g748bjh9c650ie2—10); fruitflies (http://flybase.org/staticpages/docs/nomenclature/nomenclature3.html#1.2.3.); zebrafish (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines#ZFINZebrafishNomenclatureGuidelines-1.1); man (http://www.genenames.org).

lower vertebrates/invertebrates (e.g., etherlipid synthesis). In the following paragraphs the main pathways are briefly described, whereas their specific roles, if known, will be highlighted when discussing the different models (enzymes are named according to the mouse nomenclature).

Typically, peroxisomes can β-oxidize a broad range of natural, often also xenobiotic, compounds containing a fatty acyl side chain with or without a methyl-branch, in α-position of the carboxy-group. This process consists of a sequence of four reactions, resulting in shortening of the main chain of an acyl-CoA by 2 carbons (see **Figure 1**) (Van Veldhoven, 2010). In a first step, acyl-CoA is converted into 2-*trans*-enoyl-CoA by an acyl-CoA oxidase (ACOX), thereby producing H_2O_2. The number of ACOXs varies between species and ACOXs acting on 2-methyl-acyl-CoAs (ACOX2 and ACOX3 in mammals) are stereospecific, only the 2S-isoform is desaturated, hence an additional peroxisomal enzyme, 2-methylacyl-CoA racemase (AMACR), is required to convert the 2R-isoforms. The oxidation is followed by a hydration of the double bond by a 2-enoyl-CoA hydratase, a dehydrogenation by 3-hydroxyacyl-CoA dehydrogenase, and finally a thiolytic cleavage, generating acetyl-CoA (or propionyl-CoA in case of 2-methylbranched acyl-CoA) and a shortened acyl-CoA. Generally more than one enzyme can catalyze each of these steps, either homologous proteins as is the case for ACOXs or totally different proteins, e.g., thiolases encoded by the *Acaa1* or *Scp2* genes, or activities can reside in multi-enzymes (e.g., EHHAHD, also called multifunctional protein 1 (MFP1), HSD17B4, often called MFP2), which catalyze the hydration and dehydrogenation steps in a stereoselective manner. In mammals, a well-characterized β-oxidation pathway is the formation of C_{24}-bile acids, starting from C_{27}-bile acids (cholestanoic acids). In lower vertebrates, such as reptiles, some amphibia, and lungfishes, however, no C_{24}-bile acids are found (Hofmann et al., 2010). On the other hand, the genomes of amphibia, bony fishes and various invertebrates like insects, bivalves, and sea urchins (but not nematodes), encode a peroxisomal AMACR, suggestive for a role of peroxisomes in breakdown of other isoprenoid derived carboxylates in these species.

α-Oxidation is a process whereby fatty acids are shortened by one carbon atom, amply documented for phytanic acid in man, a diet derived 3-methylbranched fatty acid, and less well-known for long chain 2-hydroxy fatty acids (Van Veldhoven, 2010) (see **Figure 2**). For phytanic acid, the process starts with the hydroxylation of phytanoyl-CoA at position 2 (by phytanoyl-CoA hydroxylase, PHYH), followed by a cleavage into formyl-CoA and pristanal, catalyzed by 2-hydroxyacyl-CoA lyase (HACL1). 2-Hydroxy long chain fatty acids do not depend on PHYH and are, after activation, shortened into a (n-1)fatty aldehyde by HACL1. This pathway is present in all mammals, and representative species of birds, reptiles, amphibian, fish, insects, nematodes, echinoderms, cnidaria, ascidia.

In contrast to the bulk of glycerolipids containing ester-linked fatty acids, a small portion of glycerolipids contains an ether bond, the precursor of which is formed by peroxisomal enzymes (see **Figure 3**). A first one, dihydroxyacetone-phosphate acyltransferase (GNPAT) generates an obligate precursor, 1-acyl-dihydroxyacetone-phosphate, a second one catalyzes the exchange of the acyl for an alcohol (alkyl dihydroxyacetone-phosphate synthase, ADHAPS). After reduction, the generated 1-alkylglycerol-3-phosphate follows the same anabolic routes as 1-acylglycerol-3-phosphate in the ER, leading to neutral and phosphoglycerolipids with a 1-alkyl group. In mammals, 1-alkyl-2-acylglycerophosphoethanolamine is desaturated just adjacent to the ether linkage, generating plasmenylethanolamine which can be converted to the choline analogue. Phospholipids with this vinylether group are better known as plasmalogens. Based on genomic information, the key enzymes GNPAT and ADHAPS are expressed in nematodes, cnidaria, echinoderms, insects, fish, amphibia, reptiles and birds. The presence of plasmalogens will however, depend on the expression of plasmanylethanolamine desaturase, an orphan enzyme not yet cloned. Besides mammals, plasmalogens have been identified in various animals including birds, amphibia, fish, insects, molluscs, marine worms, jelly fish, echinodermata, slime mold (Horrocks and Sharma, 1982).

Depending on species, peroxisomes or related organelles (glyoxysomes) are more or less actively involved in glyoxylate metabolism and in the degradation of purines (purine oxidation pathway or ureide pathway). Depending on the phylogenetic position of the species, purines are degraded till the level of ureum (amphibian, fish) or only till uric acid (man).

PEROXISOME BIOGENESIS

Proteins involved in the formation (biogenesis) of peroxisomes were first identified in yeast (Erdmann et al., 1989), and the major players in this process are rather well-conserved throughout the different kingdoms. In yeast and lower eukaryotes, however, more peroxins are found that are involved in fission/fusion processes and regulation of the number of peroxisomes[2], which is related to the fact that these species must be able to adapt their intracellular organelles quickly to changes in their environment. Briefly for animals, peroxisomal matrix proteins, synthesized in the cytosol, are captured by binding partners that recognize a specific motif within their primary sequence, either a C-terminal tripeptide, better known as Peroxisome Targeting Signal 1 (PTS1) which is recognized by PEX5, or an N-terminal nonapeptide (PTS2), bound by PEX7 (see **Figure 4**). Upstream residues of PTS1, often referred to as SKL-sequence, influence binding to Pex5, hence a more in depth analysis of the interaction has broadened PTS1 to a dodecamer (Brocard and Hartig, 2006). In all species investigated, only a minority of matrix proteins contain PTS2, and in certain species, such as nematodes (*C. elegans;* Motley et al., 2000), diatoms (*Phaeodactylum tricornutum;* Gonzalez et al., 2011), and insects (*Drosophila;* Faust et al., 2012), PEX7 is even missing. In those organisms, the classical PTS2 proteins are still associated with peroxisomes, but are decorated with PTS1 (de Vet et al., 1998; Motley et al., 2000; Faust et al., 2012).

PEX5-PTS1 cargoes dock to the peroxisomal membrane via PEX14/PEX13, and are subsequently translocated through the bilayer. At the matrical side of the membrane, the cargo is released

[2]In *sensu stricto* of the original definition (Distel et al., 1996), these proteins should not be called peroxins.

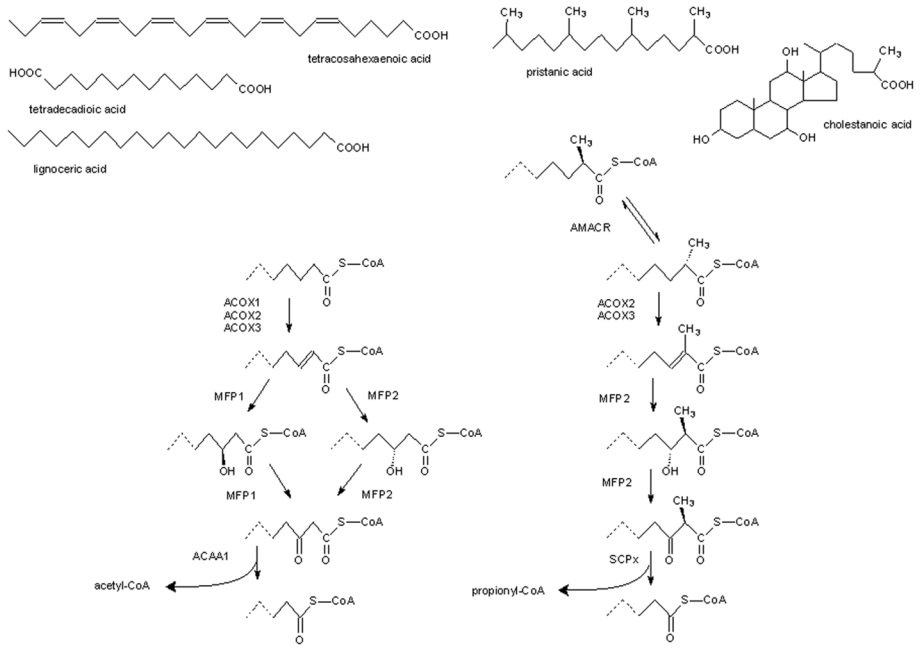

FIGURE 1 | Generalized scheme of peroxisomal β-oxidation in animals. On **top**, structures of some fatty carboxylates that, after activation (not shown), are degraded by peroxisomal β-oxidation. At the **right**, enzymatic reactions/enzymes involved in degradation of substrates containing a 2-methylbranch, based on the situation in mammals. Most of these enzymes can act on straight chain substrates, shown at the **left**, as well. The latter compounds are also recognized by more selective enzymes which do not tolerate a 2-methylbranch. ACAA1, 3-ketoacyl-CoA thiolase; ACOX, acyl-CoA oxidase; AMACR, 2-methylacyl-CoA racemase; MFP, multifunctional protein; SCPx, sterol carrier protein X-thiolase.

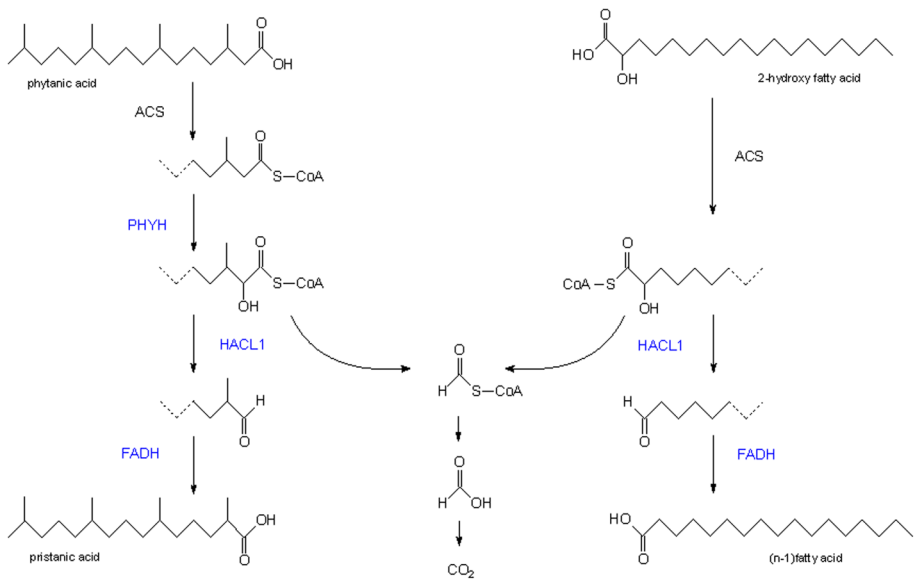

FIGURE 2 | Peroxisomal α-oxidation. Scheme of the enzymatic reactions/enzymes involved in the peroxisomal breakdown of phytanic acid (**left**) and 2-hydroxy long chain fatty acids (**right**). Enzymes in blue are associated with peroxisomes. ACS, acyl-CoA synthetase; FADH, fatty aldehyde dehydrogenase; HACL1, 2-hydroxyacyl-CoA lyase; PHYH, phytanoyl-CoA α-hydroxylase.

and PEX5 recycles to the membrane where it will undergo ubiquitination mediated by the RING-finger proteins PEX2, PEX10, and PEX12, and extracted back to the cytosol via PEX1/PEX6 in an ATP-dependent manner.

PEX7-PTS2 cargo also binds to PEX14, not directly but mediated via a long isoform of PEX5 (PEX5L) in mammals and other vertebrates (**Figure 4**). In fungi, the function of the latter is taken over by PEX18/PEX21 (Dodt et al., 2001).

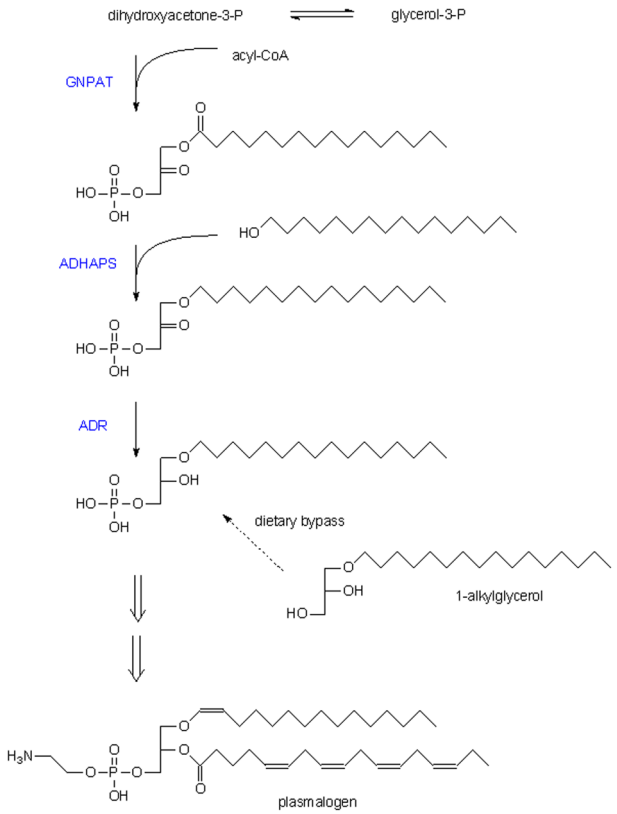

FIGURE 3 | Etherlipid biosynthesis. Scheme of the reactions involved in formation of ether lipids, starting with dihydroxyacetone-phosphate. The long chain alcohol used by ADHAPS it generally 16–18 carbons, either saturated or with one double bond. Double arrows indicate multiple steps, catalyzed by ER-associated enzymes like headgroup addition and the introduction of the double bond in the 1-alkylchain. Generally, plasmalogens are enriched in PUFA at position 2 of the glycerol moiety (shown is 1-hexadecyl-2-arachidonoyl-plasmenylethanolamine). Dietary 1-alkylglycerol can bypass the peroxisomal steps in this pathway (dashed arrow). GNPAT, dihydroxyacetone-phosphate acyltransferase; ADHAPS, alkyl dihydroxyacetone-phosphate synthase; ADR, acyl/alkyl-dihydroxyacetone-phosphate reductase.

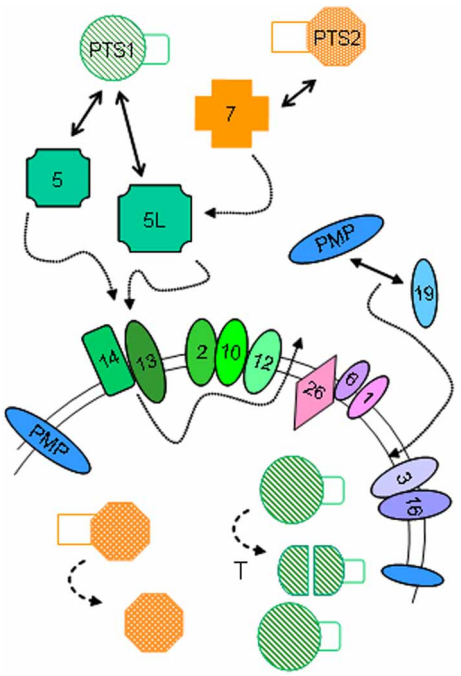

FIGURE 4 | Peroxisome biogenesis in animals. Simplified scheme for the import of PTS1-proteins, PTS2-proteins and integral peroxisomal membrane proteins (PMP) in animals and the involved peroxins, indicated by their PEX number. Role of ubiquitination of PEX5 and farnesylation of PEX19 is not shown. After import, some PTS1-proteins are processed by TYSND1, a peroxisomal protease (T), while the signal peptide of PTS2-proteins is cleaved off. Adapted from Van Veldhoven (2010).

Membrane biogenesis is depending on PEX19, PEX3, and (in animals) PEX16 (Fujiki et al., 2006). PEX19, a mainly cytosolic protein, plays a chaperone like function and binds most newly synthesized integral peroxisomal membrane proteins (PMP), and docks to PEX3, an integral peroxisomal membrane protein.

Finally, size and abundance of peroxisomes are regulated by PEX11 proteins, which play also a role in elongation of the organelles (Thoms and Erdmann, 2005; Koch et al., 2010). In mammals three isoforms are known, in lower animals only one (**Table 1**).

MODELS

In the following sections, laboratory animals in which peroxisome biogenesis has been studied will be described. In **Table 1**, gene symbols and alternative names for peroxins in these animals are listed. Given differences in life cycle and organogenesis, the development of these animals will be shortly described, and specific metabolic roles of peroxisomes, if documented, will be highlighted.

NEMATODES

A fertilized *Caenorhabditis elegans* egg develops into a small worm within the shell, in about 10–12 h. In the preceding 6 h (organogenesis/morphogenesis stage), the spheroid embryo started to elongate while its three germ layers differentiate into organs. After hatching, the post-embryonic development will start and the animal will pass, if food is present, through four larval stages (L1–L4, separated by 7–9 h) to reach sexual maturation, generally as a hermaphrodite, about 1.2 mm long, and will start to produce eggs. The cycle from egg to egg is therefore about 3 days; life span of the worm is 2 weeks.

In the adult nematode, peroxisomes are mainly present in the epithelial cells of the digestive tract, one of the largest organs, and in the pharyngeal gland (Yokota et al., 2002). In the gut, their volume density is 1.86/100 μm^2 cytoplasm, similar to that in rat liver. Similar to rodents, fibrates increase the number of peroxisomes (Yokota et al., 2002). Based on the fluorescence pattern of animals expressing CFP-SKL, larvae contain more and larger peroxisomes than adult worms (Petriv et al., 2002).

In *C. elegans*, peroxisomal β-oxidation serves to generate acyl-CoAs used for the synthesis of dauer pheromone, also called daumone, a mixture of ascarosides which are excreted

Table 1 | Overview of peroxins in animals used for peroxisomal research and in man.

Peroxin[2]	Description	Domain		*Caenorhabditis elegans*[1]	*Drosophila melanogaster*	*Danio rerio*	*Mus musculus*	*Homo sapiens*
PEX1	AAA-ATPase		gi	25153574	21355121	283046720	61657895	4505725
			NP	NP_510386.2	NP_652016.1l	NP_001164306.1	NP_082053.1	NP_000457.1
			alias	prx-1 (isoform a); C11H1.4a	FBgn0013563	793906; ZDB-GENE-070530-1	ZWS1; 5430414H02Rik; E330005K07Rik	PBD1A; PBD1B; ZWS; ZWS1
			aa (MW)	996 (111997)	1006 (113739)	1237 (136665)	1244 (136613)	1283 (142737)
PEX2	E3 ligase	Zinc RING finger	gi	133931002	21355975	189536742	254028168	4506343
			NP	NP_502201.2	NP_648210.1	XP_684073.2	NP_001156773.1	NP_000309.1
			alias	prx-2; ZK809.7	CG7081; DmelPex2; Dmel; CG7081	ZDB-GENE-070530-2	D3Ertd138e; PAF-1; PMP35; Pxmp3	PAF1; PBD5A; PBD5B; PMP3; PMP35; PXMP3; RNF72; ZWS3
			aa (MW)	273 (31194)	281 (32239)	312 (35000)	305 (34813)	305 (34765)
PEX3	PEX9 docking factor		gi	193209553	21357431	41055494	9910484	4505727
			NP	NP_001123111.1	NP_648753.1	NP_956522.1	NP_064345.1	NP_003621
			alias	prx-3; C15H9.8	CG6859; DmelPex3; Dmel\CG6859; FBgn0036484	zgc:56313; fd60g05.y1; ZDB-GENE-040426-979	1700014F15Rik; 2810027F19Rik	PBD10A; TRG18
			aa (MW)	353 (39754)	385 (43562)	364 (41427)	372 (42093)	373 (42009)
PEX5	PTS1-receptor	TPR	gi	71983707	24639189	41055947	472339081	196259772
			NP	NP_001022019.1	NP_569949.3	NP_957450.1	NP_001264259.1	NP_001124496.1
			alias	prx-5; C34C6.6	CG14815; DmelPex5; Dmel\CG14815; EG:63B12.5	PXR1; zgc:56318; ZDB-GENE-040426-981	AW212715; ESTM1; PTS1R; Pxr1; X83306	PBD2A; PBD2B; PTS1-BP; PTS1R; PXR1
			aa (MW)	502 (55344)	559 (62994)	600 (67012)	602 (66675)	602 (66699)
PEX5L	PTS1-receptor	TPR	gi	absent	absent	absent	113930737	196259774
			NP				NP_033021.2	NP_001124497.1
			alias				AW212715; ESTM1; PTS1R; Pxr1; X83306	PBD2A; PBD2B; PTS1-BP; PTS1R; PXR1
			aa (MW)				639 (70625)	(639) 70734
PEX6	AAA-ATPase		gi	17562804	78707192	326678870	21703962	194018488
			NP	NP_504268.1	NP_001027403.1	XP_001332652.4	NP_663463.1	NP_000278.3
			alias	prx-6; CELE_F39G3.7	CG11919; CG30019; DmCG11919; DmelPex6; Dmel\CG11919	ZDB-GENE-081104-252	AI132582; mKIAA4177; PAF-2; D130055I09Rik; peroxisomal-type ATPase 1	PAF-2; PAF2; PBD4A; PDB4B; PXAAA1
			aa (MW)	720 (81223)	897 (100990)	865 (94312)	981 (104418)	980 (103930)
PEX7	PTS2-receptor	WD40	gi	absent	24661084*	61806636	6679283	4505731
			NP		NP_648251.1	NP_001013550.1	NP_032848.1	NP_000279.1
			alias		CG6486; DmelPex7; Dmel\CG6486	zgc:103552; ZDB-GENE-050320-105	MmPEX7	PBD9B; PTS2R; RCDP1; RD
			aa (MW)		339 (37486)	314 (34818)	318 (35371)	323 (35761)

(Continued)

Table 1 | Continued

Peroxin[2]	Description	Domain		*Caenorhabditis elegans*[1]	*Drosophila melanogaster*	*Danio rerio*	*Mus musculus*	*Homo sapiens*
PEX10		Zinc RING-finger	gi		392894943	54400490	109150414	24797089
			NP		NP_001021200.2	NP_001005994.1	NP_001035866.1	NP_722540.1
			alias		C34E10.4a[3]	zgc:103520	AV128229; Gm142	NALD; PBD6A; PBD6B; RNF69
			aa (MW)		314 (35871)	318 (37181)		346 (39083)
PEX11A	Peroxisome elongation Peroxisome constriction		gi	17506083	??	156739285	6755034	4505717
			NP	NP_493273.1		NP_001096590.1	NP_035198.1	NP_003838.1
			alias	prx-11; CELE_C47B2.8		si:dkeyp-84g1.1; 565760; ZDB-GENE-050419-121	PEX11alpha	hsPEX11p; PEX11-ALPHA; PMP28
			aa (MW)	214 (23780)		246 (27867)	246 (28022)	247 (28222)
PEX11B	Peroxisome elongation Peroxisome constriction		gi	??	19922346	113951761	241666483	4505719
			NP		NP_611071.1	NP_001039319.1	NP_001155859.1	NP_003837.1
			alias		CG8315; DmelPex11; Dmel\CG8315	zgc:153402; 566742; ZDB-GENE-060825-289	PEX11beta; Pex11pbeta	PEX11-BETA; PEX14B
			aa (MW)		241 (27007)	266 (29496)	259 (28579)	259 (28300)
PEX11G	Peroxisome elongation Peroxisome constriction		gi	??	28571837[4]	71834488	21735445	18087833
			NP		NP_651137.3	NP_001025343.1	NP_081227.1	NP_542393.1
			alias		BcDNA:RE30473; Dmel\CG13827	63203; ZDB-GENE-050913-79	1810022F11Rik; 1810049N02Rik; Pex11g; Pex11gamma	
			aa (MW)		233 (26208)	240 (26210)	241 (27021)	241 (26505)
PEX12		RING Zinc finger, C3HC4 type	gi	17551466	24580706	41055606	19527244	4505721
			NP	NP_509908.1	NP_608546.1	NP_956499.1	NP_598786.1	NP_000277.1
			alias	prx-12; F08B12.2	CG3639; DmelPex12; Dmel\CG3639	zgc:56182; 393174; ZDB-GENE-040426-929	AI451906	PAF-3; PBD3A
			aa (MW)	359 (41158)	297 (34413)	303 (33979)	359 (40502)	359 (40666)
PEX13	Docking PTS-cargo complex	SH3	gi	17533615	20129941	41055287	31543471	4505723
			NP	NP_495513.1	NP_610850.1	NP_956939.1	NP_076140.2	NP_002609.1
			alias	prx-13; F32A5.6	CG4663; DmelPex13; Dmel\CG4663; FBgn0033812	zgc:66124; ZDB-GENE-040426-1544	2610008O20Rik	NALD; PBD11A; PBD11B; ZWS
			aa (MW)	330 (35635)	440 (46529)	416 (45338)	405 (44479)	403 (43999)
PEX14	Docking PTS-cargo complex		gi	17541806	21355205	292627105	9790153	4758896
			NP	NP_502097.1	NP_649253.1	XP_688421.4	NP_062755.1	NP_004556.1
			alias	prx-14; R07H5.1	CG4289; DmelPex14; Dmel\CG4289	559933; ZDB-GENE-060130-169	R75137	dJ734G22.2; NAPP2; PBD13A; Pex14p
			aa (MW)	258 (28025)	280 (30673)	422 (46234)	376 (41077)	377 (41106)

(Continued)

Table 1 | Continued

Peroxin[2]	Description	Domain		*Caenorhabditis elegans*[1]	*Drosophila melanogaster*	*Danio rerio*	*Mus musculus*	*Homo sapiens*
PEX16			gi	Absent	21355481	68448487	254750742	254750742
			NP		NP_649252.1	NP_001020340.1	NP_660104.2	NP_660104.2
			alias		CG3947; DmelPex16; Dmel\CG3947	im:6894523; zgc:112248		PBD8A; PBD8B
			aa (MW)		341 (39228)	335 (38424)	336 (38546)	336 (38546) (splice form; only on EST!)
PEX19	Cytosolic chaperone; PMP import receptor	CAAX-box	gi	17553610	24583827	62899043	226958490	4506339
			NP	NP_498947.1	NP_609547.2	NP_001017399.1	NP_075528.3	NP_002848.1
			alias	F54F2.8	BEST:GH03076; CG5325; DmelPex19; Dmel\CG5325	wu:fb40d12; wu:fc41h09; zgc:110675	Pxf	D1S2223E; HK33; PBD12A; PMP1; PMPI; PXF; PXMP1
			aa (MW)	282 (30857)	292 (31175)	288 (31412)	299 (32602)	299 (32676)
PEX20	Cytosolic chaperone		gi	??	386768875	Absent	Absent	Absent
			NP		NP_001245818.1[5]			
			alias		CG3696; DmelPex20; Dmel\CG3696; EK2-4; kis; Su(Pc)21AB			
			aa (MW)		5343 (575803)			
PEX23	Peroxisome proliferation; peroxisomal growth regulation		gi	Absent	24667330	Absent	Absent	Absent
			NP		NP_730508.1[6]			
			alias		CG18565; CG32226; CG6468; DmelPex23; Dmel\CG32226			
			aa (MW)		1350 (149356)			
PEX26	Anchor for PEX1 and PEX6 to peroxisome membrane		gi	??	??	41053983	21311973	189083737
			NP	??	??	NP_956214.1	NP_083006.1	NP_001121121.1
			alias			fk41g06; wu:fk41g06; zgc:64014	4632428M11Rik; AI853212	PBD7A; PBD7B; PEX26M1T; Pex26pM1T; FLJ20695
			aa (MW)			313 (34257)	305 (33885)	305 (33767)

[1] *It should be noted that in the Worm database, prx-number has been proposed as acronym for peroxisome assembly factors given confusion with pex (pachytene exit defect). However, various entries related to prx are linked to both PeroxidoRedoXins and PeRoXisome assembly factors, given use of similar acronym.*

[2] *Peroxins, not present in animals, include PEX4, PEX8, PEX15, PEX17, PEX18, PEX21, PEX22, PEX25, PEX27, PEX28, PEX29, PEX30, PEX31, PEX32, PEX34 (all present in yeasts), PEX9, PEX20, PEX23 (Yarrowia sp.), PEX24 (yeasts, plants), PEX33 (Neurospora sp.).*

[3] *The C34E10.4 locus produces a primary transcript coding for two different proteins, PEX10 (at the 5′; C34E10.4a) or WARS-2 (at the 3′; C34E10.4b).*

[4] *In addition to this entry, another PEX11 like protein (201 amino acids, MW 22671) is encoded by the fly genome. It concerns NP_995800.1 (gi 45552555), also named CG33474; Dmel\CG33474, which is most similar to PEX11G.*

[5] *One of the six different isoforms encoded by CG3696, nowadays referred to as kismet; homologous to the human CHD7 (chromodomain-helicase-DNA-binding protein 7).*

[6] *Whether NP-730508.1 represents the counterpart of Yarrowia PEX23 or yeast PEX31 is questionable; they all contain a Dysferlin domain, but likely this entry is the counterpart of TECP1 (tectonin beta-propeller repeat-containing protein 1).*

[??] *not present in database, likely absent.*

[*] *homologue, but functionality not proven.*

when the larvae are exposed to a hostile environment to block further development. Ascarosides are glycolipids and, in the case of dauer pheromone, consist of a hydroxylated medium chain fatty acid such as 6-hydroxyheptanoic acid or 8-hydroxy-2-nonenoic acid, O-glycosidically linked to ascarylose (3,6-didesoxymannose). Particularly daf-22 and dhs-28 (Butcher et al., 2009; Joo et al., 2009), the nematode counterparts of SCPX and the dehydrogenase moiety [3] of D-specific MFP2, respectively, and acox-1 (Joo et al., 2010), one the seven nematodal ACOX proteins, are required for dauer pheromone production.

Regarding peroxisome biogenesis, it should be mentioned that the genome of *C. elegans* (and other nematodes) does not encode PEX7 (Motley et al., 2000). According to Thieringer et al. (2003) PEX16 is also missing and only one PEX11 isoform is present (**Table 1**).

During various large screenings by RNAi soaking, feeding or injection experiments, different peroxins were hit, however, the phenotype of the offspring was only minimally scored and the efficacy of silencing not investigated (see **Table 2** and associated references). Moreover, these screens display some variability between approaches, are known to give rise to false negatives, and silencing is less effective in the nervous system. Efficacy can be increased by performing screens in the *rrf-3* mutant, a strain being hypersensitive to RNAi, likely due to longer half life of RNAi (Simmer et al., 2003). Overall these screens, certainly those by Simmer et al. (2003) and Sonnichsen et al. (2005), indicate that normal larval development depends on functional peroxisomes (**Table 2**).

In more in depth investigations silencing dsRNAs were injected into the gonads of young adult hermaphrodites, followed by scoring of their effect on the progeny. Rachubinski and coworkers found that RNAi inactivation of *Pex5*, *Pex12*, *Pex13,* and *Pex19* greatly reduced the percentage of adult progeny, at 3 days following injection of dsRNA, most progeny being developmentally delayed and still at the L1, L2, or L3 larval stage (Petriv et al., 2002). Targeting of *Pex6*, *Pex1*, or *Pex2* was without effect, but the employed dsRNAs did also not affect the peroxisomal import of a fluorescent PTS1-protein (CFP-SKL). In contrast, injection of dsRNA targeting *Pex5*, *Pex13,* or *Pex19*, caused a cytosolic fluorescence of the reporter. Silencing of *Pex12* resulted in fewer but larger peroxisomes.

Thieringer et al. (2003) reported similar experiments. Blocking either *Pex5*, *Pex6*, *Pex12*, *Pex13,* or *Pex19* caused an arrest of the growth of their progeny at the L1 larval stage (**Figure 5A**). The arrested worms were viable, and resumed growth after 2–8 days, likely depending on quantity and stability of injected DNA, and developed into normal worms.

Development seems less dependent on *Pex10*. During an ethyl methanesulfonate mutagenesis screen for genes affecting lipid droplets, Zhang et al. (2012) could classify surviving mutants having enlarged lipid droplets into four complementation groups, one group being linked to *Pex10*. In the mutant strain (prx-10(hj21)), PTS1 import was affected. Although not discussed in their paper, development and morphology of the worm appeared normal (based on pictures of 1 day adult). Given that the three other groups were linked to peroxisomal β-oxidation enzymes (*maoc-1*, *dhs-28*, *daf-22*, respectively, corresponding to an enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and SCP2-containing thiolase), one could wonder why only one peroxin was hit in this screen or whether the others remained undetected due to lethality or slower development.

The mechanism underlying the developmental problems was not addressed, but might indeed be caused by peroxisomal metabolic inactivity. *Pex5*(RNAi) prevents initiation of post-embryonic cell divisions, and normal cell migrations including those of neuronal cells, are blocked (Thieringer et al., 2003). This phenotype resembles that of starved larvae, therefore division might require a peroxisomal metabolite. Furthermore, larval development of nematodes seems to be dependent on ether-lipid synthesis. Eight days after injecting gonads of adults with dsRNA directed toward ADHAPS, their offspring was still in the larval stage, whereas those injected with non-specific dsRNA produced mature adults (Motley et al., 2000). Similar findings were reported by Petriv et al. (2002). Also β-oxidation might play a role in development. Upon silencing of Δ3,5-Δ2,4-dienoyl-CoA isomerase (encoded by *Y25C1A.13*), an enzyme required for degradation of polyunsaturated fatty acids, or silencing of the three ABC half-transporters (encoded by *T02D1.5*, *C44B7.9*, and *C44B7.8),* implicated in peroxisomal membrane translocation of fatty acids/acyl-CoAs, a similar phenotype is seen: no adult offspring three days after injection (Petriv et al., 2002). It should be noted, however, that silencing of peroxisomal thiolases, either the classical ones (encoded by *T02G5.4* and *T02G5.8*) or SCP2-containing thiolase (encoded by *daf-22*), had no effect (Petriv et al., 2002). Also important to mention is that the *C. elegans* dienoyl-CoA isomerase, thought to be the counterpart of mammalian ECH1 (Petriv et al., 2002), which is targeted to both mitochondria and peroxisomes, does not have a PTS1. This complicates the interpretation of these silencing experiments.

Whereas a defective peroxisome biogenesis affects early larval development, silencing of *Pex* genes at a later stage seems beneficial. An extended life span, 22.7 days compared to 16.22, was seen upon silencing of *Pex5* in L4 larvae in the *eri-1(mg366)* strain, a strain more sensitive to RNAi (Curran and Ruvkun, 2007). Similarly, Zhou et al. reported a 17% increase for *Pex13* and 8% for *Pex5* (15% for PMX4, a peroxisomal membrane protein), when silenced in 1 day old adult (Zhou et al., 2012). It is suggested that the longer survival is related to reduced generation of reactive oxygen species (ROS) when peroxisomes are less or not functional (see Fransen et al., 2012). The amount of measurable ROS is lowered in the *Pex5*, *Pex11*, or *Pex13* silenced animals. Strangely, and in contrast to most other screens, silencing of these peroxins in L1 larvae had no effect, but controls on RNAi efficacy are missing.

Summarizing, RNAi based data suggest that peroxins play a critical role in nematode development, but are less important in the adult stage. A drawback of this technique is, however, the

[3]In contrast to most other higher eukaryotes, the two catalytic domains of MFP2 are expressed as separate proteins in *C. elegans* (Huyghe et al., 2006a).

Table 2 | Overview of large scale silencing screens in *C. elegans* affecting peroxins.

References	Method	*Pex1*	*Pex2*	*Pex3*	*Pex5*	*Pex6*	*Pex10*	*Pex11*	*Pex12*	*Pex13*	*Pex14*	*Pex19*
Gonczy et al., 2000	Injection in gonads of dsRNA targeting genes of chromosome III						Slow growth					Larval arrest
Maeda et al., 2001	dsRNA soaking					Sick						
Kamath et al., 2003	Feeding *E. coli* expressing dsRNA	N	Slow growth	N	Slow growth; clear	N	Slow growth		Slow growth; clear	Slow growth	Slow growth	Slow growth
Simmer et al., 2003	Feeding *E. coli* expressing dsRNA in *rrf-3* strain	Slow growth	Slow growth	Slow growth	Larval arrest	Slow growth	Slow growth		Slow growth	Larval arrest	Larval arrest	Larval arrest
Ashrafi et al., 2003	Bacterial feeding of L1 larvae; analysis of fat content				Reduced fat content							
Rual et al., 2004	Feeding at the first larval stage (L1) *E. coli* expressing inducible hairpin RNAi	No		No	None	Long	No	No	Embryonic lethal	No	No	No
Sonnichsen et al., 2005	Injection dsRNA into young adult worms; examination phenotype of F1 progeny	No		Early larval arrest; defective early embryo-genesis	Embryonic lethal	No	Early larval lethal	No	No	Embryonic lethal	Early larval arrest	Early larval lethal
Sieburth et al., 2005	Feeding bacteria expressing dsRNA to larvae; screening adults for decreased acetylcholine secretion (aldicarb resistance)		Aldicarb resistant									

(Continued)

Table 2 | Continued

References	Method	*Pex1*	*Pex2*	*Pex3*	*Pex5*	*Pex6*	*Pex10*	*Pex11*	*Pex12*	*Pex13*	*Pex14*	*Pex19*
Fernandez et al., 2005	Soaking of L4 stage larvae; with dsRNA corresponding to ovary expressed genes; progeny						Embryonic lethal	N			Embryonic lethal	Embryonic lethal
Curran and Ruvkun, 2007	Bacterial dsRNA feeding of L4-stage larvae (*eri-1(mg366)* strain); Screening life span of adult				Extended life span; Fat content reduced							
Byrne et al., 2007	Bacterial dsRNA feeding to L3-L4 stage worms; progeny and growth				Organism develop-ment variant							
Ceron et al., 2007	Bacterial dsRNA feeding to L1 larvae (*lin-35(n2239)* strain); progeny				Larval arrest; reduced brood size							

N, no abnormalities reported; clear, animals appear unusually transparent when compared to control; long, animals are longer and thinner than control animals at the same developmental stage; sick, animals exhibit some combination of abnormal features relating to size, movement, body integrity, pigmentation, viability, fertility; larval arrest: development halted at any larval stage, failure to reach adulthood; slow growth, any variation that causes a reduction in growth rate compared to control.

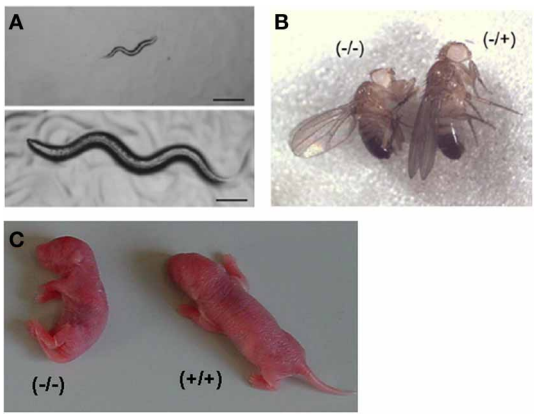

FIGURE 5 | Developmental delay in peroxisome deficient animals. (A) Developmental arrest of *C. elegans* at the L1/L2 stage by *Pex5* RNAi (top panel), compared to wild type nematode (bottom panel), being photographed 3 days after being laid. Bar, 10 μm. Reproduced/adapted with permission from Thieringer et al. (2003). **(B)** Reduced body size and weight of an adult male homozygous *pex16*[1] fruitfly compared to heterozygous animal. Taken from Nakayama et al. (2011). **(C)** Appearance of newborn mice pups, showing severe hypotonia and growth delay in a *Pex5*$^{-/-}$ pup compared to a wild type littermate.

variability. In the near future, more solid data on the role of peroxins in nematodes are expected, given the increasing availability of deletion mutants (C.elegans mutation consortium. 2012): a *Pex5* mutant (tm4948) with a 439 bp deletion is sterile[4]; a *Pex1* mutant (tm0392) with a 681 bp deletion is classified as lethal or sterile[5].

FRUITFLY

About 21–22 h after fertilization (hpf), *Drosophila* larvae will hatch from the eggs. One distinguishes 17 steps during this period, known as Bownes stage numbers. In stage 6 (180–195 min), gastrulation starts, whereas formation of the Malphigian tubes (counterpart of kidney in mammals) starts in stage 10. In the late stage 11, the stomatogastric nervous system develops. During the subsequent larval stages, three in total, most of the organs/structures of the adult fly will develop, starting from imaginal discs. At the end of the third larval stage (120 hpf), metamorphosis starts, divided in a prepupal period and a pupal period, in total 4 days. Finally, the flies emerge from the pupal case (eclosion). They start mating 12 h after emergence and will live for about a month.

Based on fatty acid analysis of certain *Pex* mutants, very long chain fatty acids (VLCFA) are degraded via peroxisomal β-oxidation in fruitflies (Chen et al., 2010). Related to purine/xanthine metabolism, it should be noted that the eye pigment formation is dependent on peroxisomes. The rosy-506 eye-color mutant lacks xanthine dehydrogenase/oxidase, which is targeted to peroxisomes (Beard and Holtzman, 1987).

The genome of *Drosophila* encodes at least 15 peroxins (Chen et al., 2010; Mast et al., 2011), being homologous to mammalian peroxins. Whether orthologous of the fungal *Pex20* and *Pex23* are expressed (Mast et al., 2011), is questionable (see comments to the related entries in **Table 1**).

Based on RNAi in *Drosophila* S2 cells expressing GFP-SKL, silencing of *Pex1*, *Pex5*, *Pex13*, *Pex16* results in import deficiency, silencing of *Pex2*, *Pex3*, *Pex6*, *Pex12*, *Pex14* in impaired import. Interfering with *Pex11* or *Pex19* affects peroxisome number (reduced) and size (larger), whereas RNAi of the putative *Pex20* or *Pex23* has an opposite effect, more peroxisomes of smaller size (Mast et al., 2011).

Although for most of these peroxins, mutants with transposon P1-insertions were present in the repositories (see **Table 3**), these were not studied in depth. According to Spradling *et al.* (Spradling et al., 1999), the *Pex2*^{f018} allele was lethal, but this was later shown to be due to a second mutation (Chen et al., 2010). More recently, a library of RNAi transgenes, expressing inverted repeats causing conditional gene inactivation, became available, covering 88% of the predicted protein coding genes (Dietzl et al., 2007). For all fly peroxins, transgenic lines are available (unpublished data), but as far as known, not evaluated.

Related to fly development, and as far as studied in detail, PEX1, PEX3, and PEX13 appear critical. P-element insertion in *Pex1* (*pex1*^{s4868}) (Chen et al., 2010; Mast et al., 2011) or in *Pex13* (*pex13*^{KG04339}) (Chen et al., 2010), X-ray mutagenized *Pex1*[1] (Mast et al., 2011) or a deletion in *Pex3*, generated by P-element imprecise excision of *pex3*^{CG6859} (Nakayama et al., 2011), are lethal[6] at the larval stage when homozygous. Expression of a wild type PEX1 rescues the *pex1*^{s4868} or *pex1*[1] mutants to survive past the second larval instar (Mast et al., 2011). *Pex1* mutant larvae displayed a delay in development, little coordinated locomotion, poor feeding, and died at the L1–L2 stage (Mast et al., 2011). Some larvae even died a few hours after hatching, being unable to crawl out of the eggshells. In the peripheral and central nervous system various abnormalities were documented. These include malformation of the ventral nerve cord (lack of or underdeveloped commissures, breaks in longitudinal connectivities), reduced number of motor neurons, disorganization of glia cells, loss and hypoplasia of peripheral neurons, malformation of eye discs. In the malphigian tubules, structural abnormalities were noticed.

A dsRNA screen was conducted in preblastoderm embryos to detect genes that affect embryonic nervous system development. Although 50% of the *Drosophila* genes were covered, only one peroxin was hit, i.e., PEX19. Silencing of *Pex19* resulted in disruption of the ventral nerve cord, misrouting of axons and disorganization of dorsal clusters of cells in the peripheral nervous system in stage 15–16 embryos (Koizumi et al., 2007).

Flies with insertional mutations in *Pex2*(*pex2*^{f0189} and *pex2*^{HP35039}) (Chen et al., 2010), *Pex12* (*pex12*^{f01300}) (Chen et al., 2010), *Pex1* (*pex1*^{S4868}) (Zhou et al., 2012) or *Pex13* (*pex13*^{KG04339}) (Zhou et al., 2012) or a deletion in *Pex10* (excision

[4]http://www.shigen.nig.ac.jp/c.elegans/mutants/DetailsSearch?lang=english&seq=4948.

[5]http://www.shigen.nig.ac.jp/c.elegans/mutants/DetailsSearch?lang=english&seq=392.

[6]The *Pex1*^{S084807} and *Pex1*^{02402} alleles were reported to be semi-lethal and in homozygous third instar larvae necrosis was observed in salivary gland cells (Burmester et al., 2000).

Table 3 | Overview of classical peroxin alleles in *Drosophila melanogaster*.

Gene	Allele	Mutagenesis method	References
Pex1	*Pex1*^{1}	X-ray	http://flybase.org/reports/FBal0031854.html
	Pex1^{s4868}	P{lacW} insertion	http://flybase.org/reports/FBti0009969.html
	Pex1^{S084807}	P{lacW} insertion (chromosome 3)	http://flybase.org/reports/FBal0095307.html
	Pex1^{02402}	P{PZ} insertion	http://flybase.org/reports/FBal0031019.html
Pex2	*Pex2*^{f0189}	PBac{WH} transposase	http://flybase.org/reports/FBal0161076.html
	Pex2^{f01075}	PBac{WH} transposase	http://flybase.org/reports/FBal0222659.html
Pex3	*Pex3*^{EY1920}	P{EPg} insertion	http://flybase.org/reports/FBal0215913.html
	Pex3^{c02356}	PBac{PB} transposase	http://flybase.org/reports/FBal0222963.html
Pex5	*Pex5*^{JC02}	P{PZ} insertion	http://flybase.org/reports/FBal0244572.html
Pex6	*Pex6*^{EY09695}	P{EPgy2} insertion	http://flybase.org/reports/FBal0176366.html
	Pex6^{f03888}	PBac{WH} transposase	http://flybase.org/reports/FBal0222729.html
Pex7		none	
Pex10	*Pex10*^{NP7003}	P{GawB} insertion	http://flybase.org/reports/FBal0225637.html
	Pex10^{c03596}	PBac{PB} transposase	http://flybase.org/reports/FBal0225639.html
	Pex10^{DP01222}	P{Mae-UAS.6.11} insertion	http://flybase.org/reports/FBal0238882.html
	Pex10^{EY23523}	P{EPgy2} insertion	http://flybase.org/reports/FBal0245128.html
	Pex10^{G5094}	P{EP} insertion	http://flybase.org/reports/FBal0220877.html
	Pex10^{MI04076}	Mi{MIC}insertion	http://flybase.org/reports/FBal0264496.html
	Pex10^{c03579}	PBac{PB} transposase	http://flybase.org/reports/FBal0225638.html
Pex11	*Pex11*^{LA00967}	P{Mae-UAS.6.11} insertion	http://flybase.org/reports/FBal0184822.html
	Pex11^{f03235}	PBac{WH} transposase	http://flybase.org/reports/FBal0184821.html
Pex12	*Pex12*^{f01300}	PBac{WH} transposase	http://flybase.org/reports/FBal0185737.html
	Pex12$^{\Delta 303}$	P{EPgy2} insertion	http://flybase.org/reports/FBal0242101.html
Pex13	*Pex13*^{e02054}	PBac{RB} transposase	http://flybase.org/reports/FBal0185582.html
	Pex13^{KG04339}	P{SUPor-P} insertion	http://flybase.org/reports/FBal0134334.html
	Pex14^{EY02900}	P{EPgy2} insertion	http://flybase.org/reports/FBal0156977.html
Pex14	*Pex14*^{EY02900}	P{EPgy2} insertion	http://flybase.org/reports/FBal0156977.html
Pex16	*Pex16*^{EY05323}	P{EPgy2}	http://flybase.org/reports/FBal0161441.html
	Pex16^{GS14106}	P{GSV6} insertion	http://flybase.org/reports/FBti0106543.html
Pex19	*Pex19*^{DP00474}	P{Mae-UAS.6.11} insertion	http://flybase.org/reports/FBal0238836.html
	Pex19^{EY21383}	P{EPgy2} insertion	http://flybase.org/reports/FBal0192628.html
Pex23		none (see also **Table 1**)	

of P-element in *pex10*^{EY23523}) (Chen et al., 2010) or *Pex16* (excision of *pex16*^{CG3947}) (Nakayama et al., 2011) are viable. Fertility, however, was reduced in *Pex2*, *Pex10*, or *Pex12* female mutants and males were sterile (Chen et al., 2010). The latter phenotype was due to an arrest in the germ cell development at the level of the spermatocyte growth stage. Similarly, male fertility was compromised in the *Pex16* mutant (Nakayama et al., 2011). Testes of this mutant were smaller and did not contain mature sperm cells, although early spermatocyte cysts were still present, due to an arrest in the maturation of spermatocytes at the young apolar stage (**Figures 6A,B**). This arrest and the fertility could be rescued by overexpression of PEX16 in the cyst cells, although germ cells still lacked peroxisomes. Expression of PEX16 in the germline cells, however, did not rescue the spermatogenesis, indicating that peroxisomes in the somatic cysts cells play an important role in spermatogenesis (Nakayama et al., 2011). This is, however, in contrast to the *Pex2* mutant in which rescue of the germ cells normalized the phenotype (Chen et al., 2010). It is suggested that VLCFA, which show an age-dependent increase in *Pex10* mutants (2.9- and 3.9-fold for whole body $C_{26:0}$ at 2 and 15 days, respectively), play a critical role in spermatogenesis in insects (Chen et al., 2010).

Although viable, the *Pex16* mutant adult flies were considerably smaller (30% females; 15% males) (**Figure 5B**) and their locomotion was affected, the latter likely being responsible for a severe reduction of their lifespan (to one third for females; to one-fourth for males) (Nakayama et al., 2011). Peroxisomes were still present in the malpighian tubule cells of *Pex16* mutant flies, but their number is greatly reduced. Not unexpectedly, the eye pigmentation was affected in *Pex16* mutants, resembling the *rosy* phenotype, and biochemically, an increase in (whole body) VLCFA levels was seen (2-fold for $C_{24:0}$ in males, 3-fold in females). Histology of brain revealed a low density of dendritic trees in the lobula plate of the optic lobe; other cells in the optic lobe and other parts of the brain were unaffected. The dendritic reduction was already visible at the pupal stage and did not aggravate with age, suggesting a developmental problem, not a degeneration. Interestingly this defect can be rescued by

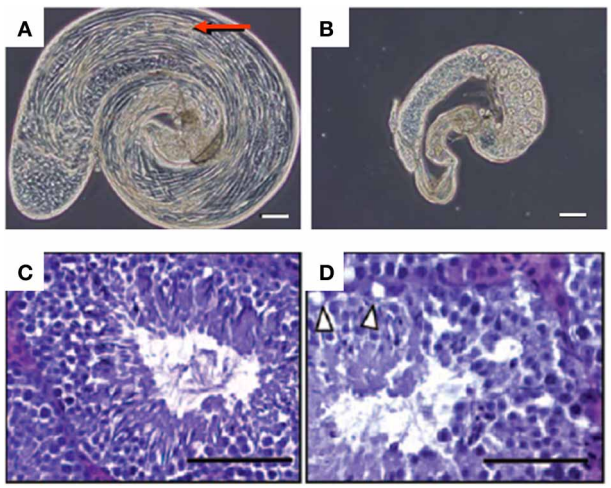

FIGURE 6 | Male fertility problems in peroxisome deficient animals. **(A,B)** Phase-contrast micrographs of testes of fruitflies, with bundles of elongated spermatids (arrow) in a wild type **(A)** and arrest of germline cell maturation in a *pex16*[1] homozygous fruitfly **(B)**. Bar, 100 μm. Taken from Nakayama et al. (2011). **(C,D)** Hematoxylin-eosin staining of 7 weeks old testis of wild type **(C)** and Sertoli PEX5 knockout mice **(D)**, the latter showing lipid droplets that were emptied during the embedding procedure in the outer layer of the seminiferous epithelium (arrowheads) and reduced numbers of spermatozoa in the lumen of the tubuli. Bar 100 μm. Taken from Huyghe et al. (2006b).

expression of PEX16 in the fat body or in differentiated neurons (Nakayama et al., 2011).

The viability of the above mentioned *Pex1* and *Pex13* mutants can be explained by the nature of the mutation, affecting the promoter and resulting in lower expression (∼20% of wild type) (Zhou et al., 2012). Interestingly, life span of these flies increased (16% in males; 13% in females), whereas their hydrogen peroxide levels were decreased. This is similar to findings reported in nematodes (see Nematodes). Along the same lines, *Pex19* expression was reported to be repressed (1.8-fold) when feeding flies 4-phenylbutyrate, a diet which extends their lifespan by 36% (Kang et al., 2002). The latter compound is known in the peroxisomal field by its ability to induce the expression of ABCD2 (Kemp et al., 1998), an ABC-transporter functionally related to ABCD1 which is mutated in X-ALD.

ZEBRAFISH

Given the translucency of the embryo and the short developmental period, zebrafish (*Danio rerio*) is an organism of choice for dynamic developmental studies. Gastrulation starts around 6 h post-fertilization (hpf), first somites are formed at 11 hpf, and at 24 hpf the embryo, surrounding the yolk sac, shows already the typical fish-like shape and tail and primary organs have been formed. In the subsequent day, the circulatory system and fins are formed. Cartilage development starts at 48 hpf, and at 3 days, fishes are self-supporting, first as larvae till 1 month of age, then juveniles till adulthood, around 90 days. Total life span is around 2 years.

Transcripts for peroxisomal matrix and membrane proteins can be detected starting at 24 hpf in the head region, whereas catalase-positive peroxisomes become visible in the liver and the pronephric duct in 4 days old fishes (Krysko et al., 2010). In adult fish, peroxisomes are most prominent in liver (Braunbeck et al., 1990; Krysko et al., 2010), renal proximal tubules (Krysko et al., 2010) and the intestinal epithelium (Krysko et al., 2010). For more information on expression in zebrafish during embryogenesis, the reader is referred to a large scale *in situ* hybridization screen (Thisse et al., 2004).

Similarly to rodents, zebrafish hepatic peroxisomes respond to peroxisome proliferators and an increased number is observed in liver when fishes are exposed to clofibrate (Venkatachalam et al., 2012) or phthalate esters (Ortiz-Zarragoitia et al., 2006).

Based on scattered information, the organelles are active in β-oxidation. Presence of ACOX1 was demonstrated (Ibabe et al., 2005; Morais et al., 2007) and the enzymes able to act on branched fatty acids, such as MFP2 (encoded by *hsd17b4*) and SCPX (encoded by *scp2a*), are expressed (Thisse et al., 2004), but apparently C_{24}-bile acids are not formed in zebrafish, in contrast to other teleost fish (Hofmann et al., 2010). Based on genomic information, fish peroxisomes can synthesize etherlipids and contain an α-oxidation pathway.

Regarding peroxisome biogenesis, all classical peroxins are expressed in *D. rerio* (see **Table 1**), and based on high throughput analysis, *Pex3*, *Pex5*, *Pex7*, *Pex10*, *Pex14*, *Pex19* are ubiquitously expressed from 24 hpf on, with higher expression in the head region (Thisse et al., 2004).

Despite the wide spread use of morpholinos to interfere with expression in zebrafish, in only few reports, as far as documented, this technique was applied to peroxisome biogenesis. Injection of morpholinos, intended to block the splice sites in *Pex3* or *Pex13*, into one-cell embryos did not affect peroxisomal import. Subsequent RNA analysis revealed that these morphilinos did not eliminate exons, instead produced a short in frame insertion (*Pex3*) or deletion (*Pex13*) (Krysko et al., 2010). Blocking of the translation of *Pex13* was more effective to reduce the number of hepatic peroxisomes, but high doses were needed and not all of the injected embryos showed such response. A *Pex5* blocking morpholino had no effect at low dose, and caused embryonal death at higher dose. Finally, overexpression via mRNA injection of an N-terminal domain of (human) PEX3, having a dominant negative effect in human fibroblasts (Soukupova et al., 1999), did not affect biogenesis (Krysko et al., 2010). Coutinho et al. (2004) did not observe any abnormalities at 32 hpf when one cell stage embryos were injected with morpholinos directed against the 5′-end of *Pex19* (notochord differentiation or pigmentation were normal), the efficacy of the morpholino was, however, not controlled.

Although technically easy, the dilution of morpholinos or mRNA upon subsequent cell divisions, combined with the turnover of peroxisomes, half life estimated at 2 days in cultured mammalian cells (Huybrechts et al., 2009), is a major obstacle in the embryonic injection approach. In the near future, more solid data might emerge from analysis of insertional zebrafish mutants.

Although tools to carry out large scale insertional mutagenesis and positional cloning in zebrafish were developed several years ago using mouse retroviral vectors (Gaiano et al., 1996; Golling et al., 2002) the number of created, annotated and available mutants, however, remains low. For a more targeted approach, engineered Zn-finger nucleases are a promising tool to create zebrafish knockouts (Foley et al., 2009).

MICE

The intra-uterine development of mice takes 20–21 days. During this period, embryos are depending on the maternal circulation with regards to most nutrients. Examples of exceptions are brain poly-unsaturated fatty acids (PUFA) that are partly dependent on local synthesis (Janssen et al., 2000). At birth, organogenesis of most organs has been completed, except formation of the cerebellum which extends into the postnatal period and maturation of gonads before adulthood. After birth, pups are nursed and milk-fed till weaning, about 3 weeks later. At 6 weeks (females) or eight (males) of age, animals become sexually active and start to breed. Lifespan, under laboratory conditions, is 18–30 months.

In mammals, peroxisomal β-oxidation serves to generate PUFA and C_{24} bile acids. The first are implicated in many brain processes such as learning, memory, behavior; the latter are required for efficient uptake of lipophilic nutrients in the intestines. This pathway also shortens VLCFA, pristanic acid and dicarboxylic fatty acids (Van Veldhoven, 2010). Removal of the toxic phytanic acid requires an active α-oxidation. Plasmalogen deficiency in mammals is linked to a specific bone developmental problem, in man known as rhizomelic chondrodysplasia punctata (RCDP), and RCDP type I is linked to PEX7 deficiency.

Currently, the following peroxins have been inactivated in mice: PEX5 (Baes et al., 1997), PEX2 (Faust and Hatten, 1997), PEX11A (Li et al., 2002a; Weng et al., 2013), PEX11B (Li et al., 2002b), PEX13 (Maxwell et al., 2003), and PEX7 (Brites et al., 2003; Braverman et al., 2010). Mice lacking both PEX11A and PEX11B were also created (Li et al., 2002b), or lacking a peroxin together with another peroxisomal protein such as *Pex7*$^{-/-}$*:Abcd1*$^{-/-}$ mice (Brites et al., 2009).

Given obvious similarities, PEX5, PEX2, and PEX13 deficiencies can be treated together, separately from the PEX7 knockout model. Considering that PEX11 proteins are not involved in peroxisome biogenesis *per se* and that this process is not affected in the *Pex11a*$^{-/-}$ and *Pex11b*$^{-/-}$ mice, but mainly their elongation and abundance, these models will not be discussed further in this chapter. Below we will summarize the main findings in the other mouse models [see also recent reviews by Baes and Van Veldhoven (2006, 2012)].

Related to PEX5, PEX2, and PEX13 deficient models, knockouts pups are born alive in the expected Mendelian ratio and without major deformities or skeletal malformations, suggesting a normal intra-uterine development (Baes et al., 1997; Maxwell et al., 2003). However, in case of PEX2 deficiency in an inbred 129 background, embryonic lethality was reported and only 20% of the pups are born (Faust and Hatten, 1997). In these three models, newborn pups are, however, growth retarded and severely hypotonic (**Figure 5C**), hence they do not feed and die 6–24 h after birth. Some *Pex2*$^{-/-}$ pups (20–30%), in a mixed Swiss Webster × 129SvEv background, survive for about 1–2 weeks (Faust and Hatten, 1997) and the postnatal survival can be improved by oral bile acid therapy (9% alive after 30 days) (Keane et al., 2007). The reason for this strain-dependent differences, although often seen in other mouse models, is not clear.

At closer inspection, there are some developmental problems, especially in the brain. Lamination of the cerebral cortex is affected due abnormal and delayed neuron migration (Baes et al., 1997; Faust and Hatten, 1997; Gressens et al., 2000). In the longer surviving *Pex2*$^{-/-}$ pups, dendritic arborization of the Purkinje cells in the cerebellum is reduced and their axons are dystrophic (Faust, 2003). Similar findings were seen in a *Pex5* and *Pex13* brain knockout (see further).

Finally, at the subcellular level, mitochondrial abnormalities were documented in liver (Baumgart et al., 2001; Keane et al., 2007) and lamellar lipid deposits were evident in the adrenocortical cells (Faust and Hatten, 1997).

Biochemically, various peroxisome dependent parameters are abnormal in pups with these *Pex* gene inactivations [accumulation of VLCFA, lack of plasmalogen, abnormal bile acids, shortage of docosahexaenoic acid (DHA)]. Changes in brain PUFA composition have been proposed to modify α-synuclein (Yakunin et al., 2010), which could contribute to the neuropathology. In whole brain extracts of these three models, Yakunin et al. (2010) showed increasing oligomerization and phosphorylation of α-synuclein. Such changes trigger intraneuronal deposition of α-synuclein (Lewy bodies), being a hallmark of synucleopathies such as Parkinson disease.

A different phenotype is seen in PEX7 deficient mice (Brites et al., 2003). Embryonic lethality is not seen, but these pups are also hypotonic and growth impaired (15–30% lower body weight at birth), and the majority (70%) dies before weaning (50% after 1 day, likely due to the hypotonia). The surviving animals do live till adulthood and longer, but males are infertile, the seminiferous epithelium being devoid of spermatogonia and spermatocytes. In brain, a delay in neuronal migration is seen, and ossification of distal bone elements of the limbs, skull and vertebrae, is defective. The amount of white, but not brown, adipose tissue is reduced (Brites et al., 2011). Bilateral cataracts develop 2 weeks after birth (Brites et al., 2011), the time pups open their eye lids. Biochemically, plasmalogens are depleted, phytanic acid cannot be degraded, and VLCFA oxidation is impaired in fibroblasts, but increased VLCFA levels are only found in spleen, spinal cord and neonatal brain (Brites et al., 2009).

In *Pex7* hypomorphic mice, in which *Pex7* transcripts are reduced to 5%, lifespan is normal (Braverman et al., 2010). The mice are still smaller, but are fertile. Their tissue content of plasmalogens is low but not absent, DHA in RBC is lowered and phytanic acid accumulates. Pathological findings include endochondral ossification defects, abnormalities in lens fibers and eye cataract (Braverman et al., 2010).

Feeding 6-weeks old *Pex7*$^{-/-}$ mice with 1-*O*-octadecylglycerol, an etherlipid which is bypassing the

peroxisomal biosynthetic steps (see **Figure 3**), reveals that several phenotypic abnormalities are related to plasmalogen deficiency. The diet restores plasmalogen levels in non-nervous tissues. In parallel, testicular pathology is ameliorated (spermatogenesis was restored, although mature spermatozoa were still not detectable), and adipocytes displayed a normal size and fat content. When giving 1-*O*-octadecylglycerol to newborn pups, via supplementing it to the diet of the mother, testicular degeneration was prevented and cataract formation was absent or only unilateral and reduced to a small nuclear cataract (Brites et al., 2011). In the hypomorphic *Pex7*$^{-/-}$ mice, such treatment did not affect the cataracts (Braverman et al., 2010).

Severe bone abnormalities, a major hallmark in patients with PEX7 deficiency as reflected in their name (RCDP), are not observed in mice. Upon closer investigation, a delay, however, in endochondral bone formation was reported in both complete (Brites et al., 2003) and hypomorphic PEX7 (Braverman et al., 2010) deficient mice, likely due to a delayed maturation of chondrocytes at the pre-hypertrophic state, but further mechanistic insights were not generated.

Given the lethality of peroxin knockouts, especially of those with affected PTS1-import, developmental and behavioral studies are limited. This can be circumvented by conditional knock-out whereby peroxisomes are removed in specific tissues and/or at a certain stage. Tissue-specific removal of peroxisomes can be established by crossing mice containing a floxed *Pex* gene (Baes et al., 2002) with mice expressing cre in a promoter-specific manner. The promoter also determines the time point from when on the *Pex* gene is irreversibly inactivated in the targeted cells and their descendents. This technology was applied for *Pex5* creating mice lacking peroxisomes in the central nervous system (CNS) [*nestin-Cre*, in neural precursors from embryonic (E) day 11 (Hulshagen et al., 2008)], hepatocytes [alfafoetoprotein-*Cre*, from E10 (Krysko et al., 2007) and albumin-*Cre*, from birth (Peeters et al., 2011)]. By using a similar approach, brain specific PEX13 knockouts were obtained *(nestin-Cre*) (Müller et al., 2011).

Pex5 was further inactivated in specific cell types by using appropriate Cre-expressing mice: Sertoli cells (*Amh-Cre*, from E14) (Huyghe et al., 2006b), oligodendrocytes (*Cnp-Cre*, from E14) (Kassmann et al., 2007), principal neurons in the forebrain (*Nex-Cre*, from E12) (Bottelbergs et al., 2010), and astrocytes (*Gfap-Cre* from E13) (Bottelbergs et al., 2010). The specific inactivation of PEX5 in adipocytes failed due to the non-selectivity of the *aP2* promoter driving Cre expression (Martens et al., 2012).

Overall, these studies indicate that absence of peroxisomes in adipose (Martens et al., 2012), neurons (Bottelbergs et al., 2010), astrocytes (Bottelbergs et al., 2010), or Sertoli cells (Huyghe et al., 2006b) does not compromise life span. Postnatal thriving, however, requires functional liver and brain peroxisomes. Moreover, absence in liver results in life threatening development of hepatocarcinomas (Dirkx et al., 2005), absence in brain shortens life span considerably to 6 months with 20% dead before 3 weeks for *Pex5-loxP:nestin-cre* (Hulshagen et al., 2008) or 35 days for *Pex13-loxP:nestin-cre* (Müller et al., 2011) mice. Of the different models with specific brain cell inactivation, the oligodendrocyte knockout represents the worst outcome: almost none of the affected animals survive 1 year of age (Kassmann et al., 2007). Its phenotype resembles that of a total deficiency of peroxisomes in the brain, but with delayed onset of demyelination, axonal loss and neuroinflammation. The latter encompasses a strong activation of the innate immune system with microglia reactivity and increased expression of pro-inflammatory markers (Kassmann et al., 2007; Bottelbergs et al., 2012). The biochemical factor(s) contributing to or causing this phenotype remain unclear. To which extent peroxisomal metabolites can be transferred from one cell type to another in brain, or from the body to the brain, is not fully established, but an important role of peroxisomes in neurons or astrocytes in pre- and postnatal life can be excluded.

For more information about these models, and how peroxin deficiencies affect brain, liver and testis, we refer to recent reviews (Baes and Van Veldhoven, 2006, 2012; Baes and Aubourg, 2009). It should be stressed that part of the pathology seen in these mouse models might be related to the, not yet completely understood, interplay between peroxisomes, their metabolites and other organelles. As initially observed in PEX5 (Baumgart et al., 2001) and PEX2 knockouts (Keane et al., 2007), and further documented in the *albumin-Cre/Pex5-loxP* mice (Dirkx et al., 2005), absence of peroxisomes in hepatocytes affects their mitochondria severely. Structural alterations are seen in the inner mitochondrial membrane, and its potential is collapsed. Activities of complex I, III, and V are reduced. In addition, lipid droplets and ER stress are noticed. Based on the upregulation of ATF3, ATF4, ATF6, and CHOP, the unfolded protein response pathway is activated in absence of peroxisomes (Dirkx et al., 2005). Similar findings were seen in liver of surviving PEX2 pups, the integrated stress response mediated by PERK and ATF4 signaling being activated (Kovacs et al., 2009). It is postulated that perturbed peroxisomal β-oxidation metabolites (e.g., bile acid (intermediates), dicarboxylic acids), are causative factors given the fact that ER stress is also seen in mice with β-oxidation defects (Huang et al., 2011).

CONCLUSION

Although peroxisomes are not essential for cell functioning and survival, at the multicellular level they are indispensable as demonstrated by the different animal models treated in this chapter.

A common feature in animals with peroxisome biogenesis defects is a developmental delay, smaller size at hatching/birth and limited to very short lifespan (**Figure 5**). The reason for the delay is not clear. In most models, organogenesis seems to proceed normal, but the central nervous system appears sensitive to absence of peroxisomes (abnormal cerebellar lamination and delayed neuron migration in mice; malformation of the ventral nerve cord in fruitfly; block of neuron cell migration in L1 stage in nematodes). In a later stage of life, neuronal problems are manifested in reduced locomotion (larvae of insects) or coordination and motor skills (mice). In nematodes, normal larval development is dependent on ether lipids. In mice (and man), plasmalogen deficiency is compatible with prenatal

development but the newborns exhibit already several abnormalities.

With regard to the nervous system, an intriguing question is to which extent myelinization/demyelinization and axonal integrity are linked to peroxisomes. Myelin, formed by the oligodendrocytes, is indeed enriched in metabolites related to peroxisomes (plasmalogens, VLCFA). It is therefore surprising that myelination is initially normal when peroxisomes are ablated from oligodendroglia and that in adulthood myelin becomes destabilized. Importantly, as both in the total brain and the oligodendrocyte knockout, degenerated axons are observed surrounded with a normal myelin sheet, it was postulated that oligodendroglial peroxisomes serve to support axons independent of myelination. This is further endorsed by the finding that peroxisomes are abundant in paranodes (Kassmann et al., 2011), sites where glia and axons interact. In this context, one should recall that in species in which axons are not myelinated such as fruitfly, neuronal abnormalities are seen when peroxisomes are ablated (Nakayama et al., 2011).

Another remarkable finding, although not studied in all models, is the male sterility, documented at least in *Drosophila* and in mice knockouts (**Figure 6**). In fly, peroxisomes of the cysts cell appear to be important for spermatogenesis (Nakayama et al., 2011), which is mirrored in mice where peroxisomes are necessary in the Sertoli cells (Huyghe et al., 2006b). In insects, the infertility was linked to accumulation of VLCFA, in mice experimental evidence points toward both an accumulation of VLCFA and VLCFA-PUFA (Huyghe et al., 2006b). The importance of normal peroxisomal β-oxidation for male fertility was further confirmed in ACOX1 (Fan et al., 1996) and MFP2 knockout mice (Huyghe et al., 2006b). In addition, a depletion of ether lipids also causes male infertility in PEX7 (Brites et al., 2011) and GNPAT (Rodemer et al., 2003) knockout mice.

Finally, related to aging and neurodegenerative diseases, and the emerging role of peroxisomes in ROS signaling (Titorenko and Terlecky, 2011; Fransen et al., 2012) scattered information derived from the animal models discussed above, suggest that less active peroxisomes in adulthood could positively contribute to longevity. This seems, however, in conflict with the general concept that the metabolic activity of these organelles becomes compromised during aging. On the other hand, it would be consistent with studies on the importance of catalase in aging. Improving the removal of peroxide in peroxisomes, by expressing an engineered catalase with a higher affinity for PEX5, delays the appearance of senescence markers in human fibroblasts (Koepke et al., 2007). Hence, not the peroxisomal metabolic activity, but the ratio of ROS-generation/removal (Fransen et al., 2013), might be a determining factor in aging.

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Expanding functional repertoires of fungal peroxisomes: contribution to growth and survival processes

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It has long been regarded that the primary function of fungal peroxisomes is limited to the β-oxidation of fatty acids, as mutants lacking peroxisomal function fail to grow in minimal medium containing fatty acids as the sole carbon source. However, studies in filamentous fungi have revealed that peroxisomes have diverse functional repertoires. This review describes the essential roles of peroxisomes in the growth and survival processes of filamentous fungi. One such survival mechanism involves the Woronin body, a Pezizomycotina-specific organelle that plugs the septal pore upon hyphal lysis to prevent excessive cytoplasmic loss. A number of reports have demonstrated that Woronin bodies are derived from peroxisomes. Specifically, the Woronin body protein Hex1 is targeted to peroxisomes by peroxisomal targeting sequence 1 (PTS1) and forms a self-assembled structure that buds from peroxisomes to form the Woronin body. Peroxisomal deficiency reduces the ability of filamentous fungi to prevent excessive cytoplasmic loss upon hyphal lysis, indicating that peroxisomes contribute to the survival of these multicellular organisms. Peroxisomes were also recently found to play a vital role in the biosynthesis of biotin, which is an essential cofactor for various carboxylation and decarboxylation reactions. In biotin-prototrophic fungi, peroxisome-deficient mutants exhibit growth defects when grown on glucose as a carbon source due to biotin auxotrophy. The biotin biosynthetic enzyme BioF (7-keto-8-aminopelargonic acid synthase) contains a PTS1 motif that is required for both peroxisomal targeting and biotin biosynthesis. In plants, the BioF protein contains a conserved PTS1 motif and is also localized in peroxisomes. These findings indicate that the involvement of peroxisomes in biotin biosynthesis is evolutionarily conserved between fungi and plants, and that peroxisomes play a key role in fungal growth.

Keywords: peroxisome, fungi, Woronin body, biotin, mitochondria

INTRODUCTION

Peroxisomes are ubiquitous organelles in eukaryotic cells and typically contain enzymes involved in the β-oxidation of fatty acids and detoxification of reactive oxygen species. Additionally, peroxisomes are known to have various physiological functions based on their roles in diverse metabolic activities. For example, mammalian peroxisomes participate in the lipid biosynthesis such as ether phospholipids, and in the oxidation of amino acids and polyamines (Wanders and Waterham, 2006). In plants, peroxisomes are involved in the glyoxylate cycle (Mano and Nishimura, 2005), photorespiration (Reumann and Weber, 2006), male-female gametophyte recognition (Boisson-Dernier et al., 2008) and biosynthesis of the hormones jasmonic acid and auxin (Weber, 2002; Woodward and Bartel, 2005). Peroxisomes also play important roles in higher eukaryotes, with defects in peroxisome biogenesis resulting in severe human disease, such as Zellweger syndrome, neonatal adrenoleukodystrophy, and Refsums disease (Waterham and Ebberink, 2012). In plants, loss of peroxisomal function causes embryo lethality, suggesting that peroxisomes have an essential role in growth and development (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Tzafrir et al., 2004; Fan et al., 2005).

The primary role of fungal peroxisomes is the β-oxidation of fatty acids, as fungal mutants lacking peroxisomes fail to grow in minimal medium containing fatty acids as the sole carbon source (Erdmann et al., 1989; Hynes et al., 2008). Peroxisomes are also required for methanol metabolism in methylotrophic yeasts, including *Pichia pastoris* (van der Klei et al., 2006). In filamentous fungi, peroxisomes are also involved in secondary metabolism including the biosynthesis of penicillin, AK (*Alternaria kikuchiana*) toxin, and paxilline (Saikia and Scott, 2009; Imazaki et al., 2010; Bartoszewska et al., 2011), plant pathogenicity (Kimura et al., 2001; Asakura et al., 2006), and sexual development (Bonnet et al., 2006; Peraza-Reyes et al., 2008). While fungal peroxisomes are known to proliferate massively on oleate and acetate, inducing substrates for this organelle (van der Klei and Veenhuis, 2006), it is apparent that many peroxisomes constitutively exist in the cell of filamentous fungi under the normal growth condition e.g., on glucose (Tanabe et al., 2011). The delayed growth and aberrant organelle morphologies observed in peroxisome-deficient mutants (Bonnet et al., 2006; Idnurm et al., 2007; Hynes et al., 2008) suggest that peroxisomes have fundamental roles for the growth of filamentous fungi. However, the molecular mechanisms underlying these severe growth effects remain unknown.

In this review, evidence for the roles of peroxisomes in fungal growth, particularly the involvement of the Woronin body, a peroxisome-derived organelle with wound-healing function, and the recently identified function of peroxisomes in vitamin biosynthesis are presented.

THE WORONIN BODY, AN ORGANELLE SPECIFIC TO PEZIZOMYCOTINA SPECIES, DIFFERENTIATES FROM PEROXISOMES

Species of Pezizomycotina (filamentous ascomycetes) grow via elongation of the hyphal tip to form straight primary hyphae with branches. The hyphae are divided into distinct cells by the formation of septa, and thus filamentous fungi are characterized by multicellularity. The septum is proposed to have several possible functions, including increasing the mechanical integrity of hyphae and division of mycelium into sections that undergo distinct developmental processes. However, septa do not completely separate adjacent cells in the hyphae due to the presence of a septal pore, which allows the passage of cytoplasm and organelles between adjacent cells (Markham, 1994; Freitag et al., 2004; Lew, 2005; Tey et al., 2005; Ng et al., 2009). This intercellular communication resembles that found in higher eukaryotes, such as gap junctions in animal cells and plasmodesmata in plant cells, and suggests that filamentous fungi possess a cell-to-cell channel that modulates responses to environmental changes and development processes necessary for multicellularity.

Cytoplasmic continuity between adjacent cells through the septal pore is associated with catastrophic risk of cytoplasmic loss by adjacent cells due to hyphal lysis. This risk was clearly demonstrated by the exposure of the filamentous fungus *Aspergillus oryzae* grown on agar medium to hypotonic shock, which caused most of the hyphal tips to burst and lose the cytoplasmic constituents (**Figure 1A**) (Maruyama et al., 2005). However, as evidenced by differential interference contrast (DIC) and fluorescence microscopy, ∼80% of the immediately adjacent cells retained their cytoplasmic constituents (**Figure 1B**) (Maruyama et al., 2005), allowing these cells to initiate regrowth by creating a new hyphal tip (Maruyama et al., 2006; Maruyama and Kitamoto, 2007). This process represents a type of defense system that aims to promote the survival of these multicellular organisms by preventing the excessive loss of cytoplasm upon hyphal lysis.

The Woronin body is a unique organelle present in Pezizomycotina species that plugs the septal pore upon hyphal lysis and prevents excessive cytoplasmic loss from the cell adjacent to the lysed cell (**Figure 2A**) (Markham and Collinge, 1987). This organelle has two morphologically distinct subclasses; it is generally observed by transmission electron microscopy as a spherical electron-dense structure in the vicinity of the septum (**Figure 2B**), although a limited number of species, such as *Neurospora crassa*, form hexagonal crystalline Woronin bodies that are occasionally visible by light microscopy (Markham, 1994).

Jedd and Chua (2000) first identified Hex1 as a major protein of the Woronin body in *N. crassa*. Genes encoding the Hex1 protein are conserved in Pezizomycotina species (Jedd and Chua, 2000; Asiegbu et al., 2004; Curach et al., 2004; Soundararajan et al., 2004; Maruyama et al., 2005; Beck and Ebel, 2013). Self-assembly of Hex1 provides the Woronin body with a mechanically solid core that provides resistance to the protoplasmic streaming pressure arising from hyphal lysis (Jedd and Chua, 2000; Yuan

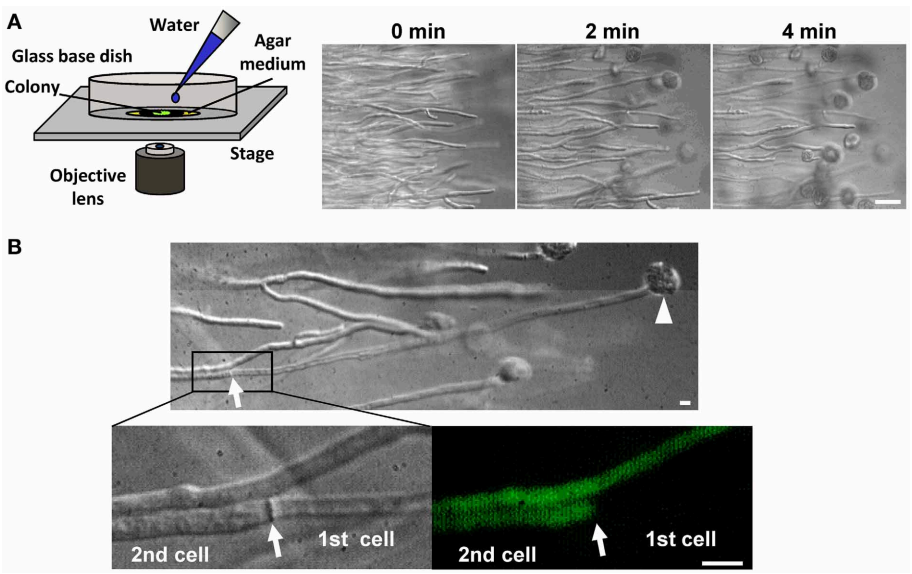

FIGURE 1 | Hyphal tip bursting upon hypotonic shock in the filamentous fungus *A. oryzae*. (A) Time-lapse observation of hyphal tip bursting upon hypotonic shock. Hyphal tips at the edge of a colony grown on agar medium were observed by DIC microscopy before and after flooding hyphae with water. Bar: 50 μm. **(B)** Excessive loss of cytoplasmic constituents is prevented upon hyphal tip bursting induced by hypotonic shock. The cytoplasm was labeled by EGFP. An arrowhead and arrow indicate a burst hyphal tip and the adjacent septum, respectively. Note that the cell (2nd) adjacent to the lysed cell (1st) retains its cytoplasmic constituents, as determined by DIC and fluorescence microcopy. Bar: 10 μm.

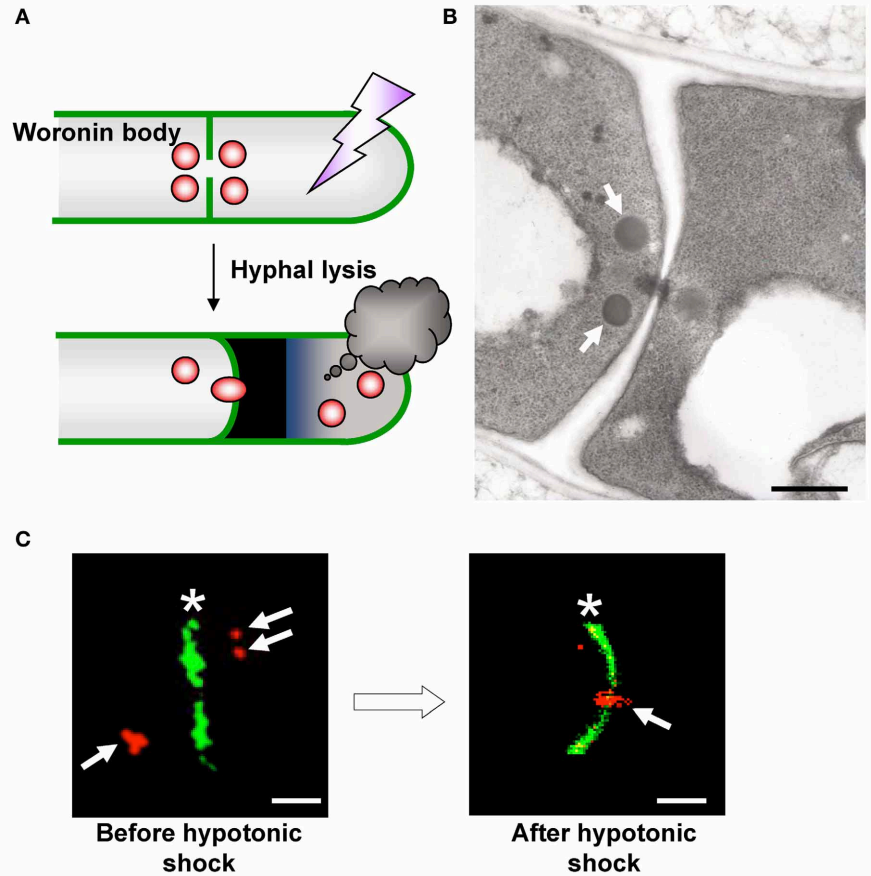

FIGURE 2 | Morphology and function of the Woronin body. (A) Schematic model of Woronin body function. **(B)** Transmission electron microscopic observation of Woronin bodies (arrows) in *A. oryzae* (Maruyama et al., 2005). Bar: 500 nm. **(C)** Confocal images of Woronin bodies (red, arrows) and septa (green, asterisks) before (left) and after (right) hyphal tip bursting induced by hypotonic shock. Woronin bodies and septa were fluorescently labeled by expressing DsRed2–AoHex1 and RNase T1–EGFP fusion proteins, respectively (Maruyama et al., 2005). Bar: 2 μm.

et al., 2003). Phosphorylation of Hex1 has a role in the formation of the multimeric core of the Woronin body (Tenney et al., 2000; Juvvadi et al., 2007). Deletion of the *hex1* gene results in the disappearance of Woronin bodies and is associated with severe cytoplasmic bleeding upon hyphal lysis (Jedd and Chua, 2000; Tenney et al., 2000; Maruyama et al., 2005). In the case of *A. oryzae*, *hex1* deletion (Δ*hex1*) significantly reduces the ability of this strain to prevent excessive cytoplasmic loss (Maruyama et al., 2005, 2010; Escaño et al., 2009). Using fluorescence microscopy, Woronin bodies were demonstrated to plug the septal pore adjacent to a lysed cell upon hyphal lysis in the *A. oryzae* wild-type strain (**Figure 2C**) (Maruyama et al., 2005). Recently, Bleichrodt et al. (2012) reported that the Woronin body reversibly closes the septal pore during normal growth of *A. oryzae*, a function that contrasts the behavior of this organelle conventionally observed during hyphal lysis. In addition, although wild-type *A. oryzae* has heterogeneous distribution of hyphae and gene expression activity, the absence of Woronin bodies results in uniform activity distribution of different cells (Bleichrodt et al., 2012). Collectively, Woronin bodies impede cytoplasmic continuity between adjacent cells during normal growth and help maintain hyphal heterogeneity in mycelia. This function of Woronin bodies may represent the most primitive way to regulate cell-to-cell channels in multicellularity by a simple plugging behavior similar to that upon hyphal lysis. Additionally, the roles of Woronin bodies in conidiation (asexual spore formation), survival under nitrogen starvation and efficient plant pathogenesis were reported (Yuan et al., 2003; Soundararajan et al., 2004).

A relationship between peroxisomes and the Woronin body is suggested from the fact that Hex1 contains peroxisomal targeting signal sequence 1 (PTS1) at the C-terminus (Jedd and Chua, 2000). Time-lapse imaging demonstrated that Woronin bodies bud from peroxisomes in *N. crassa* (Tey et al., 2005) and that Woronin body biogenesis requires the presence of peroxins that mediate peroxisomal protein import (Ramos-Pamplona and Naqvi, 2006; Managadze et al., 2007; Liu et al., 2008). The peripheral membrane peroxisomal protein Pex11 is implicated in peroxisomal proliferation and division (Erdmann and Blobel, 1995; Marshall et al., 1995), and in the absence of Pex11, filamentous fungi only contain few enlarged peroxisomes (**Figure 3A**, EGFP-PTS1) (Hynes et al., 2008; Escaño et al., 2009; Opaliński et al., 2012). It was also demonstrated that ability

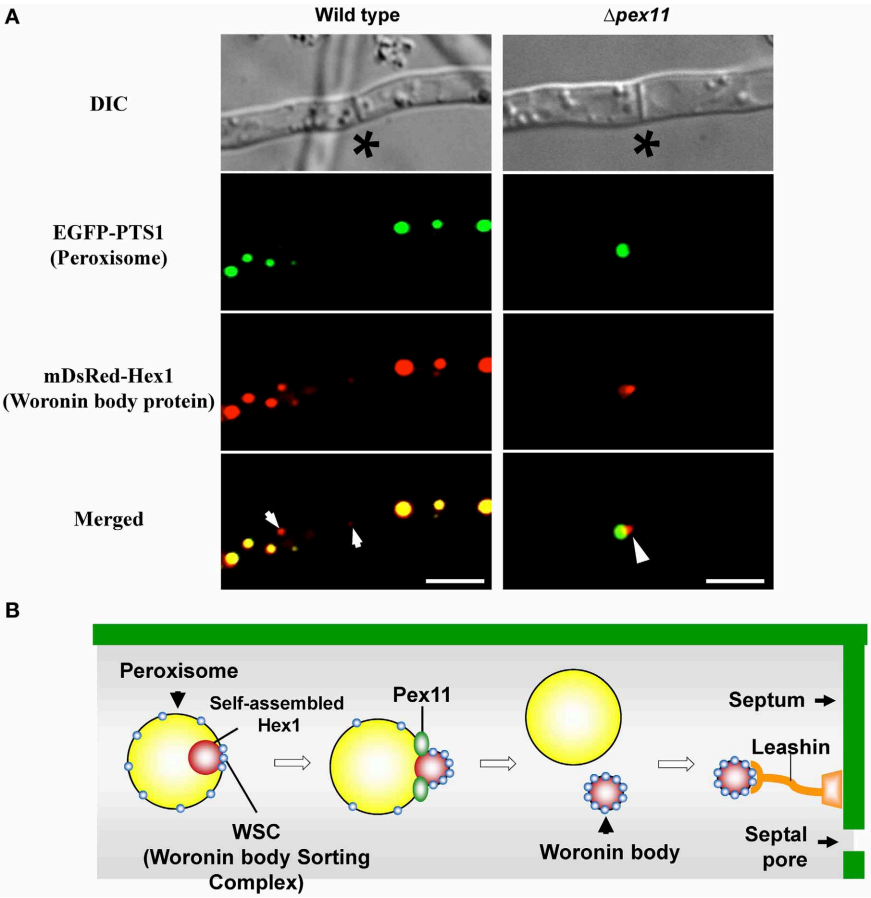

FIGURE 3 | Differentiation of the Woronin body from peroxisomes. **(A)** Fluorescence microscopic analysis of wild-type and Δ*pex11* strains of *A. oryzae* expressing EGFP-PTS1 and mDsRed-AoHex1 fusion proteins for visualization of peroxisomes and the major Woronin body protein, respectively (Escaño et al., 2009). Asterisks denote septa and arrows indicate Woronin bodies (red) independent of peroxisomes (green). Arrowheads represent assembly of Hex1 attached to the matrix side of the peroxisome. Bars: 5 μm. **(B)** Schematic model of Woronin body differentiation from peroxisomes.

of Pex11-deficient strain of *A. oryzae* to prevent the excessive loss of cytoplasm is reduced by ∼30% compared to wild type (Escaño et al., 2009), indicating that Pex11 is involved in Woronin body function. Under fluorescence microscopy, Woronin bodies are typically observed as small dots independent of peroxisomes (**Figure 3A**, mDsRed-AoHex1). In the absence of Pex11, however, the Woronin body protein Hex1 forms a structure that attaches to the matrix side of the peroxisomal membrane, but the mature Woronin body fails to differentiate from peroxisomes (**Figure 3A**) (Escaño et al., 2009). The Pezizomycotina-specific protein WSC (Woronin body sorting complex) recruits the Hex1 assembly to the matrix side of the peroxisomal membrane and facilitates the budding of the Woronin body (Liu et al., 2008). It has been suggested that Pex11 elongates the peroxisomal membrane to facilitate the division of peroxisomes by dynamin-related proteins (Koch et al., 2003, 2004; Schrader, 2006). Heterologous expression of Hex1 in the yeast *Saccharomyces cerevisiae* suggested that dynamin-related proteins participate in the budding of Woronin bodies from peroxisomes (Würtz et al., 2008). ApsB, a component of the microtubule-organizing center (MTOC), has been shown to interact with Hex1 and to localize to peroxisomes via peroxisomal targeting signal sequence 2 (PTS2) (Zekert et al., 2010). Hex1 physically associates with the essential matrix import peroxin Pex26 and promotes the enrichment of Pex26 in the membranes of differentiated peroxisomes (Liu et al., 2011). After Woronin bodies differentiate from peroxisomes, evidence suggests that the Pezizomycotina-specific protein Leashin (LAH) tethers the Woronin bodies to the vicinity of the septum (Ng et al., 2009). A schematic model of Woronin body differentiation from peroxisomes is presented in **Figure 3B**. Although a number of proteins functionally/spatially related to the Woronin body have been identified (Engh et al., 2007; Fleissner and Glass, 2007; Kim et al., 2009; Maruyama et al., 2010; Lai et al., 2012; Yu et al., 2012), the molecular mechanism for Woronin body biogenesis remains to be completely resolved.

INVOLVEMENT OF PEROXISOMES IN BIOTIN BIOSYNTHESIS IN FUNGI

Biotin is an essential cofactor involved in a number of carboxylation and decarboxylation reactions (Knowles, 1989). In

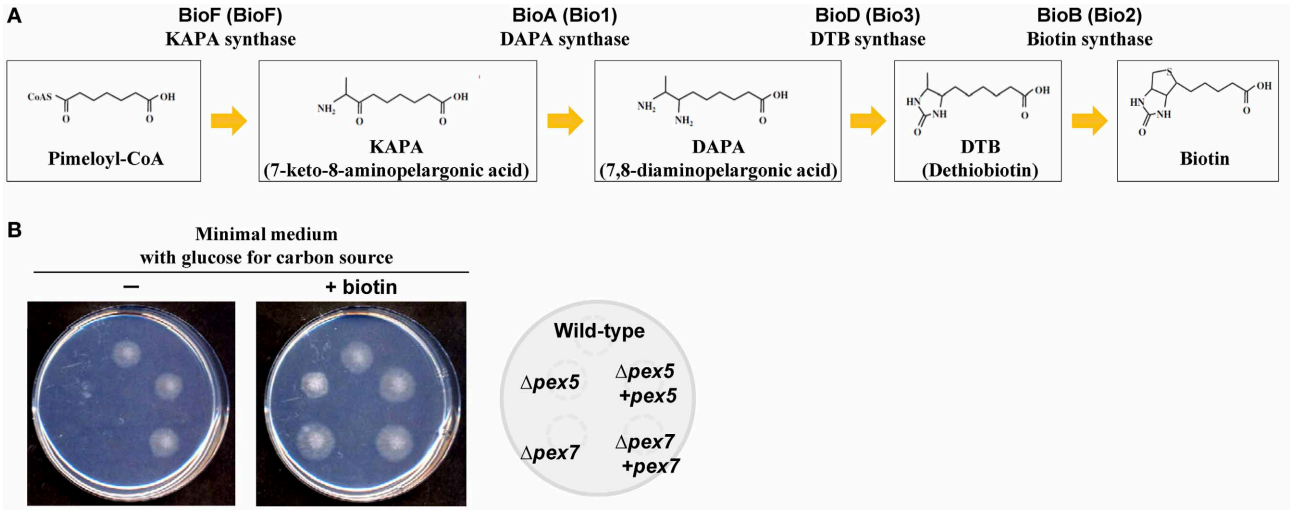

FIGURE 4 | Biotin biosynthetic pathway and biotin auxotrophy in peroxisome-deficient strains. (A) The final four reactions of the biotin biosynthetic pathway and involved enzymes from fungi and plants (in parentheses). **(B)** Growth impairment of strains defective in peroxisomal targeting signal receptors (Δ*pex5* and Δ*pex7*) grown on minimal medium containing glucose as the sole carbon source. Growth of the wild-type, Δ*pex5,* and Δ*pex7* strains and the complemented strains (indicated to the right) on medium with and without biotin.

eukaryotes, plants and numerous fungal species are capable of synthesizing biotin. The studies of plants and fungi have revealed that the final four reactions in the biosynthetic process, which convert pimeloyl-CoA to biotin, are conserved (**Figure 4A**) (Streit and Entcheva, 2003). In plants, the enzymes BioF, Bio1, Bio3, and Bio2 mediate the final four steps of biotin biosynthesis. It was previously reported that BioF, a 7-keto-8-aminopelargonic acid (KAPA) synthase catalyzing the conversion of pimeloyl-CoA to KAPA, is localized to the cytoplasm (Pinon et al., 2005). The final three reactions converting KAPA to biotin occur in mitochondria. The *BIO3* and *BIO1* genes are unidirectionally aligned and expressed as a chimeric transcript, resulting in the production of Bio3-Bio1 as a bifunctional protein catalyzing desthiobiotin (DTB) synthase and 7, 8-diaminopelargonic acid (DAPA) synthase reactions (Muralla et al., 2008). Bio3-Bio1 contains a mitochondrial targeting sequence (MTS) and localizes in mitochondria (Muralla et al., 2008; Cobessi et al., 2012). The Bio2 protein, a biotin synthase catalyzing the conversion of DTB to biotin, also contains a MTS and must be mitochondrially localized for biotin prototrophy (Baldet et al., 1997; Picciocchi et al., 2001; Arnal et al., 2006). It was therefore suggested that plant biotin biosynthesis occurs in both the cytoplasm and mitochondria (Rébeillé et al., 2007).

In *Aspergillus* species, mutants of the *pex5* and *pex7* genes are defective in protein import into the peroxisomal matrix due to the lack of PTS1 and PTS2 receptors, respectively (Hynes et al., 2008; Tanabe et al., 2011). These mutants fail to grow in minimal medium containing oleic acid as the sole carbon source due to defective peroxisomal β-oxidation; however, unlike the corresponding yeast mutants, the mutants of *Aspergillus* species also exhibit growth defects when grown on glucose (Hynes et al., 2008; Tanabe et al., 2011). Surprisingly, the growth defects are restored by the addition of biotin (**Figure 4B**). In fungi, biotin is synthesized through the sequential activities of three Bio proteins: BioF, a KAPA synthase; BioD/A, a chimeric protein composed of DTB and DAPA synthases; and BioB, a biotin synthase (**Figure 4A**) (Magliano et al., 2011a,b; Tanabe et al., 2011). The BioD/A protein localizes in mitochondria, suggesting that this is where KAPA is converted to biotin (Tanabe et al., 2011). Protein sequence analysis of fungal BioF proteins revealed that the C-terminal PTS1 sequences are conserved in ascomycete and basidiomycete species (**Figure 5A**). Consistent with these findings, BioF protein localizes in the peroxisomes via PTS1 (**Figure 5B**), and the peroxisomal targeting of this KAPA synthase is required for biotin biosynthesis (Magliano et al., 2011a; Tanabe et al., 2011). Yeast species appear to have lost the gene encoding BioF, as evidenced by their biotin auxotrophy, although several species have reacquired biotin prototrophy by horizontal gene transfer and gene duplication followed by neofunctionalization (Hall and Dietrich, 2007).

A new model for biotin biosynthesis in fungi is proposed in **Figure 6**. In this biotin biosynthesis pathway, the production of pimeloyl-CoA may involve proteins containing PTS1 and PTS2 (Tanabe et al., 2011), while peroxisomal β-oxidation is also involved (Magliano et al., 2011a). Ohsugi et al. (1988) reported that pimelic acid, a putative pimeloyl-CoA precursor, were produced from long chain fatty acids such as oleic acid in yeasts, which may support a relevance of β-oxidation to supplying a precursor substrate for biotin biosynthesis. In peroxisomes, KAPA is first synthesized from pimeloyl-CoA by BioF protein and is then likely transported from peroxisomes to mitochondria, where it serves as a substrate for the final series of biosynthesis reactions that convert KAPA to biotin by the action of the BioD/A and BioB proteins. Thus, functionally coupling between peroxisomes and mitochondria appears to be required for biotin biosynthesis.

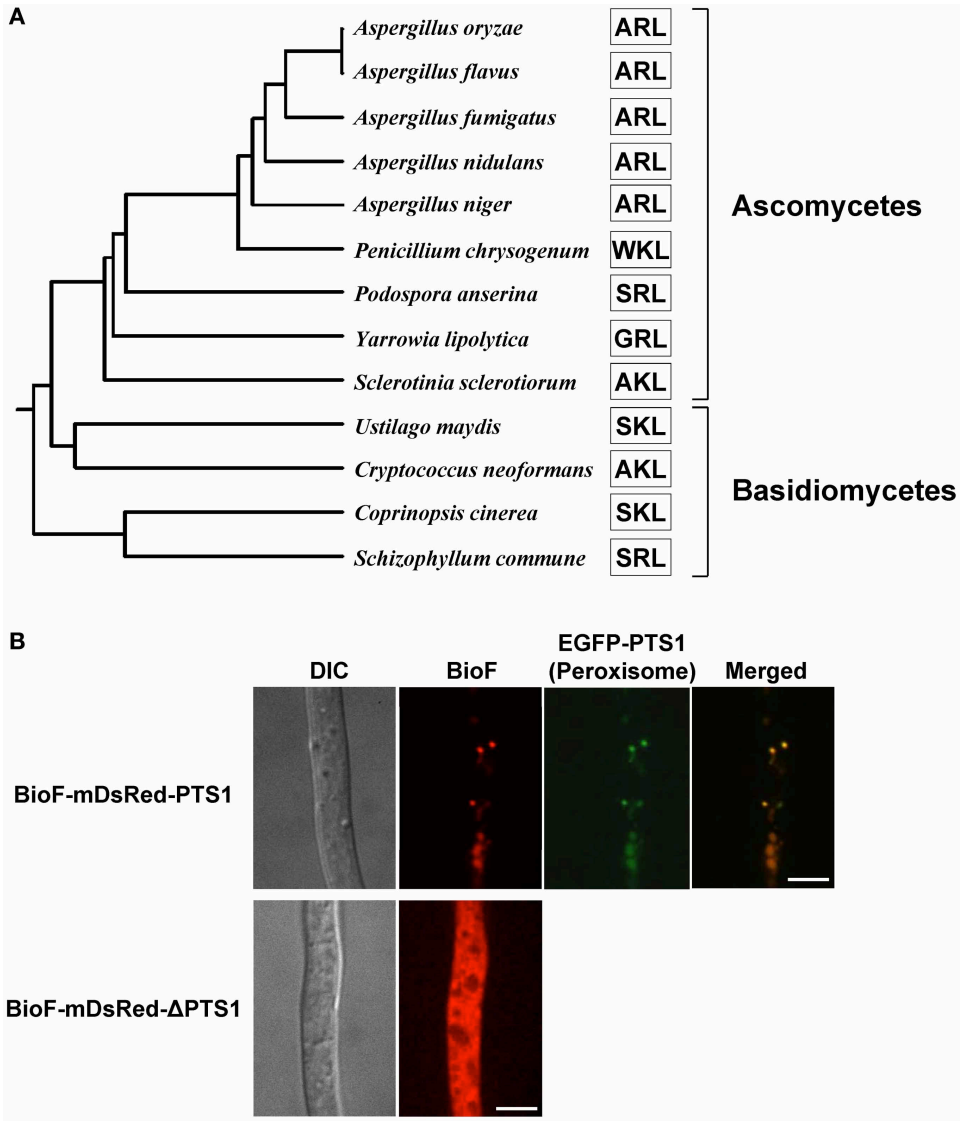

FIGURE 5 | Phylogenetic relationship and peroxisomal localization of fungal BioF proteins. (A) Phylogenetic analysis of fungal BioF proteins. The amino acid residues of the C-terminal peroxisomal targeting signals (PTS1) are indicated by open boxes. The full-length amino acid sequences of the fungal BioF proteins were aligned using the Clustal W program (version 2.1), and then the phylogenetic tree was constructed. The Genbank accession numbers for the sequences used in the analysis are as follows: *Aspergillus oryzae*, XP_001817022.1; *Aspergillus flavus*, XP_002383037.1; *Aspergillus fumigatus*, XP_747713.1; *Aspergillus nidulans*, ACR44939.1; *Aspergillus niger*, XP_001396737.1; *Penicillium chrysogenum*, XP_002563821.1; *Podospora anserina*, XP_001903515.1; *Yarrowia lipolytica*, XP_504066.1; *Sclerotinia sclerotiorum*, XP_001590700.1; *Ustilago maydis*, XP_757344.1; *Cryptococcus neoformans*, XP_566616.1; *Coprinopsis cinerea*, XP_001836705.2; and *Schizophyllum commune*, XP_003028193.1. **(B)** Peroxisomal localization of fungal BioF protein. Note that BioF (BioF-mDsRed-PTS1) co-localizes with peroxisomes (EGFP-PTS1), but disperses in the absence of PTS1 (BioF-mDsRed-ΔPTS1) (Tanabe et al., 2011). Bars: 5 μm.

CONSERVED PEROXISOMAL LOCALIZATION OF BIOF PROTEIN AND ITS POSSIBLE RELEVANCE TO FUNGAL GROWTH/DEVELOPMENTAL PROCESSES

As described above, plant BioF protein functions as a KAPA synthase and was shown to be cytosolic by GFP fusion at the C-terminus (Pinon et al., 2005). Phylogenetic analysis revealed that BioF proteins from various plant species possess PTS1 at the C-terminus (**Figure 7A**) (Tanabe et al., 2011; Maruyama et al., 2012), suggesting that the peroxisomal localization of BioF proteins is conserved throughout the plant kingdom. An N-terminal GFP-BioF fusion protein co-localizes with peroxisomes, and deletion of PTS1 causes cytosolic localization, suggesting that BioF is localized to peroxisomes via the PTS1 sequence (**Figure 7B**) (Tanabe et al., 2011).

Plant biotin-auxotrophic mutants exhibit embryo lethality, indicating that biotin biosynthesis is vital for plant growth and development (Schneider et al., 1989; Shellhammer and Meinke, 1990; Patton et al., 1998; Tzafrir et al., 2004; Arnal et al.,

2006). Embryo development also requires peroxisomal functions (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Tzafrir et al., 2004; Fan et al., 2005). Tanabe et al. (2011) suggested that fungi and plants use an evolutionarily conserved pathway for biotin biosynthesis that involves both peroxisomes and mitochondria. These findings suggest that biotin biosynthesis might be one of the reasons why peroxisomal deficiency results in embryo lethality. The *Aspergillus* peroxisome-deficient strains showing biotin auxotrophy exhibit abnormal polar growth (Tanabe et al., 2011), and impairment of sexual development by peroxisomal malfunction was reported in filamentous fungi (Bonnet et al., 2006). These similarities in the fungal and plant phenotypes indicate that growth and developmental defects due to peroxisomal deficiency may be partially or entirely attributed to biotin auxotrophy. More extensive studies will provide insight into the importance of biotin biosynthesis and peroxisomal function during growth and development of fungi and plants.

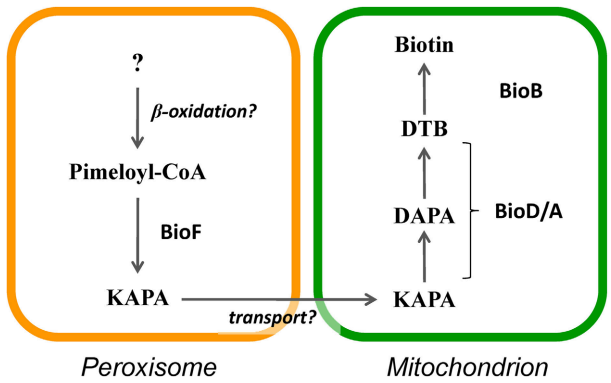

FIGURE 6 | Model of subcellular compartmentalization of the biotin biosynthetic pathway in eukaryotes.

CONCLUSION

The primary function of fungal peroxisomes was long thought to be limited to the β-oxidation of fatty acids. During the two past decades, an increasing number of studies have unmasked the functional diversity of fungal peroxisomes (Pieuchot and Jedd, 2012), including the very recent findings that peroxisomes contain siderophore biosynthetic enzymes and are involved in iron acquisition (Gründlinger et al., 2013), and that several glycolysis enzymes possess cryptic PTS1 motifs that are activated by alternative splicing and stop codon read-through

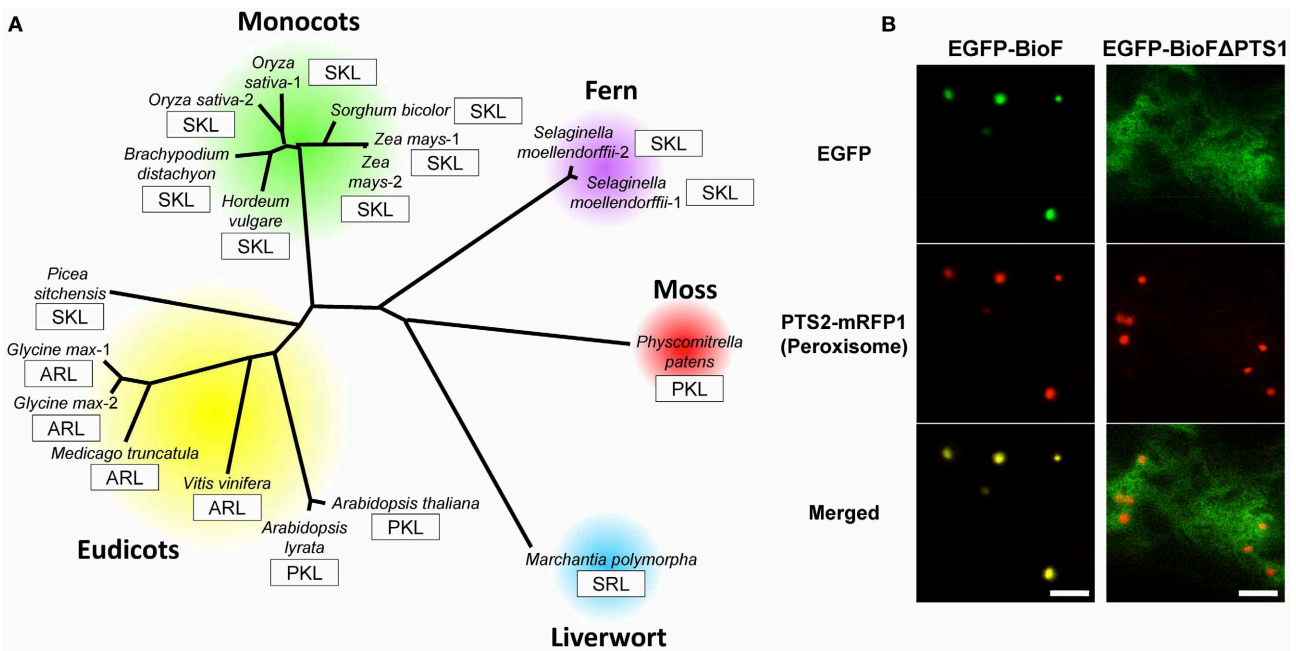

FIGURE 7 | Phylogenetic relationship and peroxisomal localization of plant BioF proteins. (A) Phylogenetic analysis of plant BioF proteins (Maruyama et al., 2012). The amino acid residues of C-terminal peroxisomal targeting signals (PTS1) are indicated by open boxes. The full-length amino acid sequences of the plant BioF proteins were aligned using the method described in **Figure 5**. The Genbank accession numbers for the sequences used in the analysis are as follows: *Arabidopsis thaliana*, NP_974731.1; *Arabidopsis lyrata*, XP_002871105.1; *Oryza sativa*-1, BAD87813.1; *Oryza sativa*-2, NP_001065381.1; *Hordeum vulgare*, BAK03504.1; *Brachypodium distachyon*, XP_003574335.1; *Sorgham bicolor*, XP_002467492.1; *Zea mays*-1, ACG35792.1; *Zea mays*-2, ACG35881.1; *Selaginella moellendorffii*-1, XP_002969752.1; *Selaginella moellendorffii*-2, XP_002981364.1; *Physcomitrella patens*, XP_001769874.1; *Picea sitchensis*, ABR18106.1; *Vinis vinifera*, XP_002268950.1; *Medicago truncatula*, XP_003598166.1; *Glycine max*-1, XP_003527547.1; and *Glycine max*-2, XP_003522881.1. The amino acid sequence of the BioF protein of *Marchantia polymorpha* was confirmed by PCR amplification and cDNA sequencing based on information obtained from the *Marchantia* expression sequence tag database (Maruyama et al., 2012). **(B)** Peroxisomal localization of plant BioF protein. Note that BioF (EGFP-BioF-PTS1) co-localizes with the peroxisomes (PTS2-mRFP1), but disperses in the absence of PTS1 (EGFP-BioF-ΔPTS1) (Tanabe et al., 2011). Bars: 5 μm.

(Freitag et al., 2012). The present review has described the findings that demonstrate the fundamental involvement of fungal peroxisomes in the regulation of multicellular growth and the biosynthesis of the essential vitamin biotin. Further investigations, including proteomic/metabolomic approaches and genomic bioinformatics, will lead to a comprehensive understanding of the newly emerged functions of fungal peroxisomes.

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Peroxisomes and sexual development in fungi

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Peroxisomes are versatile and dynamic organelles that are essential for the development of most eukaryotic organisms. In fungi, many developmental processes, such as sexual development, require the activity of peroxisomes. Sexual reproduction in fungi involves the formation of meiotic-derived sexual spores, often takes place inside multicellular fruiting bodies and requires precise coordination between the differentiation of multiple cell types and the progression of karyogamy and meiosis. Different peroxisomal functions contribute to the orchestration of this complex developmental process. Peroxisomes are required to sustain the formation of fruiting bodies and the maturation and germination of sexual spores. They facilitate the mobilization of reserve compounds via fatty acid β-oxidation and the glyoxylate cycle, allowing the generation of energy and biosynthetic precursors. Additionally, peroxisomes are implicated in the progression of meiotic development. During meiotic development in *Podospora anserina*, there is a precise modulation of peroxisome assembly and dynamics. This modulation includes changes in peroxisome size, number and localization, and involves a differential activity of the protein-machinery that drives the import of proteins into peroxisomes. Furthermore, karyogamy, entry into meiosis and sorting of meiotic-derived nuclei into sexual spores all require the activity of peroxisomes. These processes rely on different peroxisomal functions and likely depend on different pathways for peroxisome assembly. Indeed, emerging studies support the existence of distinct import channels for peroxisomal proteins that contribute to different developmental stages.

Keywords: peroxisomes, peroxins, fungi, sexual development, meiosis, organelle biogenesis, cell differentiation

INTRODUCTION

Peroxisomes are single membrane-bound organelles that are highly dynamic and versatile. They are present in most eukaryotic organisms and involved in a number of essential metabolic pathways. Peroxisomes have an important role in lipid metabolism (Wanders et al., 2010) and are implicated in the homeostasis of reactive oxygen species (ROS) (Fransen et al., 2012). Ubiquitous metabolic pathways, like the β-oxidation of fatty acids, reside in peroxisomes throughout eukaryotes (Poirier et al., 2006). In addition, peroxisomes perform many diverse metabolic activities. Some of these activities have a relatively broad distribution among eukaryotes, like the glyoxylate cycle in plants, fungi and some protists (Huang et al., 2004; Kunze et al., 2006). Others are highly specific, like penicillin biosynthesis in the fungus *Penicillium chrysogenum* (Bartoszewska et al., 2011).

Peroxisomes are now recognized as important signaling organelles. Signaling molecules are generated by metabolic process in peroxisomes (Nyathi and Baker, 2006; Joo et al., 2010; Del Rio, 2011), which can modulate the activity of key signaling proteins (Li et al., 2000). Signaling proteins are also targeted directly into peroxisomes, where they integrate external signals to trigger specific cell developmental responses (Szoor et al., 2010). Furthermore, peroxisomes function as a scaffold for the assembly of specific macromolecular signaling complexes, which participate in the orchestration of complex signaling networks (Dixit et al., 2010; Horner et al., 2011).

Fungi provide a notable example of the functional versatility of peroxisomes. In addition to the ubiquitous roles shared with most eukaryotes, peroxisomes in fungi participate in metabolic pathways like methanol assimilation (Van Der Klei et al., 2006), biotin (Tanabe et al., 2011) and siderophore biosynthesis (Grundlinger et al., 2013). Moreover, several core glycolytic enzymes localize to peroxisomes in fungi (Freitag et al., 2012), a property until recently believed to be restricted to some Euglenozoa protists (Gualdron-Lopez et al., 2012). In addition, the fungal peroxisome is implicated in secondary metabolism and participates in the formation of metabolites like β-lactam antibiotics (penicillins, cephalosporin) and mycotoxins (AK-toxin, paxilline, aflatoxins) (Bartoszewska et al., 2011; Martin et al., 2012). Remarkably, peroxisomes impact the fungal cell dynamics by functions beyond their metabolic activity. For example, ascomycete fungi possess a specialized type of peroxisome, the Woronin body, which serve as plugs for septal pores that interconnect hyphal cell compartments (Jedd, 2011). Also, a subclass of peroxisomes has been implicated in the organization of the microtubule cytoskeleton in *Emericella nidulans* (anamorph: *Aspergillus nidulans*) (Zekert et al., 2010).

In agreement with their functional versatility, peroxisomes participate in diverse developmental processes, such as gametophyte recognition during fertilization in *Arabidopsis*

thaliana (Boisson-Dernier et al., 2008) and the host-related morphogenic transitions of *Trypanosoma brucei* (Szoor et al., 2010). In animals, developmental processes like spermatogenesis (Chen et al., 2010; Nakayama et al., 2011; Baes and Van Veldhoven, 2012) and nervous system development (Faust et al., 2005; Baes and Van Veldhoven, 2006, 2012; Mast et al., 2011; Nakayama et al., 2011) require peroxisome activity, and their deficiencies cause severe and highly complex diseases in humans (Waterham and Ebberink, 2012). Fungi have provided numerous examples of developmental processes that depend on peroxisomes (reviewed in Peraza-Reyes et al., 2010). Different developmental events underlying the formation of asexual spores rely on peroxisomes. They also participate in developmental processes that define some fungal lifestyles, including the differentiation of infective structures, appresoria, in phytopathogenic fungi, in the yeast-mycelial transition of some dimorphic fungi, and in the development of nematode-trap cells by nematophagous fungi. The focal point of this review is the role of peroxisomes in the developmental process of fungal sexual reproduction.

PEROXISOME BIOGENESIS, AN OVERVIEW

Peroxisome formation is mediated by conserved proteins known as peroxins, which are denoted by the Pex acronym. Peroxisomes can multiply by growth and division from pre-existing peroxisomes or be formed by budding from specific domains of the endoplasmic reticulum (ER) (Dimitrov et al., 2013). Formation of peroxisomal membranes and insertion of proteins into peroxisome membranes depend on the peroxins Pex3 and Pex19. On the other hand, the import of proteins into the luminal space of peroxisomes relies on a second group of peroxins, which are mostly peroxisome-membrane associated proteins (Liu et al., 2012; Pieuchot and Jedd, 2012; Theodoulou et al., 2013). Elimination of Pex3 and Pex19 abrogates peroxisome formation. In contrast, deletion of the peroxins that mediate the import of peroxisome matrix proteins results in peroxisome remnants devoid of luminal proteins.

Two highly conserved import pathways drive the peroxisome matrix protein import. These pathways are defined by the import receptors Pex5 and Pex7. These peroxins recognize the peroxisome proteins in the cytosol, by means of their peroxisome targeting sequences (PTS1 and PTS2, respectively), and mediate their import into the organelle. The activity of the receptor Pex7 depends on accessory proteins known as PTS2-coreceptors (Schliebs and Kunau, 2006). In plants, animals and probably some fungi, Pex5 provides the PTS2-coreceptor activity. In contrast, most studied fungi harbor additional specific peroxins to fulfill this task, like the yeast paralogous Pex18 and Pex21, and their filamentous-fungi functional equivalent Pex20.

Both import pathways converge at a peroxisome membrane-associated complex known as the importomer. This complex consists of docking and RING-finger subcomplexes and facilitates the translocation of proteins across the peroxisome membrane. Interestingly, the import receptor Pex5 itself, along with a docking-complex peroxin Pex14 forms a transient and highly dynamic channel, which likely constitutes the site of protein translocation across the peroxisomal membrane (Meinecke et al., 2010). Import receptors and their cargos are imported to peroxisomes, and after releasing their cargo proteins in the peroxisome lumen, import receptors are translocated back to the cytosol. This process is mediated by the peroxisomal receptor export machinery, or exportomer (Platta et al., 2013), and allows the receptors to be used in subsequent rounds of import. Failure to export the receptors results in a blockage of the import process.

SEXUAL DEVELOPMENT IN FUNGI

Sexual reproduction in fungi exhibits a great diversity of reproductive strategies and mating systems (for review Casselton and Feldbrugge, 2010; Debuchy et al., 2010; Ni et al., 2011). The sexual life cycle of a model ascomycete fungus is shown in **Figure 1**. This process involves the alternation of haploid and diploid phases, which is sustained by the succession of karyogamy and meiosis, and provides the potential for genetic exchange. In general terms, sexual reproduction in fungi involves the differentiation of specialized mating structures that, after recognition of compatible partners, fuse to produce a zygote. In some fungi, karyogamy, and thus zygote formation, is concurrent to plasmogamy. However, in many fungal species, namely in Ascomycota and Basidiomycota (the Dikarya subkingdom), plasmogamy and karyogamy are separated in time by the propagation of a dikaryotic stage, which amplifies the number of karyogamies and meiocytes obtained from a unique fecundation event. Ultimately, meiosis takes place and for the majority of fungi the nuclear products of meiosis are packaged into sexual spores.

Sexual reproduction involves important changes in cellular architecture and often requires differentiation of multiple cell types. For many fungi, this process takes place inside of complex multicellular fruiting bodies. The formation of these structures occurs by a series of diverse cellular processes, including fusion, septation, branching, aggregation and adhesion of hyphae (Kues, 2000; Lord and Read, 2011). Therefore, sexual reproduction in fungi implicates a precise spatiotemporal coordination between various cell developmental events and progression through karyogamy and meiosis. Peroxisomes play an important role in the orchestration of these complex developmental processes.

PEROXISOMES ARE INVOLVED IN THE DEVELOPMENT OF SEXUAL REPRODUCTIVE STRUCTURES

PEROXISOMES FACILITATE NUTRIENT CHANNELING TO SUSTAIN THE FORMATION OF SEXUAL REPRODUCTIVE STRUCTURES

Sexual reproduction in fungi frequently takes place when cells have exhausted the external nutrients and reach stationary phase. Under these circumstances, sexual development is mainly sustained by nutrients provided by the pre-existing vegetative cells from where the differentiated cells arise. The translocation of nutrients from vegetative hyphae is of particular importance for the formation of fruiting bodies, large and complex structures that in many fungi emerge from the substrate to grow into the air (Wosten and Wessels, 2006). The nutrients provided by the mycelium are derived largely from reserve compounds, such as carbohydrate and lipid reserves, or are generated by cell auto-assimilative processes, like autophagy (Bartoszewska

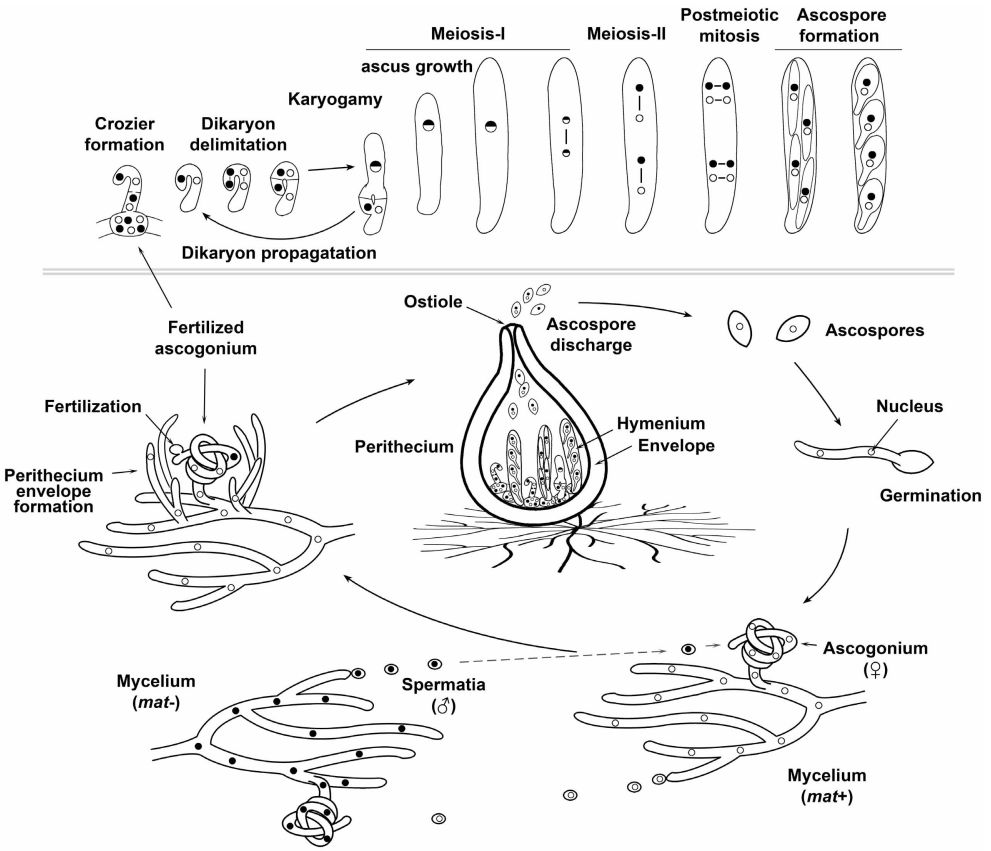

FIGURE 1 | The sexual life cycle of a model filamentous ascomycete fungus. Sexual reproduction of most filamentous Ascomycetes takes place inside of multicellular fruiting bodies, which consist on sexual tissues (the hymenium) surrounded by a protective envelope. The hymenium is derived from mating and after karyogamy and meiosis produces sexual spores (ascospores), whereas the envelope tissues originate from maternal vegetative hyphae and are sterile. In a number of Ascomycetes, pyriform-shaped sexual fructifications are known as perithecia (singular perithecium). The sexual life cycle of filamentous Ascomycetes initiates by the differentiation of female organs (ascogonia), which originate as curved branches arising from vegetative hyphal cells. Ascogonia then become surrounded by aggregated hyphae, which eventually develop the perithecial envelope. Ascogonia can be fertilized by hyphae or asexual spores, but some fungi differentiate specialized cells that act as male gametes (spermatia). In heterothallic (self-sterile) fungi, fertilization only takes place between reproductive structures that differ genetically at their mating type (denoted in the figure as *mat*+ and *mat*− and illustrated by nuclei with different shading). After fertilization, the male gametic nucleus is delivered into the ascogonium, which contains the female gametic nuclei. This results in the formation of the hymenium. The upper inset illustrates the development of the hymenium from the dikaryotic stage to ascospore formation (from left to right): the two gametic nuclei (of opposite mating type in heterothallic fungi) are isolated in pairs in specialized hook-shaped cells called croziers. After coordinated mitoses (lines linking nuclei represent spindles) and septa formation, three cells are formed in each crozier: an upper binucleated and two flanking uninucleated cells. The two uninucleated cells fuse to produce a new dikaryotic crozier, which propagates the dikaryotic stage, whereas the upper dikaryotic cell undergoes karyogamy and develops into an ascus (the meiocyte). Meiosis takes place in this upper cell. Finally, the haploid nuclear products issued from meiosis are packaged into ascospores. In the figure, which illustrates the development of *P. anserina*, the eight nuclei issued from a post-meiotic mitosis are enclosed two by two into ascospores resulting in asci with four binucleated ascospores. Ascospores maturate inside the original mother ascus, from which they are ultimately forcibly ejected out.

and Kiel, 2011). Peroxisome metabolism can play an important role in channeling some of these nutrients, most notably the lipids.

Storage lipids, which are primarily triacylglycerides, accumulate inside of lipid droplets (also known as lipid bodies) (Murphy, 2012). These organelles are derived from the ER and exhibit a close association with mitochondria and peroxisomes, which degrade fatty acids released from triglycerides to produce energy and biosynthetic metabolites via fatty acid β-oxidation and the glyoxylate cycle (refer **Figure 2** for an outline on these metabolic pathways). Interestingly, association of peroxisomes with lipid droplets is so intimate that peroxisomes even invade the core of the lipid droplets by protrusive structures, pexopodia, that may facilitate the transfer of lipids into peroxisomes (Binns et al., 2006).

Early research on the regulation of intermediate metabolism in fungi revealed that the sexual fruiting bodies of basidiomycetes like *Coprinopsis cinerea* and *Schizophyllum commune* contain very low levels of isocitrate lyase activity, a glyoxylate cycle specific enzyme. In contrast glycolysis, together with the tricarboxylic acid (TCA) cycle, represent the major pathways for carbohydrate metabolism (Cotter et al., 1970; Schwalb, 1974; Moore and

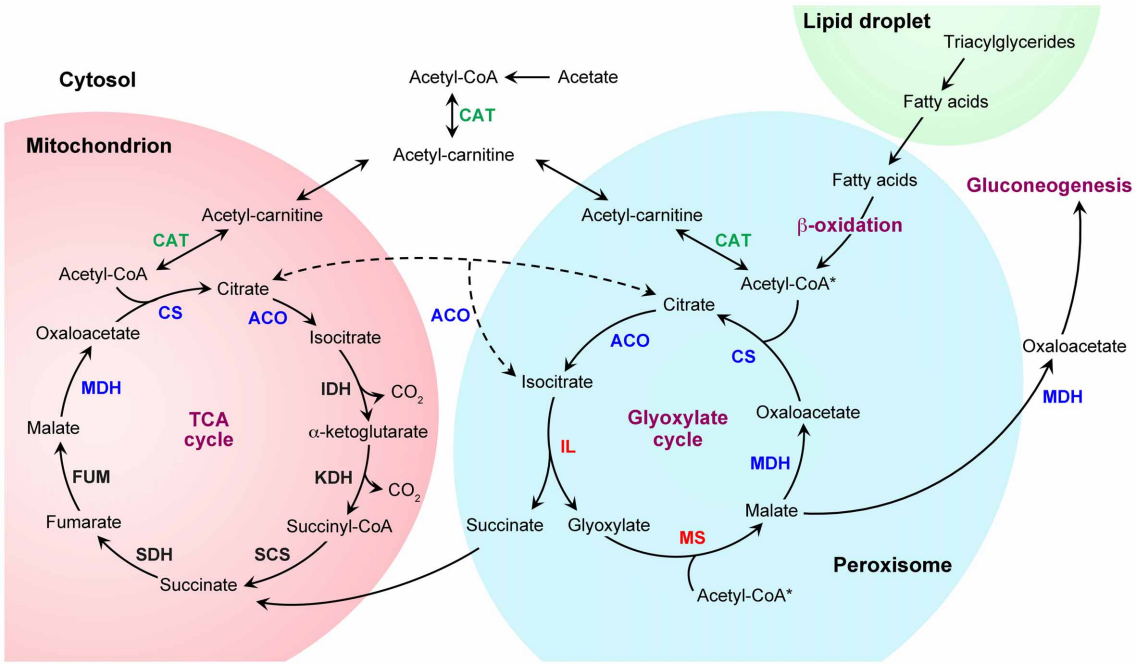

FIGURE 2 | Metabolic pathways for fatty acid and acetate utilization. Triacylglycerides, which accumulate inside of lipid droplets, are converted to fatty acids and transferred to peroxisomes for catabolism. Fatty acids are converted to acetyl-coenzyme A (-CoA) by the fatty acid β-oxidation pathway. In ascomycete yeasts like *Saccharomyces cerevisiae*, the β-oxidation pathway is exclusively peroxisomal (Hiltunen et al., 2003; Shen and Burger, 2009). However, in most other fungi, this pathway occurs in peroxisomes and mitochondria (Maggio-Hall and Keller, 2004; Boisnard et al., 2009; Shen and Burger, 2009; Kretschmer et al., 2012a,b; Patkar et al., 2012) (only the peroxisomal pathway is illustrated). The anaplerotic glyoxylate cycle then allows the conversion of acetyl-CoA to four-carbon dicarboxylic acids by bypassing the decarboxylation reactions of the tricarboxylic acid (TCA) cycle (note that two reactions can feed acetyl-CoA into the glyoxylate cycle, asterisks). The glyoxylate shunt intermediates can replenish the TCA cycle or serve as precursors for gluconeogenesis (shuttling between compartments of some intermediates, such as malate and oxaloacetate, is not illustrated). The glyoxylate cycle consists of five enzymatic reactions, of which three are shared with the TCA cycle. Depending on the fungal lineage, these three enzymatic steps (blue font) can take place in peroxisomes, mitochondria or the cytosol (except for citrate synthase, for which no cytosolic form has been described so far). In contrast, the specific enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase (red font), are typically peroxisomal (Kunze et al., 2006; Hynes, 2010; Strijbis and Distel, 2010). The most notable exception is *S. cerevisiae*, which harbors cytosolic isocitrate lyase (Taylor et al., 1996). Peroxisomal β-oxidation-produced acetyl-CoA can also be transferred to mitochondria for energy generation via TCA cycle. This transport depends on the interconversion of acetyl-CoA and acetyl-carnitine by carnitine acetyl-CoA transferases (green font). Acetyl-carnitine can then be imported into mitochondria by an acyl-carnitine carrier protein (Strijbis and Distel, 2010). Fatty acyl-CoA β-oxidation intermediates may also be transferred to mitochondria for further β-oxidation and acetyl-CoA generation within mitochondria (not shown). Cytosolic acetyl-CoA, which can be produced from C2 compounds, can also be transferred via acetyl-carnitine to mitochondria and peroxisomes for metabolism via the TCA and glyoxylate cycles. Only relevant enzymes are shown, abbreviations are as follows: ACO, aconitase; CAT, carnitine acetyltransferase; CS, citrate synthase; FUM, fumarase; IDH, isocitrate dehydrogenase; IL, isocitrate lyase; KDH, α-ketoglutarate dehydrogenase; MDH, malate dehydrogenase; MS, malate synthase; SCS, succinyl CoA synthetase; SDH, succinate dehydrogenase.

Ewaze, 1976). Two main scenarios could account for these observations. First, the glyoxylate cycle, as well as the β-oxidation of fatty acids, could be dispensable for fruiting body development. Alternatively, fatty acid β-oxidation and the glyoxylate shunt could take place in the mycelium that produces the fruiting bodies, and the glyoxylate cycle-derived intermediates could be transported into the developing fructifications. A number of lines of evidence now indicate that both of these scenarios occur in fungi.

In the brown-rot basidiomycete *Fomitopsis palustris*, comparison of the enzymatic activities of the TCA and glyoxylate cycles suggests that the glyoxylate bypass has no prominent role within fruiting bodies. Furthermore, even when both specific enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, exhibit high enzymatic activity in young mycelium; they are significantly down-regulated much before fruiting body formation. Thus, no correlation is observed in this fungus between the glyoxylate cycle activity and the developmental stages where sexual fruiting bodies are produced (Yoon et al., 2002a).

In contrast, in the white-rot basidiomycete *Flammulina velutipes* (the Enokitake mushroom), the activity of malate synthase and isocitrate lyase are significantly higher in the mycelial mats producing fruit bodies compared to equivalent mycelia than do not produce fructifications. In addition, the mycelial activity of malate synthase increases during fruiting-body development (Yoon et al., 2002b). This suggests an important role for the glyoxylate cycle in the vegetative cells supporting fruiting-body formation in *F. velutipes*.

The participation of the glyoxylate cycle in the formation of sexual reproductive structures has been demonstrated in the

cereal pathogen *Gibberella zeae* (anamorph: *Fusarium graminearum*). In this ascomycete, the transcript levels of the isocitrate lyase-encoding gene, *ICL1,* are high in mycelia before sexual development, and become negligible in late stages of fruiting-body (perithecium) development. In this fungus, the repression of *ICL1* occurs after perithecia induction. Furthermore, deletion of *ICL1* results in a severe reduction in the number of produced perithecia (Lee et al., 2009). These data indicate that in this fungus the glyoxylate cycle is, in fact, required for fruiting body formation, and suggest a channeling of glyoxylate cycle-derived intermediates from the vegetative mycelium into developing perithecia.

The glyoxylate shunt in *G. zeae* is required for fatty acid utilization (Lee et al., 2009); thus, the requirement of the glyoxylate cycle for perithecia formation may, indeed, be related to the mobilization of storage lipids. A critical event for reserve lipid utilization is the shuttling between peroxisomes and mitochondria of β-oxidation-produced acetyl-coenzyme A (-CoA) to allow the production of energy via the TCA cycle. This transport depends on the carnitine acetyltransferase-mediated acetyl-carnitine shuttle (**Figure 2**) (Strijbis and Distel, 2010). *G. zeae* mutants defective for a peroxisomal/mitochondrial carnitine acetyltransferase (CAT) produce fewer perithecia than a wild-type strain (Son et al., 2012). This finding is consistent with a role for the fatty acid β-oxidation pathway in sustaining fruiting-body formation. Interestingly, in these mutants, perithecia are produced only by some sectors of the mycelial colonies. This suggests that nutrients may be channeled into specific sectors of the mycelium to sustain the formation of limited numbers of fructifications when reserve nutrient mobilization is inefficient.

A genome-wide analysis of gene expression during sexual development in *G. zeae* revealed an expression pattern for the triacylgliceride metabolism genes that is consistent with lipid accumulation before sexual reproduction and lipid oxidation early during fruiting-body development (Guenther et al., 2009). This expression pattern is characterized by an up-regulation in mycelium preceding sexual development of the majority of lipid biosynthesis genes, which are subsequently repressed when perithecia start forming. This down-regulation of lipid biosynthesis genes occurs concomitantly with an up-regulation of lipid oxidation genes, which include the genes for the β-oxidation of fatty acids. Most peroxin-encoding genes followed the expression pattern of the lipid oxidation genes, suggesting an involvement of peroxisomes in this process (Guenther et al., 2009). Guenther and coworkers also observed high levels of lipid droplet accumulation in hyphae competent for sexual reproduction. This accumulation of lipids is notable in the specialized hyphae from where perithecia emerge, as well as in perithecium initials themselves, which are filled with densely packed lipid droplets (Guenther et al., 2009). Altogether, these data indicate that reserve lipids accumulate in the vegetative phase preceding sexual development and are later oxidized by β-oxidation to produce, along with the glyoxylate cycle, energy and biosynthetic intermediates to support sexual fruiting body formation in *G. zeae*. Consistent with participation of peroxisomes in these processes, perithecia formation is also severely reduced in mutant strains defective for peroxisome formation (Min et al., 2012).

PEROXISOMES MAY FACILITATE NUTRIENT CHANNELING FROM ASEXUAL TO SEXUAL DIFFERENTIATED MULTICELLULAR STRUCTURES

A nice example of the diversity of developmental systems leading the formation of reproductive structures in fungi is observed in the ascomycete *Sclerotinia sclerotiorum*. In this devastating plant pathogen, sexual fruiting bodies (referred to as apothecia) consist on large cup-shaped structures formed by a stipitate disc where the sexual tissues (the so-called hymenium) are embedded. In this fungus, apothecia do not arise from the mycelium, but from a second differentiated multicellular structure known as the sclerotium. Sclerotia are asexual resistance structures that consist of densely packed hyphal aggregates coated by a rind of highly melanized hyphae (Erental et al., 2008).

In *S. sclerotiorum*, differentiation of apothecia is affected by disruption of *pth2*, which encodes a peroxisomal CAT. The elimination of this protein results in fructifications with short stipes and disks that do not fully expand (Liberti et al., 2013). Since *pth2* is also required for fatty acid β-oxidation, it is possible that fatty acid utilization is also required for apothecia formation. Moreover, since the resources supporting sexual fruiting-body formation arise from sclerotia and not from mycelia, peroxisomes may also be important for nutrient channeling between distinct multicellular fungal structures. However, the identity of the sclerotial reserve nutrients supporting apothecium formation is not well-understood. Whereas a high sclerotium lipid content and the presence of hyphae with a rich content in lipid bodies has been documented (Calonge, 1970; Kosasih and Willetts, 1975), it has also been suggested that sclerotium carbohydrates rather than lipids sustain apothecium formation (Weete et al., 1970; Coley-Smith and Cooke, 1971). Consequently, it has been postulated that the role of Pth2 in apothecium formation could be regulatory rather than nutritional (Liberti et al., 2013). Further research should provide relevant information on this issue.

PEROXISOMES MAY ALSO FACILITATE NUTRIENT CHANNELING BETWEEN DIFFERENT CELL TYPES WITHIN FRUCTIFICATIONS: EVIDENCE FROM THE TRUFFLES

In the ecto-mycorrhizal fungus *Tuber borchii* (the whitish truffle), the transcripts for isocitrate lyase and malate synthase are much more abundant in fruiting bodies at different stages of maturation than in the vegetative mycelium (Lacourt et al., 2002; Abba et al., 2007). Likewise, an enhanced accumulation of the transcripts coding for glyoxylate cycle enzymes is observed in the fruiting bodies of *Tuber melanosporum* (the Perigord black truffle) (Ceccaroli et al., 2011). This suggests that in these Ascomycetes the glyoxylate cycle is required for later stages of fruiting-body development, which could include fruiting-body enlargement, meiotic development and ascospore (the meiotic-derived sexual spore of Ascomycetes) differentiation. Interestingly, in *T. borchii* lipid droplets are abundant in the vegetative cells in young fructifications. The lipid droplets are not observed in these cells after fruiting-body maturation but do accumulate in mature ascospores (Abba et al., 2007). This suggests that a relocation of

metabolic resources also takes place between different cell types of the fruiting bodies along their development in some fungi.

LIFESTYLE-RELATED IMPLICATION FOR PEROXISOMES IN FRUITING BODY DEVELOPMENT, AN EXAMPLE FROM PHYTOPATHOGENIC FUNGI

Importantly, fungi discussed above differ in their phylogenetic origin and in their lifestyle and reproductive systems. Thus, different requirements for the fatty acid β-oxidation and glyoxylate cycle during development may reflect diverse ways evolved in fungi with different lifestyles to sustain fruiting-body development. This is also evident when we consider the correlation between sexual development and the plant infection cycle in *G. zeae*. During the infection of wheat, formation of perithecium initials by the fungus begins when the fungus-colonized plant tissues begin to senesce. The perithecium initials function as overwintering structures on plant debris, and resume their development to form perithecia and ultimately produce ascospores that infect new plants at permissive temperatures (Trail, 2009). Notably, the transcriptional regulation pattern of the genes involved in lipid biosynthesis and oxidation observed during sexual development in culture also occurs during the equivalent developmental stages *in planta* (Guenther et al., 2009). This indicates that lipid reserves that accumulate in hyphae and perithecium-initials during plant colonization are used for overwintering and later oxidized to support perithecium development upon favorable climatic conditions.

On the other hand, the transcriptional regulation pattern of the lipid metabolism genes during development in *G. zeae* is similar, but not completely alike, in the related cereal pathogen *Gibberella moniliformis* (anamorph: *Fusarium verticillioides*). In contrast to *G. zeae*, the induction of lipid biosynthesis genes prior sexual development in *G. moniliformis* is moderate, and this fungus harbors elevated transcript levels of lipid oxidation genes even before sexual development (Sikhakolli et al., 2012). These differences could be interpreted in terms of their reproductive system. While sexual development seems to be critical for the homothallic (self-fertile) *G. zeae* infection cycle, the heterothallic (self-sterile) *G. moniliformis* seems to be sexually less prolific. The constantly high transcript levels of lipid oxidation genes in *G. moniliformis* could indicate that these genes also participate in the asexual spore formation process, which precedes sexual development and is more profuse than in *G. zeae* (Sikhakolli et al., 2012).

ADDITIONAL ROLES FOR PEROXISOMES DURING FRUITING BODY FORMATION

The formation of sexual fruiting bodies in saprophytic ascomycetes like *Aspergillus nidulans*, *Podospora anserina* and *Neurospora crassa* is affected when peroxisome biogenesis is compromised (Bonnet et al., 2006; Managadze et al., 2007; Hynes et al., 2008). *A. nidulans* produce small fruiting bodies (cleistothecia) in homozygous crosses whenever the PTS1 peroxisome matrix protein import is impaired. This phenotype is not observed in *pexG* (*pex7*) mutants, where only the PTS2 import pathway is affected. In *A. nidulans*, isocitrate lyase import into peroxisomes depends on PexG and peroxisomal localization of malate synthase (a PTS1-containing protein) is not essential for a functional glyoxylate cycle (Hynes et al., 2008). Moreover, deletion of *acuJ*, which encodes a peroxisomal/mitochondrial CAT essential for acetate and fatty-acid utilization, has no detectable developmental defects (Hynes et al., 2011). These observations suggest that the deficiency in cleistothecia formation resulting from defective PTS1 peroxisome protein import is not caused by an impaired β-oxidation/glyoxylate cycle-dependent nutrient channeling. Thus, there may be additional functions for peroxisomes in fruiting body development in *A. nidulans*.

In *P. anserina*, deletion of the PTS1 receptor PEX5 results in a reduction in the size and number of perithecia produced by this fungus (Bonnet et al., 2006). This developmental phenotype is not produced by defects in β-oxidation of fatty acids (Boisnard et al., 2009). Thus, in *P. anserina* and *A. nidulans*, peroxisomes have additional roles during fruiting body development beyond the β-oxidation pathway. However, unlike *A. nidulans*, deletion of *P. anserina pex7*, which encodes the PTS2 receptor PEX7, partially suppresses the perithecium development phenotype observed upon deletion of *pex5* (Bonnet et al., 2006). This indicates that mislocalization of all proteins that undergo PEX5- and PEX7-dependent import into peroxisomes is less detrimental compared to mislocalization of peroxisomal matrix proteins that require PEX5 for import. Furthermore, the perithecium developmental phenotype of *P. anserina pex5* mutants displays a maternal effect: it is observed both in homozygous and heterozygous (to a wild-type strain) crosses whenever the *pex5* mutant acts as female partner (Bonnet et al., 2006). This indicates that peroxisomes are required for the development of maternally derived tissues. These tissues could be concerned with the formation of the perithecium envelope (see **Figure 1**), which in ascomycete fungi like *P. anserina* is exclusively of maternal origin (Debuchy et al., 2010).

In *N. crassa*, peroxisomes could be required at a very early stage of perithecium development, as mutant strains deficient for the docking-complex peroxin PEX14 are female sterile and do not produce perithecia (Managadze et al., 2007). Interestingly, no similar phenotype has been associated to other fungal mutations affecting peroxisome biogenesis, including *pex14* deletion in *P. anserina* (Peraza-Reyes et al., 2008). This may indicate fundamental differences in sexual determination even in closely related fungi.

PEROXISOMES ARE INVOLVED IN THE FORMATION OF SIGNALING MOLECULES THAT REGULATE SEXUAL DEVELOPMENT

An additional function of peroxisomes during sexual reproduction stems from their participation in the formation of signaling molecules. In *A. nidulans*, the formation of sexual reproductive structures and asexual spores is affected by mutations that abolish PTS1 peroxisome import. These mutations also exacerbate the sexual development phenotype of a *veA1* mutant and result in very decreased cleistothecia formation (Hynes et al., 2008). The *veA1* mutation affects the velvet protein VeA, a regulatory protein that coordinates the balance between sexual and asexual development (Bayram and Braus, 2012). The development of this fungus is also coordinated by *psi* factors, fatty acid-derived oxylipin pheromones. *Psi* factors are secondary metabolites produced by hydroxylation of oleic, linoleic and linolenic acids. The ratio of *psi* factors determines the balance between asexual and

sexual development (for review, Tsitsigiannis and Keller, 2007). In *A. nidulans*, oleic acid stimulates the production of cleistothecia and reduces asexual sporulation. Importantly, deletion of PexF (Pex6, an exportomer peroxin) inhibits oleate-stimulated cleistothecium formation. These observations suggest that the peroxisome metabolism affects the levels of oxylipin *psi* factors, which control the balance between sexual and asexual development in *A. nidulans* (Hynes et al., 2008).

The acyl-CoA-binding protein Acb1 of *Pichia pastoris* is another signaling molecule whose formation depends on peroxisomes. Acb1 is a conserved protein that is secreted and proteolytically processed to produce an extracellular signaling peptide. In the yeast *P. pastoris*, Acb1 secretion is induced by nitrogen starvation and is required for ascospore formation (Manjithaya et al., 2010). Interestingly, Acb1 secretion occurs by an unconventional pathway that relies on autophagosome-like vesicles and not on the classical ER/Golgi secretory system (for review, Rabouille et al., 2012). Importantly, Acb1 secretion requires peroxisome biogenesis and formation of medium-chain fatty acyl CoA inside peroxisomes. Thus, it has been postulated that Acb1 binding to medium-chain fatty acyl CoA produced within peroxisomes is critical for Acb1 secretion (Manjithaya et al., 2010). Intriguingly, however, mutants of *P. pastoris* defective for the importomer, which is required for Acb1 secretion (Manjithaya et al., 2010), do not display sexual cycle defects (Waterham et al., 1996). Therefore, the precise participation of peroxisomes during this process awaits further examination. Nonetheless, these observations provide an interesting example of how the peroxisome function can impact different cellular processes implicated in the formation of signaling molecules important for sexual development.

INVOLVEMENT OF PEROXISOMES IN MATING, AN EXAMPLE FROM THE VIRULENCE-RELATED SEXUAL DEVELOPMENT OF A PLANT PATHOGEN

Ustilago maydis (the cuitlacoche or corn smut) is a basidiomycete plant pathogen that completely depends on the infection of its host (maize) to complete its sexual cycle (reviewed in Vollmeister et al., 2012). Haploid yeast-like cells of this fungus proliferate saprophytically by budding. However, in order to infect its host and to undergo sexual reproduction, the growth pattern must be switched to produce filamentous cells. This transition requires the pheromone-mediated recognition of mating partners, and results in the formation of conjugation hyphae. These cells undergo plasmogamy by fusing their tips and generate a dikaryotic hypha, which is the infectious cell type of *U. maydis*. The dikaryotic hyphae proliferate in the plant and ultimately differentiate into diploid teliospores (resting spores competent to undergo meiosis of some Basidiomycetes), which are produced inside of fungus-induced plant tumors (Vollmeister et al., 2012).

A third example of a signaling role for peroxisomes is observed in the virulence-related sexual cycle of *U. maydis*. This example also illustrates an interesting connection between peroxisomes in sexual-development and the infection cycle of a plant pathogen. Fatty acids, as well as their hydroxylated derivatives (which are components of the plant cuticle cutin), induce filamentation in *U. maydis* (Klose et al., 2004; Mendoza-Mendoza et al., 2009). Interestingly, the switch to filamentous growth induced by fatty acids depends on the β-oxidation pathway and, depending on the fatty acid, mitochondrial and/or peroxisomal pathways are required for this induction (Klose and Kronstad, 2006; Kretschmer et al., 2012a).

The ability to mate also depends on the β-oxidation of fatty acids. The mating ability of cells deficient for the peroxisomal β-oxidation is reduced in heterozygous crosses, and weak in homozygous crosses; whereas it is only slightly reduced in homozygous crosses of cells deficient for the mitochondrial β-oxidation (Kretschmer et al., 2012a). These observations indicate that the developmental switch resulting in hyphal growth in *U. maydis* is regulated by intermediate metabolites or fatty acid derivatives that are produced by the fatty acid β-oxidation. They also reveal a role for peroxisomes in the differentiation process upholding mating in *U. maydis*. Interestingly, proliferation of dikaryotic hyphae in the host tissues and the formation of plant tumors is also reduced by deficiencies in the β-oxidation pathway (Klose and Kronstad, 2006; Kretschmer et al., 2012a). Moreover, the differentiation of teliospores is significantly delayed when there are defects in peroxisomal β-oxidation (Klose and Kronstad, 2006). Whether this developmental delay results from the lower hyphae proliferative efficiency or from additional roles for β-oxidation in teliospore differentiation remains to be established. Nevertheless, altogether, these observations indicate that the fatty acid β-oxidation has a profound and complex impact on the virulence-related sexual development of *U. maydis*.

PEROXISOME INVOLVEMENT IN MEIOTIC DEVELOPMENT AND SEXUAL SPORULATION

PEROXISOME DYNAMICS AND ASSEMBLY

Peroxisome dynamics is differentially regulated during meiotic development and sexual spore formation

The first report that peroxisome number can be highly variable between fungal cells was published nearly 30 years ago. Veenhuis and collaborators (1984) observed that during nematode infection the trap cells of the nematophagous fungus *Arthrobotrys oligospora* were filled with peroxisomes, while vegetative cells from where trap cells emerge were not. This observation indicated that the dynamics of peroxisomes is regulated during development. Peroxisome dynamics during sexual development has been studied in the yeast *Saccharomyces cerevisiae* and in the filamentous fungus *P. anserina*. The sexual cycle of *S. cerevisiae* is initiated when haploid cells of opposing mating types encounter each other and fuse to produce a zygote, which propagates asexually by budding. When diploid cells are exposed to nutritional limitations, they undergo meiosis and pack their resulting haploid nuclei into ascospores. The four resulting ascospores—the tetrad—are encased inside the original mother cell, the ascus [for a comprehensive review on *S. cerevisiae* sporulation see Neiman (2011)].

After mating, peroxisomes from each parental cell are transferred into the zygote, where no mixing of their contents is observed (Motley and Hettema, 2007). Then, during sporulation, peroxisomes are observed throughout the two meiotic divisions and they partition to the four cellular products of meiosis. Thus, peroxisomes are partitioned during sporulation.

During this process, the number and distribution of peroxisomes are not significantly altered in *S. cerevisiae* (Gurvitz et al., 1998). Interestingly, the number, size and localization of peroxisomes is tightly regulated during the sexual development in filamentous Ascomycetes. In *P. anserina* (please refer to **Figure 1** to appreciate the sexual development of this fungus), the sexual differentiated female organs (ascogonia) contain peroxisomes that are mainly round or elongated, and have an even distribution along the septated hyphae. This arrangement is similar to the one observed in vegetative hyphae from where ascogonia are formed (Peraza-Reyes et al., 2009). After fertilization, the dikaryotic crozier cells contain few peroxisomes (Berteaux-Lecellier et al., 1995; Peraza-Reyes et al., 2011), but their number increases importantly after karyogamy in the ascus (meiocyte).

After nuclear fusion, the diploid nucleus enters meiosis and the ascus elongates from 10 to over 150 microns (see **Figure 1**). Hundreds of peroxisomes can be observed in the young growing asci, a large portion of these peroxisomes is clustered at the tip of the cell. By the end of meiotic prophase-I, asci reach their final length and peroxisomes are more evenly distributed along the cell. The number of peroxisomes remains constant from metaphase-I to ascospore formation. However, peroxisomes proliferate during ascospore maturation (Berteaux-Lecellier et al., 1995; Bonnet et al., 2006; Peraza-Reyes et al., 2009, 2010). Altogether, these observations indicate that peroxisome dynamics is highly regulated during sexual development in filamentous fungi, implying a differential requirement for these organelles throughout sexual development progression. These observations also suggest that the location where peroxisomes act is important for the development of specific sexual cells.

Peroxisome assembly is also differentially regulated during meiotic development and sexual spore formation

Remarkably, the activity of the protein machinery that drives the import of proteins into peroxisomes is also tightly coupled with sexual development. A critical component of the protein translocation machinery for peroxisome matrix protein import is PEX14 (Azevedo and Schliebs, 2006; Liu et al., 2012). This protein is a conserved component of the docking complex and has been implicated in formation of the protein translocation channel in *S. cerevisiae* (Meinecke et al., 2010). PEX14 is required for peroxisome matrix protein import in vegetative hyphae of *P. anserina*. Interestingly, its requirement for import during the sexual cycle is cell specific (Peraza-Reyes et al., 2011). PEX14 is necessary for import in dikaryotic croziers, in asci after meiotic metaphase-I and in ascospores. However, it is not essential for the growing meiotic-prophase-I asci (**Figure 3**) or for early stages of ascospore differentiation. Thus, the translocation channel for protein import can be assembled in absence of PEX14 during specific stages of sexual development. This could indicate that the constitution of the import channel differs at distinct developmental stages. Alternatively, there is evidence that Pex5 constitutes the central core of the peroxisome translocation channel (Salomons et al., 2000; Kerssen et al., 2006; Meinecke et al., 2010) and Pex14 could facilitate its assembly. Therefore, it is possible that assembly of the translocation channel could be differentially regulated during sexual development. Under both scenarios, the differential requirements for PEX14 during sexual development may reflect differences in the functional state of the translocation machinery (**Figure 3**).

Meiotic prophase-I, the stage when recombination occurs, is a critical stage of sexual reproduction. The observation that peroxisome import takes place at this stage even in absence of PEX14

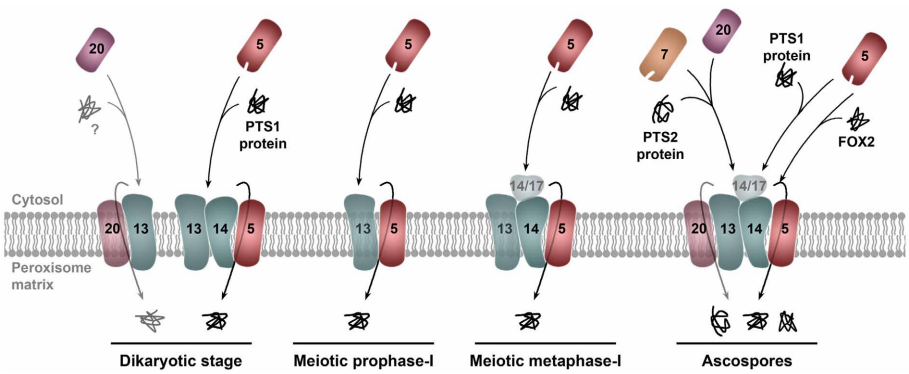

FIGURE 3 | Model for the regulation of the docking/translocation machinery for peroxisome matrix protein import during sexual development in *P. anserina*. The peroxins of the docking/translocation machinery are differentially required during sexual development in *P. anserina*. PEX14 (only the peroxin number is indicated) is required for peroxisome matrix protein import at the dikaryotic stage, in asci after the first meiotic metaphase and in ascospores; but not during the first meiotic prophase or in the early stages of ascospore differentiation (not depicted). PEX14/17 is involved in the import of matrix proteins in meiosis after metaphase-I, but it is not required for import in young differentiating ascospores (not illustrated). Upon ascospore maturation, PEX14/17 is required for the import of PTS1-containing proteins, but not for the PEX5-dependent import of proteins missing PTS1 signals, like the peroxisomal fatty acid β-oxidation multifunctional enzyme FOX2. The third docking protein, PEX13, is likely required for the activity of the docking/translocation machinery throughout meiotic development; however, absence of this protein blocks meiocyte formation. Thus, its function during meiotic development cannot be assessed (depicted with translucent shading). In addition to PEX13 (and unlike PEX5, PEX7, PEX14, or PEX14/17), PEX20 is also required for meiocyte formation, which suggests an alternative import pathway required at the dikatyoric stage for meiocyte formation. PEX20 could provide a pore-forming activity (illustrated by a translucent membrane location) additional to PEX5 for the translocation of proteins across the peroxisomal membrane (see text and Peraza-Reyes et al., 2011 for details).

could indicate an important role for peroxisomes at this stage. Interestingly, a second *P. anserina* importomer peroxin is required at some but not all stages of sexual development. This peroxin, PEX14/17, is related to but not redundant with PEX14 (Peraza-Reyes et al., 2011). Furthermore, PEX14/17 and PEX14 are not required at the same stages of sexual development, and elimination of PEX14/17 affects the specificity of the import at certain developmental stages; notably, PEX14/17 is required in ascospores for the import of PTS1-containing proteins but not for the import of PEX5 cargos that lack PTS1 signals (**Figure 3**). Since neither PEX14 nor PEX14/17 is absolutely required for import at meiotic prophase-I and in young differentiating ascospores, additional importomer components should sustain the activity of the docking/translocation machinery at these stages. One such component could be the third docking-complex peroxin PEX13 (**Figure 3**). Interestingly, this protein is required for meiocyte formation *per se* (see below), which has precluded analyzing its role in import during meiotic development. Altogether, these observations indicate that the functional state of the importomer is regulated during sexual development in *P. anserina*, and suggest that an additional regulation of the peroxisome constitution and function during development is exerted by a differential modulation of the protein complex that selectively drives the import of proteins into the organelle.

PEROXISOMES ARE REQUIRED FOR MEIOTIC DEVELOPMENT

A role for peroxisomes in the initiation of meiotic development

Consistent with their differential dynamics and assembly regulation, peroxisomes are required for specific processes of meiotic development. Notably, one such process is the induction of meiotic development *per se*. In *P. anserina*, the RING-finger peroxin PEX2 was discovered as a protein required for the transition from the pre-karyogamy dikaryotic and mitotic phase to the karyogamy and meiotic phase of the life cycle (Simonet and Zickler, 1972, 1978; Berteaux-Lecellier et al., 1995).

Mutant strains defective for *pex2* are sterile in homozygous crosses and their sexual development is blocked at the dikaryotic stage prior to formation of asci (meiocytes) and ascospores (**Figure 4C**, compare to **A**). In these mutants, the dikaryotic crozier cells differentiate normally (illustrated in **Figure 4D** for a *pex20* mutant, which exhibits the same developmental phenotype as *pex2* mutants, see below) and the coordinated mitoses that lead to dikaryon formation are synchronous with their spindle and spindle pole bodies (SPBs, the nuclear-embedded functional analogs of centrosomes) correctly formed (Simonet and Zickler, 1972). Like in a wild-type strain, these mitoses result in an upper dikaryotic cell flanked by two uninucleated cells, which will further fuse to form a new basal bi-nucleated cell. Normally, the upper dikaryotic cell develops into a meiocyte and the basal cell differentiates a new crozier, which perpetuates the dikaryotic stage (see **Figure 1**). This latter event is not affected in the *pex2* mutants; however, the two nuclei of the top dikaryotic cell do not fuse. Instead, they divide mitotically to produce another crozier cell (see arrow in **Figure 4D**). Consequently, mutant perithecia become filled with crozier "trees" (**Figure 4C**) in which no diploid stage can be detected (Simonet and Zickler, 1972, 1978; Berteaux-Lecellier et al., 1995).

In many fungi, karyogamy and meiosis are coupled processes. They are intimately associated to the cell differentiation process driving asci formation. Therefore, peroxisomes could be required either for karyogamy or meiosis *per se*, or they could be implicated in determining a differentiated cellular state competent to trigger karyogamy and meiosis.

The haploid nuclei of vegetative hyphae in *P. anserina* occasionally fuse and produce diploid nuclei. The frequency of this vegetative nuclear fusion is not affected upon *pex2* mutation, suggesting that the nuclear fusion *per se* is not impaired by this peroxisomal dysfunction (Berteaux-Lecellier et al., 1995). In filamentous Ascomycetes, the pre-meiotic DNA replication precedes karyogamy (reviewed in Zickler, 2006) and meiosis-specific proteins are incorporated into chromosomes during this phase (Storlazzi et al., 2008). Thus, the "decision" to undergo meiosis is taken before karyogamy in these fungi. Therefore, peroxisomes may be implicated in a prekaryogamy process necessary to induce meiosis rather than in karyogamy itself.

Does meiotic development require an alternative peroxisome import pathway?

In *P. anserina*, PEX2 localizes to peroxisomes during vegetative and sexual cycles, including crozier cells (Peraza-Reyes et al., 2008, 2011). Furthermore, meiocyte differentiation is also impaired by deletion of either *pex3* or *pex19*, which encode the peroxins implicated in peroxisome membrane assembly (Peraza-Reyes et al., 2011). This indicates that peroxisomes themselves are required for meiocyte formation. PEX2 and the entire RING-finger complex are required for meiocyte differentiation (Peraza-Reyes et al., 2008). Intriguingly, neither of the receptors PEX5 and PEX7 is required for karyogamy or meiosis initiation, and even double mutants lacking both import receptors can differentiate meiocytes and undergo karyogamy and meiosis (Bonnet et al., 2006). Further research revealed that the *P. anserina* docking-complex peroxin PEX13 is also required for meiocyte formation, whereas PEX14 (**Figure 4B**) and PEX14/17 are not (Peraza-Reyes et al., 2008, 2011).

These puzzling results suggested an unconventional/additional import pathway operating in *P. anserina* independently of the known import receptors. This hypothesis was further strengthened by the discovery that the PTS2-coreceptor PEX20 (**Figure 4D**) is essential for meiocyte formation (Peraza-Reyes et al., 2011). This finding, along with the notion that PTS2-correceptors display important functional similarities with PEX5 (Schafer et al., 2004; Schliebs and Kunau, 2006; Hensel et al., 2011), suggests that PEX20 could act as import receptor on its own (see **Figure 3**). Altogether, these data strongly suggest that meiocyte formation in *P. anserina* relies on a novel peroxisome import pathway, mediated by PEX20 and dependent on the docking protein PEX13 and the RING-finger complex.

The precise mechanism by which matrix proteins are imported into peroxisomes is not well-understood. Most notably, in contrast to the PTS1 import pathway, very little is known about the protein translocation channel for the PTS2 pathway. Nevertheless, based on their functional similarity with Pex5, the PTS2-coreceptors are appealing candidates for pore-forming activity (Schafer et al., 2004; Erdmann and Schliebs, 2005). It is, therefore,

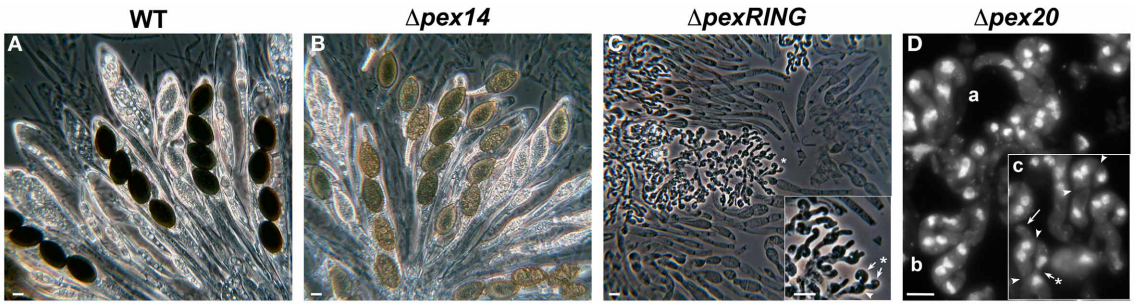

FIGURE 4 | Examples of sexual development defects produced by deficient peroxisome biogenesis in *P. anserina*. Sexual fruiting bodies of *P. anserina* wild-type **(A)** or Δ*pex14* **(B)** homozygous crosses contain asci with four ascospores. In absence of PEX14, ascospore pigmentation is deficient **(B)**. In contrast, perithecia from RING-finger-complex **(C**, here a Δ*pex2*Δ*pex10*Δ*pex12* strain**)** or Δ*pex20* **(D)** mutants contain only crozier-shaped dikaryotic cells. Image in **(D)** shows the nuclei distribution in these cells (DAPI staining). Lowercase letters indicate progressive developmental stages of crozier differentiation: **(a)** binucleated young croziers bending. **(b)** After simultaneous mitoses, each crozier contains four nuclei. **(c)** Septa formation (arrowheads) delimitates a dikaryotic upper cell from uninucleate lateral and basal cells. The uninucleated cells fuse (asterisk arrow) and the lateral-cell nucleus migrates into the basal compartment. Instead of undergoing karyogamy and meiosis as in the wild type, the nuclei from the upper dikaryotic crozier cell will be engaged in the formation a new crozier (arrow). This results in crozier "trees" where no asci are produced, as shown in **(C)**. Note how each original crozier (inset in **C**, arrowhead) produces two new croziers (arrows). Scale bars, **A–C**: 10 μm; **D**: 5 μm.

tempting to speculate that PEX20 provides a second pore-forming activity in filamentous Ascomycetes. This would indicate that *P. anserina* has distinct translocons for proteins that contribute to different stages of sexual development.

Interestingly, a genome-wide analysis of meiotic factors in *S. cerevisiae* revealed a requirement for the PTS2-correceptor Pex21p in meiotic development (Marston et al., 2004). In addition, Pex2p, Pex12p, and Pex13p (importomer peroxins), Pex6p and Pex22p (exportomer peroxins), and Pex25p (implicated in peroxisome division and biogenesis from the ER) were also identified as required for meiotic development. In contrast, deletion of the import receptors Pex5p and Pex7p, as well as the second PTS2-correceptor, Pex18p (a Pex21p paralog), was not observed to affect meiosis in this screen (Marston et al., 2004). These data could indicate a specialization for meiotic development for one of the two PTS2-coreceptors of *S. cerevisiae*. So far, no clear ortholog of *PEX20* has been observed in basidiomycete fungi (Kiel et al., 2006 and our personal observations); however, these fungi possess a Pex5/Pex20 fusion protein (Kiel et al., 2006), which could provide a separate pathway for peroxisome import. Whether this protein is implicated in sexual development in basidiomycete fungi remains undetermined.

The peroxisomal function required to induce meiosis in *P. anserina* remains elusive. The fatty acid β-oxidation pathway (either peroxisomal or mitochondrial) is not required for meiocyte formation (Boisnard et al., 2009). Likewise, simultaneous deletion of the five catalase-encoding genes in *P. anserina* does not impair meiocyte differentiation (Bourdais et al., 2012), which suggests that this process is not severely affected by deregulating the ROS homeostasis. The elimination of the peroxins that impair meiocyte formation in *P. anserina* results in different meiotic development defects in *S. cerevisiae* (Marston et al., 2004), and does not affect ascospore formation in *P. pastoris* (Waterham et al., 1996). Therefore, peroxisomes could participate in a specific function required for the differentiation of croziers/asci in filamentous fungi. However, the meiotic development of *A. nidulans* is not affected upon deletion of Pex2 or Pex13 (Hynes et al., 2008, 2010). This indicates that the involvement of peroxisomes in meiotic initiation is restricted to specific fungi, and suggests that the factors controlling meiotic entry among diverse fungi are different. Since the mating system of the homothallic *A. nidulans* differs from that of heterothallic *P. anserina*, one such difference could be related to the mating-type that governs sexual reproduction.

Involvement of peroxisomes in the distribution of meiotic-derived nuclei

In addition to meiotic initiation, progression through meiotic/post-meiotic development also requires peroxisome activity. In the homothallic *G. zeae*, the elimination of the import receptor Pex5 or the exportomer component Pex6 compromises asci and ascospore differentiation. The perithecia that are produced by mutant strains defective for these peroxins frequently lack asci and do not develop mature ascospores (Min et al., 2012). In *P. anserina*, the number of asci with well-delineated ascospores present in perithecia is also reduced by elimination of PEX5. In this fungus, delayed meiosis and nuclear misplacement during post-meiotic mitosis results in many degenerated asci, in which ascospores contain either no nuclei or an abnormal number of nuclei. These nuclear distribution abnormalities could result from incorrect spindle positioning during the second meiotic division or from impaired nuclear migration during post-meiotic mitosis (Bonnet et al., 2006). In *P. anserina*, elimination of the import receptor PEX7 and elimination of PEX5 have similar effects on asci development, although defects in *pex7* mutants are less severe compared to those observed in *pex5* mutants. In addition, the nuclear distribution defect of *pex5* mutants also displays a maternal effect (Bonnet et al., 2006). This indicates that the defect in nuclear positioning during asci development is not due to a cell autonomous effect, and suggests that maternally derived cells facilitate efficient progression through meiosis/post-meiotic development in a peroxisome-dependent manner.

In *A. nidulans*, microtubule-cytoskeleton and nuclear dynamics depend on the protein ApsB. This protein is a component of microtubule-organizing centers at the SPBs and at a septum-associated microtubule-organizing center, which is specific to fungal cells. In addition, ApsB localizes to a subpopulation of peroxisomes and it has been postulated that peroxisomes may deliver ApsB to its septal localization (Zekert et al., 2010). Thus, peroxisomes could have additional roles in regulating nuclear distribution during the sexual cycle. Actually, elimination of ApsB in *A. nidulans* results in misshapen ascospores produced in low numbers and exhibiting very low viability (Clutterbuck, 1994). Nevertheless, this phenotype has not been observed in any of the analyzed peroxisome biogenesis deficient mutants (Hynes et al., 2008, 2010). Therefore, the contribution of the peroxisomal form of ApsB to ascospore differentiation could be minor, or redundant systems could ensure ApsB correct localization during sexual development. Further research to better understand the involvement of peroxisomes in cytoskeleton organization could reveal additional roles for these organelles in meiotic development.

INVOLVEMENT OF PEROXISOMES IN SEXUAL SPORULATION

The glyoxylate cycle, sensing the metabolic potential to promote sexual spore formation

Interestingly, in *S. cerevisiae* the glyoxylate cycle is a central pathway linking the catabolic and biosynthetic metabolism, and also senses the metabolic potential of the cell to promote developmental decisions, like sexual spore formation. Sexual sporulation in *S. cerevisiae* is controlled by the nutritional status. Nitrogen starvation in media containing non-fermentable carbon sources, substrates like acetate that require glyoxylate bypass, induces sporulation (Neiman, 2011). In addition, progression through sporulation is under the control of the nutritional status. For instance, if nutrients are exhausted once meiosis has initiated, two ascospores are produced inside each ascus instead of four.

Interestingly, the number of spores an ascus will form is determined by a metabolic product of the glyoxylate cycle (Nickas et al., 2004). Ascospore formation relies on the activity of the SPBs. At the second meiotic division, the composition of the SPBs changes and they are converted from microtubule nucleation centers into membrane nucleation platforms (referred to as meiosis-II outer plaques) that direct the formation of the membranes that will surround each ascospore (Neiman, 2011). Upon carbon depletion, only one (the daughter) of the two SPBs of each spindle is transformed into a meiosis-II outer plaque, which results in the formation of two spores. Accumulation of a glyoxylate cycle-derived intermediate, which may be cytosolic oxaloacetate, triggers the modification of the two mother SPBs into meiosis-II outer plaques allowing the formation of four ascospores (Nickas et al., 2004). This indicates that the asymmetric spindle pole function is regulated by the metabolic state of the cell and that the glyoxylate cycle plays a major monitoring role in this process. This underscores the importance of the glyoxylate cycle beyond its metabolic function. Importantly, the localization of glyoxylate cycle enzymes in *S. cerevisiae* varies according to nutrient availability. For example, these enzymes localize primarily to peroxisomes during growth on fatty acids. In contrast, most of them localize to cytosol upon growth on C2 compounds. The oxaloacetate-consuming citrate synthase, which has mitochondrial and peroxisomal isoforms in yeast, is probably the only exception (Kunze et al., 2002, 2006; Lee et al., 2011; Chen et al., 2012). This indicates that different conformations of the glyoxylate cycle may be optimal to sustain different tasks, and suggests that the location where the intermediates of this pathway are produced could contribute to different cellular roles.

Peroxisome metabolism is required for sexual spore maturation and germination

Research on *P. anserina* sexual development revealed that peroxisome number increases importantly during ascospore maturation (Berteaux-Lecellier et al., 1995). A similar observation was done from asexual spores of *G. zeae* (Seong et al., 2008). Interestingly, the abundance of peroxisomes in the asexual spores of this fungus is correlated with a high number of lipids droplets in their vicinity, which disappear during germination. These observations suggest that peroxisomes are involved in spore maturation and germination, where they could drive the mobilization of the spore reserve lipids.

Ascospores of *P. anserina* peroxisomal mutants are green colored instead of black (**Figure 4**, compare **A** and **B**) display a low germination rate and a flimsy mycelium after germination (Berteaux-Lecellier et al., 1995; Bonnet et al., 2006; Peraza-Reyes et al., 2008; Boisnard et al., 2009). Melanin constitutes an important component of fungal sexual spores (Gomez and Nosanchuk, 2003). This pigment can be produced by the dihydroxynaphthalene melanin biosynthesis pathway from either acetyl-CoA or malonyl-CoA (Langfelder et al., 2003; Ramos-Pamplona and Naqvi, 2006; Coppin and Silar, 2007). Therefore, β-oxidation pathway could provide precursors for melanin biosynthesis. Melanization of ascospores in *P. anserina* (Berteaux-Lecellier et al., 1995; Bonnet et al., 2006; Peraza-Reyes et al., 2008) and appressoria in phytopathogenic fungi is affected by defects in peroxisome function (Kimura et al., 2001; Ramos-Pamplona and Naqvi, 2006). Indeed, melanization is also deficient in mutants defective for peroxisome β-oxidation pathway (Wang et al., 2007; Boisnard et al., 2009), indicating a role for this pathway in melanization. Importantly, a *P. anserina* mutant defective in both peroxisomal and mitochondrial β-oxidation pathways produces green ascospores, while a mutant defective for the first step of melanin biosynthesis gives rise to white ascospores (Coppin and Silar, 2007). Thus, β-oxidation contributes to, but it is not the only metabolic pathway providing precursors for melanin biosynthesis. Additional sources of melanin precursors could also explain why teliospores of *U. maydis mfe2* mutants, affected in peroxisome β-oxidation, only display delayed melanization (Klose and Kronstad, 2006). Interestingly, plant pathogenic fungi could obtain these biosynthetic precursors from the infected plants (Guenther et al., 2009).

Considering that melanin helps to harden the spore cell wall, the less-pigmented ascospores of *P. anserina* peroxisomal mutants should be fragilized. Indeed, deeper studies have shown that the germination defect of these ascospores is caused by their increased fragility (Boisnard et al., 2009). This is in line with the observation that *A. nidulans* peroxisomal mutant ascospores, for which no melanization defect has been reported, as well as the teliospores

of *U. maydis mfe2* mutant, which only harbor a delayed melanization, germinate efficiently (Klose and Kronstad, 2006; Hynes et al., 2008).

Germination of *P. anserina* peroxisomal mutant ascospores gives rise to a spindly mycelium with reduced growth rate (Berteaux-Lecellier et al., 1995; Bonnet et al., 2006; Peraza-Reyes et al., 2008; Boisnard et al., 2009). This phenotype disappears when glucose is added to the germination medium (Berteaux-Lecellier et al., 1995). Similarly, glucose addition suppresses the germination defect of *Aspergillus fumigatus* asexual spores defective for isocitrate lyase or malate synthase (Olivas et al., 2008). This suggests that when external resources are limiting, growth of germinative mycelia in these fungi can be sustained by spore reserve compounds, whose mobilization requires peroxisome activity.

In *N. crassa*, absence of isocitrate lyase compromises ascospore germination, which underscores the importance of glyoxylate cycle in this process. Furthermore, in this fungus addition of sucrose or TCA cycle intermediates does not improve ascospore germination of isocitrate lyase mutants (Flavell and Fincham, 1968). Interestingly, triacylglycerides constitute a major metabolic resource for germinating ascospores in *N. crassa* (Goodrich-Tanrikulu et al., 1998), whereas they represent only a minor proportion of the asexual spore lipids (Bianchi and Turian, 1967). Consistently, isocitrate lyase-deficient asexual spores of *N. crassa* do not exhibit germination defects (Flavell and Fincham, 1968).

Transfer of β-oxidation-derived acetyl-CoA into mitochondria for energy generation may be important for ascospore germination. However, elimination of CATs has an ambiguous impact on ascospore germination in different fungi. While ascospore germination is slightly reduced in *S. sclerotiorum pth2* null strains (Liberti et al., 2013), deletion of its ortholog exerts no developmental defect in *A. nidulans* (Hynes et al., 2011), and results in precocious germination in *G. zeae* (Son et al., 2012). Furthermore, defective glyoxylate cycle or peroxisome assembly does not affect sexual or asexual spore viability in *A. nidulans* (Armitt et al., 1976; Gainey et al., 1992; Hynes et al., 2008). Altogether, these data indicate that requirement for peroxisomes during spore germination depends on the constitution and metabolic resources of a spore, which can importantly vary between different fungi and depending on the sexual or asexual origin of spores.

Research on the role of peroxisomes in the sexual spores of fungal lineages that are not ascomycetes is scant. However, the presence of malate synthase activity in basidiospores (the meiotic-derived sexual spores of Basidiomycetes) of different lineages of Agaricomycetes—the mushroom-forming fungi—suggests a widespread occurrence of the glyoxylate cycle is these spores (Ruch et al., 1991). Furthermore, similarly to asexual spores of *G. zeae*, cytological and ultrastructural analyses have revealed lipid stores in basidiospores, which are resolved as numerous lipid droplets in close proximity to microbodies that are probably peroxisomes and mitochondria (Ruch and Motta, 1987; Ruch et al., 1991). These observations suggest that the fatty acid β-oxidation and the glyoxylate cycle could also be important for lipid reserve mobilization during the germination of basidiomycete sexual spores.

A role for peroxisomes in forcibly ascospore discharge

Research in *G. zeae* uncovered an additional involvement for peroxisomes in asci development, which is not related to meiotic development or ascospore formation *per se* (Son et al., 2012). After meiosis completion and ascospore formation, the remaining original ascus cell consists of a sac-like structure, which encases the ascospores. In many filamentous ascomycetes, tubular asci are perforated at their tips upon spore maturation and then act as "water cannons," which forcibly expel their ascospores out of the ascus (for review, Trail, 2007). In *G. zeae* mutants lacking the peroxisomal/mitochondrial carnitine acetyltransferase (CAT1) the maturation of asci and ascospores is normal; however, ascospore discharge is considerably reduced. This phenotype is aggravated by deletion of the gene encoding a second carnitine acetyltransferase (CAT2), which localizes to cytosol and peroxisomes (Son et al., 2012).

Ascospore ejection relies on the turgor pressure generated inside asci after an increase in its osmolyte concentration drives an influx of water (Trail, 2007). In the CAT mutants, ascospores remain clustered in *cirri* at the perithecium ostiole (see **Figure 1**) instead of been effectively expelled out from perithecia (Son et al., 2012). This suggests a deficient turgor pressure generation inside asci. The precise function of CATs in this process remains elusive. However, since turgor pressure generation probably consumes high levels of ATP (Son et al., 2012), the defect in ascospore ejection in CAT mutants could reflect the need for acetyl-CoA, which is required for mitochondrial ATP production. Defects in ascospore ejection have also been observed in *P. anserina* strains deficient for β-oxidation in mitochondria or for the PTS2 receptor PEX7. However, the ejection of ascospores in these mutants appears to be only delayed (Bonnet et al., 2006; Boisnard et al., 2009). These observations indicate that, in addition to their formation and germination, peroxisomes also participate in the dispersal of sexual spores.

CONCLUDING REMARKS

Fungi represent a large group of organisms, which exhibit high diversity in terms of lifestyles and reproductive strategies. Peroxisomes contribute to this diversity by providing metabolic versatility, which allows fungi to colonize a broad range of environments. The functional versatility provided by peroxisomes extends to the orchestration of developmental processes, like sexual reproduction. Remarkably, peroxisomes have been directly or indirectly implicated in most major developmental events driving sexual reproduction in fungi, including the formation of sexual regulatory signaling molecules, mating, meiotic induction and progression, as well as the differentiation, dispersal and germination of sexual spores. Moreover, the developmental processes accompanying sexual reproduction, like the differentiation and sustenance of the sexual reproductive structures, also critically require the activity of peroxisomes. Interestingly, during these processes peroxisomes allow metabolic relocation not only between somatic cells and sexual reproductive structures, but also between different cell types within the sexual fructifications, and between different multicellular differentiated structures. However, peroxisome involvement in the sexual cycle varies among fungal species and little conservation is so far apparent

for many peroxisome developmental functions. Although further comparative research is required to better appreciate the occurrence of such diverse developmental roles, these observations underscore the versatility of peroxisomes in fungi, which seem to be remarkably adaptable and capable of adopting different roles. They underline also the functional diversity of peroxisomes, which clearly plays an important role in the diversity of the developmental systems that have evolved in fungi over 1 billion years of evolution.

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