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CHAPTER 1

Introduction

Abstract

Peroxisomes are ubiquitous multifunctional organelles, which are found in most eukaryotic cells. A conserved function of peroxisomes is β-oxidation of fatty acids and scavenging of reactive oxygen species generated from diverse metabolic pathways. In yeast, peroxisomes are mainly formed by fission of pre-existing ones but can also form de novo from the endoplasmic reticulum. Their number, size, and function vary between organisms and environmental conditions. Peroxisome biogenesis and degradation must be orchestrated to achieve peroxisome homeostasis. Multiple quality control mechanisms are employed in the cell to ensure proper peroxisome functions and its protection from oxidative damage. These include the function of antioxidant enzymes and molecular chaperones. Dysfunctional organelles can be removed by selective autophagy.

Here, the current knowledge on peroxisome functions, redox regulation and peroxisome proliferation is presented, with a focus on studies performed on yeasts as model systems.

Introduction

Peroxisomes are very dynamic, single membrane bound, multifunctional organelles, which play an important role in several cellular processes. They are found in almost all eukaryotic cells. The name "peroxisomes" was proposed by De Duve and Baudhuin (1966) based on the presence of several enzymes. which produce and degrade hydrogen peroxide (H_2O_2).

Apart from H_2O_2 detoxification, β-oxidation of fatty acids is a highly conserved function of these organelles. In yeast and filamentous fungi, peroxisomes also play a role in the primary metabolism of unusual carbon and nitrogen sources like fatty acids, methanol, and primary amines and in the biosynthesis of secondary metabolites, such as biotin and antibiotics [1,2]. In certain filamentous fungi, specialized peroxisomes known as Woronin bodies are found, which plug septal pores in hyphae during injury [3]. These organelles originate by budding from normal peroxisomes. Plant peroxisomes perform multiple functions including lipid metabolism, photorespiration, nitrogen metabolism, reactive oxygen species (ROS) detoxification and synthesis of some plant hormones [4,5]. In animal cells, peroxisomes are among others involved in α- and β-oxidation of long-chain fatty acids, the degradation of purines and in the biosynthesis of bile acids and ether lipids such as plamalogens. Plasmogens are the most abundant phospholipid present in the myelin sheath that covers nerve cells in the brain [6].

Interestingly, recently in mammals, non-metabolic peroxisome functions like anti-viral innate immunity and anti-viral signaling, have also been identified [7]. Using organelle proteomics and high throughput mutant screens, novel peroxisome proteins and functions are still being discovered.

Peroxisome morphology, abundance and function depends on the environment and the cell type. Proteins involved in the biogenesis and maintenance of peroxisomes are called peroxins and are encoded by *PEX* genes. So far, 36 *PEX* genes have been discovered. Most peroxins are involved in matrix protein import, membrane protein insertion and regulation of peroxisome numbers [8].

In yeast, functional peroxisomes are essential for growth of cells on oleic acid or methanol. In humans, mutation in several *PEX* genes are linked with Peroxisome Biogenesis Disorders (PBDs), which affect brain development and can result in death at an early age [9]. Most peroxisome enzyme deficiencies are linked with the development and progression of severe clinical disorders [10]. In plants, the absence of peroxisomes can cause lethal phenotypes as they are required for embryogenesis and subsequent seedling germination [11].

This contribution, summarizes the current knowledge in the areas of peroxisome function, redox regulation and proliferation in yeast.

Peroxisomes and oxidative stress

In eukaryotic cells, mitochondria and peroxisomes are the main intracellular sources of ROS. Peroxisomes are considered as an important source of ROS due to their oxidative metabolism. In addition to ROS producing processes, peroxisomes accommodate several defense mechanisms and antioxidant enzymes to maintain the redox balance.

ROS can cause damage to vital cellular macromolecules such as nucleic acids, proteins and lipids. The damage caused by ROS in living cells is named oxidative stress. Accumulation of damaged components leads to ageing and age-related diseases. In humans, an imbalance in the cellular redox state causes a considerable risk for development of various diseases such as neurodegeneration, diabetes, aging, and cancer [12,13]

Peroxisomes and mitochondria are crucial for the maintenance of the redox balance in the cell. Recent studies impart strong support to the idea that peroxisomes and mitochondria share a redox-sensitive relationship involving complex signalling pathways. Peroxisome-derived oxidative stress may activate signalling pathways that result in increased mitochondrial stress-causing mitochondria-mediated cell death [14,15].

The peroxisome as a cellular source of ROS species

An important feature of peroxisomal metabolic pathways in the production of ROS, which includes superoxide anion (O_2^-) and H_2O_2 [16]. H_2O_2 is produced by peroxisomal oxidases, but in mammalian and plant peroxisomes, H_2O_2 can also be formed by a dismutation reaction of O_2^- catalyzed by superoxide dismutases via the hydroperoxyl radical ($O_2^- + H^+ \rightarrow HO_2^-$; $2HO_2^- \rightarrow H_2O_2 + O_2$). Yet, superoxide dismutases have not been reported to be present in yeast peroxisomes. Also, the hydroxyl radical ($^{\cdot}OH$), which is the most highly reactive and toxic form of ROS, can be formed in peroxisomes. This occurs by the Fenton reaction involving metal ion (e.g. iron/copper) – catalysed decomposition of H_2O_2 ($H_2O_2 + O_2^- \rightarrow O_2 + OH^- + {}^{\cdot}OH$) [17]. Transition metals, such as iron and copper, are usually present in a complex with peroxisomal enzymes as a cofactor. These metal ions can be freed and activate the formation of hydroxyl radicals under certain conditions.

The number of ROS producing peroxisomal proteins is vast and well-characterized in man [12,18,19]. Contrastingly, the yeast *Saccharomyces cerevisiae* contains only one peroxisomal enzyme (acyl-CoAoxidase, Pox1), which produces H_2O_2. Yeast species, which have more oxidases can be advantageous as model organisms to study peroxisomal ROS homeostasis. Methylotrophic yeasts such as *Hansenula polymorpha* and *P. pastoris* contain several peroxisomal oxidases such as alcohol oxidase, acyl-CoA oxidase, amine oxidase, urate oxidase and D-amino acid oxidase [20]. Notably, during the growth of methylotrophic yeast on methanol for each oxidized methanol molecule, one molecule of H_2O_2 is produced, which makes these organisms perfect models to study the impact of peroxisomal ROS and the role of peroxisomal antioxidant enzymes in aging.

Peroxisomal antioxidant defense systems

Peroxisomes employ a number of ROS detoxification systems to protect the cells from oxidative stress. Antioxidant enzymes such as peroxisomal catalase (CAT), glutathione peroxidase, peroxiredoxin I and Pmp20 degrade H_2O_2 and copper-zinc-dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) detoxify superoxide anions [19]. An imbalance in the production and scavenging of ROS can cause damage to peroxisomes or leakage of ROS from the organelles to the cytosol causing oxidative damage to other cellular components.

The role of catalase

Catalase (CAT) is the main antioxidant enzyme of peroxisomes and decomposes H_2O_2 into O_2 and H_2O. In yeast, the absence of peroxisomal CAT inhibits growth on specific carbon sources (e.g. methanol and oleate) that are metabolized by peroxisomal enzymes [21,22]. Also, the peroxisomal localization of CAT is indispensable for methylotrophic growth, because of a block in the import of CAT into peroxisomes obstructs the growth of *H. polymorpha* cells on methanol [23]. In fungi, peroxisomal CAT also protects cells from externally added H_2O_2 [24].

In *S. cerevisiae*, the absence of peroxisomal CAT extends the lifespan of this organism by elevating intracellular H_2O_2 levels, which results in an increase of superoxide dismutase (Sod2) activity and a decrease of the superoxide levels [25]. In *H. polymorpha,* the effect of peroxisomal CAT deletion on chronological aging differs depending on the growth conditions. The absence of peroxisomal CAT results in a reduced chronological lifespan when cells are grown on media containing methylamine as a sole nitrogen source. Contrarily, the lifespan of CAT deficient cells increased relative to wild type cells when cells were grown in medium supplemented with methanol. The positive effect on lifespan was due to the compensatory activation of other antioxidant enzymes, including cytochrome c peroxidase and superoxide dismutase [26]. Future studies using a yeast model with a broader range of H_2O_2 producing enzymes would explain the relation between peroxisomal H_2O_2 production and redox balance in a better way.

Reduced CAT enzyme activity in human tissue leads to hypocatalasemia which causesthe premature onset of several age-related diseases [27].

All peroxisomal CAT proteins most likely possess a relatively weak peroxisomal targeting signal 1 (PTS1). The weak targeting signal allows the protein to properly fold before its import into peroxisomes [23]. The PTS1 is recognized by the cycling PTS1 receptor, Pex5 which is recycled back into the cytosol by a process requiring monoubiquitination of the N-terminal cysteine residue. Pex5 has a redox-sensitive conserved cysteine residue, which regulates protein function in *Pichia pastoris* [28] and man [29]. Oxidation of this cysteine residue causes blockage of monoubiquitination, which serves as a signal for lysine polyubiquitination and proteasomal degradation of Pex5 [29]. CAT appears to be increasingly mislocalized to cytosol with the increase in cellular oxidative stress. Defects in the import of CAT causes decreased peroxisomal CAT enzyme activity in aging human fibroblasts [30]. In fact, restoration of CAT import by the expression of an alternative CAT with a stronger PTS1 caused reduced H_2O_2 production in ageing fibroblasts [31]. It was recently demonstrated that inefficient CAT import, when coupled with the role of redox-regulated import receptor Pex5, may constitute a cellular defense mechanism of retaining CAT in the cytosol under oxidative stress conditions [31]. These data indicate the importance of efficient import of CAT to peroxisomes for its biological function.

Peroxisomal Peroxiredoxins

The process of ROS detoxification also involves peroxiredoxins and thioredoxin-dependent peroxidases, which are conserved from yeast to humans [32].

Based on the number of cysteine residues involved in the catalytic cycle, two types of peroxiredoxins are known named as 1-Cys and 2-Cys peroxiredoxins. It is well established that mammalian Prdx5 is a 2-Cys enzyme and is targeted to peroxisomes by virtue of a PTS1

targeting signal. In addition, this enzyme has been found in other cellular compartments including the cytosol, mitochondria, and nucleus [33]. The peroxisomal Prdx5 was shown to provide antioxidant protection against oxidative stress induced by exogenous H_2O_2 [34]. Also, *S. cerevisae* contains a peroxisomal 2-Cys peroxiredoxin, Gpx1, which is also present at other cellular locations [35]. On the contrary, methylotrophic yeasts contain a 1-Cys type peroxiredoxin (Peroxisomal membrane protein 20, Pmp20), which is localized to peroxisomes [36,37]. In *Candida boidini* and *H. polymorpha*, the absence of Pmp20 results in a growth defect on media containing methanol as a carbon source. CbPmp20 displays glutathione peroxidase activity with various peroxides such as alkyl hydroperoxide and H_2O_2 as substrates. Therefore, it has been hypothesized that the main function of this enzyme is to remove lipid hydroperoxides [37]. In *H. polymorpha* apart from the severe growth defect in methanol medium, the absence of HpPmp20 resulted in mislocalization of peroxisomal matrix proteins to the cytosol, ROS accumulation, lipid peroxidation and necrotic cell death [36]. Together, these observations signify the importance of this peroxisomal antioxidant-enzyme for cellular viability.

Pexophagy

In order to maintain peroxisome homeostasis, the formation of new peroxisomes, as well as the removal of damaged peroxisomes, is critical. The pathway for the removal of superfluous or damaged peroxisomes by autophagy is referred to as pexophagy [38].

In yeast, pexophagy can be induced upon shifting cells from oleic acid or methanol containing medium, which leads to massive peroxisome proliferation, to a carbon source that is not metabolized by peroxisomal enzymes (such as glucose). Pexophagy is also induced when nutrients are generally lacking [39]. Pexophagy is induced in animal cells by amino acid starvation, treatment of cultured cells with hypolipidemic drugs or non-classical peroxisome proliferators, such as 4-phenylbutyrate [40,41].

Pexophagy can occur via macro- or microautophagy-related pathways. Macropexophagy involves the formation of a pexophagosome, which subsequently fuses with the vacuole, resulting in degradation of entire the organelle [42]. Micropexophagy is initiated by the formation of protrusions from the vacuole followed by direct engulfment by the vacuoles [43]. Several Atg proteins are have been identified that are required for micro- and macropexophagy [44]. Similar to other types of selective autophagy pathways, pexophagy requires specific cargo receptor and adaptor proteins. Atg30 was the first pexophagic adaptor protein to be identified in *Pichia pastoris* [45]. Atg36 was found as an adaptor protein for pexophagy in *S. cerevisiae* [46]. Both Atg30 and Atg36 are recruited to the peroxisomal membrane via Pex3 [47]. Both Atg30 and Atg36 undergo Hrr25-mediated phosphorylation, which allows them to interact with the common adaptor protein Atg11 and, thus, assisting in the formation of the autophagosomal membrane structure targeting the peroxisomes [47].

Contrary to yeast, mammals do not have a pexophagy-specific cargo receptor such as Atg30 or Atg36, but instead, use NBR1 and p62 (also referred to as SQSTM1) as pexophagy adaptors [48]. The proteins p62 and NBR1 are selectively degraded by autophagy. They act as cargo receptors for the autophagic degradation of ubiquitinated substrates [49]. Overexpression of Pex3 was reported to facilitate ubiquitination of peroxisomes and pexophagy induction [50]. More

recent studies have implicated the ubiquitination of mammalian Pex5 [51,52] as well as Pmp70 [40] in pexophagy.

Removal of non-functional organelles is essential for cellular-lifespan because shorter chronological lifespan and elevated levels of ROS have been observed in cells defective in autophagy [53,54]. Pexophagy inhibition results in the accumulation of peroxisomes with an impaired redox equilibrium in mammalian cells. This is because the accumulation of functionally compromised peroxisomes causes an imbalance between preserved H_2O_2 generating peroxisomal acyl-CoA oxidase and dysfunctional CAT, which leads to enhanced intra-peroxisomal oxidave stress [55]. Recent findings in *H. polymorpha* and *S. cerevisae* indicated that peroxisome fission is important for pexophagy [56,57]. It has been speculated that the fragmentation of damaged organelles promotes the engulfment of the organelles by autophagosomes [57]. These studies indicate the importance of pexophagy for the regulation of cellular homeostasis in the organism from yeast to humans.

Peroxisome proliferation

Peroxisomes are maintained in the cell through tight regulation of peroxisome biogenesis and peroxisome turnover by autophagy. Being a dynamic organelle, peroxisome shape, size and number depend on the metabolic needs of the cell. When yeast cells are grown at peroxisome repressing conditions (glucose containing medium), peroxisomes are smaller and lesser in number. On the contrary, when glucose-grown cells are shifted to the medium containing oleic acid or methanol which are metabolized by peroxisomal enzymes, peroxisome proliferation is stimulated [58].

Signalling pathways

Signalling pathways responsible to induce the expression of peroxisomal genes, regulate peroxisome proliferation. External stimuli such as fatty acids activate these signalling pathways leading to an increase in peroxisome number. In *S. cerevisiae*, transcription factors ScPip2 and ScOaf1p heterodimerize and bind to oleate response elements (OREs) present in peroxisomal gene promoters to activate their transcription. ScOaf1p contains a functional fatty acid binding domain [59]. *S. cerevisiae* also contains Adr1, an additional Zn-finger transcription factor, which senses the carbon status and controls expression of peroxisomal genes. It binds to upstream activating sequence 1 (UAS1) promoter sites under glucose derepression and oleate induction conditions [60]. In *H. polymorpha* the transcription factor Mpp1, which belongs to the Zn-cluster protein family, effects peroxisome proliferation. Mpp1 is a necessary for growth on methanol containing media [61].

Filamentous fungi contain two transcription factors, FarA and FarB, which activate the transcription of genes involved in peroxisome biogenesis and fatty acid degradation [62]. In plant, peroxisome proliferation can be induced by ROS, UV radiation, light and salt stress. The transcriptional activation of peroxisomal genes, e.g. *PEX11*, involves the induction of the far-red light receptor phyA as well as the binding of bZip transcription factor, HYH to

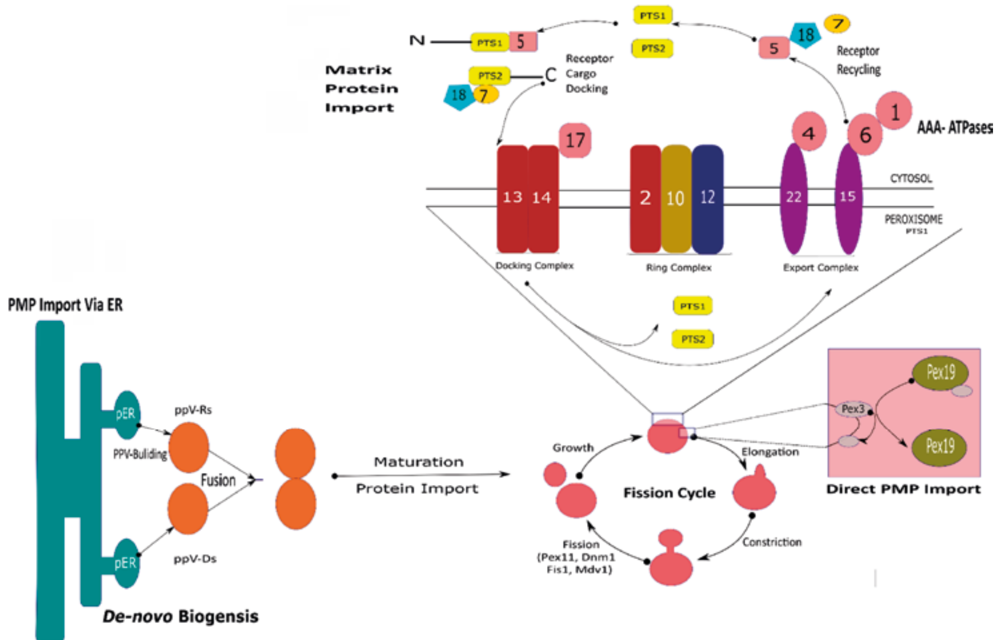

Figure 1. Hypothetical model of peroxisome biogenesis and proliferation. Peroxisome fission involves peroxisome membrane remodelling in the presence of Pex11 which results in elongation of the organelle followed by membrane constriction. Afterwards, proteins of fission machinery (Pex11, Dnm1, Mdv1, Fis1) work together for the division of peroxisomes which results in a new nascent organelle and a mature organelle (mother cell). De novo biogenesis requires fusion of two pre-peroxisomal vesicles (ppV) which are derived from the ER. PMPs are indirectly imported to the ER and are targeted to pER sub-domains of ER. The ppVs bud from the pER and fuse heterotypically. The nascent peroxisomes then import matrix proteins, eventually forming a mature peroxisome. Matrix protein import includes 4 main steps - 1. Cargo recognition in the cytosol: PTS1 and PTS2 containing peroxisomal matrix proteins are recognized in the cytosol by the cytosolic receptor proteins, Pex5 and Pex7 and coreceptor Pex20, respectively. 2. Receptor-cargo complex recognition: The PTS/receptor complex docks at the peroxisome membrane via the docking complex (Pex13, Pex14, Pex17). 3. Cargo translocation: The docking and the RING-finger complex (Pex2, Pex10, and Pex12), together form the importomer complex which functions in cargo release into the peroxisome lumen. 4. Receptor recycling: Following cargo release, the PTS receptor proteins are recycled back into the cytosol for another round of import by using components of export complex. PMPs are also imported directly via cytoplasm-to-peroxisome pathway. These PMPs have mPTSs (PMP targeting signals) and their import is dependent upon Pex19. Pex3 acts as a docking factor and once the PMP is inserted into the peroxisome membrane, Pex19 recycles back to the cytosol for next round of PMP import.

the gene promoter [63]. In mammals, apart from the major pathway which is the peroxisome proliferator-activated receptor α (PPARα) – dependent pathway, extracellular signals such as ROS and growth factors have also been reported to activate peroxisome proliferation [12,64].

The peroxisome fission machinery

Peroxisomes fission is mediated by Pex11 along with other factors including dynamin-related proteins (DRPs or dynamin-like proteins (DLPs) in mammals) [65–67].

The role of Pex11 family proteins

Pex11 represents a highly conserved protein with a well accepted role in the regulation of peroxisome size and number [64,68,69]. Pex11 was the first identified protein involved in peroxisome fission. The absence of Pex11 results in enlarged and a lower number of peroxisomes, whereas its overproduction leads to an increased number of peroxisomes [70,71]. More than one Pex11 paralog have been identified in most organisms. For instance, five Pex11-related proteins occur in *A. thaliana* whereas three Pex11-like proteins (Pex11α, Pex11β and Pex11γ) are present in mammals [72].

The induction of *PEX11* expression is a prevalent, initial event in peroxisome fission in yeasts, plants and mammals during peroxisome proliferation [64]. It is the most abundant peroxisomal membrane protein in oleate acid grown *S. cerevisiae*. In addition to peroxisome fission, Pex11 participates in several other functions, which include peroxisome inheritance [73], re-distribution of peroxisomal membrane proteins (PMPs) on the peroxisome membrane during organelle fission [74], fatty acid oxidation and transport of small molecules [75,76].

In *S. cerevisae*, three members of the Pex11 family have been identified, namelyPex11, Pex25 and Pex27. Like for *PEX11*, Deletion and overexpression of *PEX25* or *PEX27* affects peroxisomes size and number [77–79]. It was demonstrated that Pex34 plays a role in proliferation of peroxisomes in cooperation with Pex11 family members in *S. cerevisiae* [80]. Pex11C and Pex25 are the two Pex11 paralogs present in *H. polymorpha*. The functions of these proteins are still obscure. A recent study in *P. pastoris* has identified a new PMP, Pex36, which is required for growth on methanol but not an essential protein for peroxisome proliferation. However, a *pex36 pex25* mutant is showed a severe peroxisome biogenesis defect [81].

Organelle fission by the DRP machinery

Upon Pex11 mediated tubulation of peroxisomes, peroxisomes constrict by a yet unknown mechanisms. Finally, the organelle divides, mediated by DRPs [82–84]. Remarkably, peroxisomes and mitochondria share key fission proteins such as Drp1 and Fis1, adding to the "peroxisome-mitochondria connection" [85]. Similar to Pex11 deficient cells, mutants lacking DRPs display abnormal peroxisome morphologies and a severe reduction in peroxisome numbers [86,87].

In *S. cerevisae*, the DRPs Dnm1 and Vps1 participate in peroxisome fission. Dnm1 is important at peroxisome inducing growth conditions, whereas Vps1 is required at glucose repressing conditions[84]. The absence of Dnm1 or Vps1 results in decrease in peroxisome numbers whereas absence of both causes a full blockage in peroxisome fission resulting in the presence of one big peroxisome per cell. On contrary, in *H. polymorpha* only Dnm1 but not Vps1 is required for peroxisome fission and a mutant lacking Dnm1 displays a single large peroxisome with long protrusions extending from mother cell to the daughter cell and partition via cytokinesis [67]. The observation that only a single peroxisome was present in *S. cerevisae*

dnm1 vps1 or *H. polymorpha dnm1* cells supports the idea of growth and fission being the main mechanism for peroxisome proliferation.

Posttranslational modifications

A number of recent studies highlighted the role of protein phosphorylation in the regulation of peroxisome dynamics [88,89]. Protein phosphorylation regulates peroxisomal matrix and membrane protein import, proliferation, inheritance and degradation. It has been shown that phosphorylation of Pex11 is important for peroxisome fissionin *S. cerevisiae* and *P. pastoris* [90,91]. However Pex11 phosphorylation has no influence on peroxisome fission in *H. polymorpha* [90–92]. Phophorylation of Pex14, which belongs to the docking complex for the matrix import receptors Pex5 and Pex7, have been reported to counteract peroxisome proliferation by playing a role in pexophagy [93,94].

In addition to phosphorylation, other post translational modifications can also regulate peroxisome dynamics. Protein ubiquitination has been demonstrated to affect peroxisome protein import and autophagy by targeting Pex5 [95]. Furthermore, ubiquitination of other peroxins has been described to influence protein import and peroxisome degradation. For example, *H. polymorpha* PMP Pex3 is ubiquitinated and degraded by the proteasome system when methanol grown cells are shifted to glucose containing media. Pex3 degradation triggers autophagic degradation of peroxisomes via pexophagy [96,97]. It was recently demonstrated that *H. polymorpha* Pex13 is ubiquitinated and undergoes degradation in wild type cells [98].

Alterations in peroxisome proliferation are often associated with liver diseases, neurological dysfunction, as well as cellular ageing [69,99]. Understanding the mechanism of peroxisome division and proliferation will contribute towards the therapeutic approaches to improve cellular function in health and diseases.

De novo formation of peroxisomes

As yet, the existence of two pathways for peroxisome biogenesis has been proposed. Initial studies suggested that peroxisomes divide by growth and division of pre-existing organelles similar to the semi-autonomous organelles of endosymbiont origin like mitochondria and chloroplasts [100]. The second pathway of peroxisome biogenesis suggests that peroxisomes are formed *de novo* from the endoplasmic reticulum (ER) [101,102]. The *De novo* pathway has slower kinetics but it results in peroxisomes with all “new” material whereas growth and fission is a faster process, which requires the pre-existing peroxisomes [103]. However, there is still a lot of debate in regard to the existence of these models. The initial idea of peroxisomes forming from the ER was based on the observation that connections are present between ER and peroxisomes in mouse dendritic cells [104].

This concept was also proposed for *pex3* and *pex19* yeast mutants, which for long were thought to completely lack peroxisome structures. Because functional complementation of these mutants with the corresponding genes resulted in formation of peroxisomes, these organelles were assumed to be formed *de novo* [105–108]. In addition, fluorescence microscopy

studies revealed that the newly introduced Pex3 protein was first detected at the ER before appearing at the peroxisomes, suggesting the involvement of ER in *de novo* formation of peroxisomes. This conclusions was further supported by the fact that the N-terminus of Pex3 contains a signal sequence typical for ER membrane proteins [109]. However, the fact that the peroxisomes are formed upon re-introduction of Pex3-GFP under control of a strong inducible promoter in *pex3* cells cannot be ignored as such conditons can cause mistargeting of the protein. Trafficking of Pex3 and other PMPs invariably via the ER to peroxisomes in WT cells is still debated.

It was recently demonstrated that *H. polymorpha* and *S. cerevisae pex3* cells harbor pre-peroxisomal vesicles (PPVs), which mature into functional peroxisomes upon reintroduction of the *PEX3* gene suggesting that peroxisomes do not form *de novo* from the ER [110,111] . Electron microscopy analysis revealed that PPVs are located in close proximity to ER, which may have led to the conclusion that Pex3 travels via ER. So far, PMPs are never observed on the ER in WT cells under normal conditions, which suggests an ER independent route. The mechanism of PPVs formation and maturation into functional peroxisomes is still not clear.

The role of the ER in peroxisome biogenesis was demonstrated through the use of a photo-activatable Pex16-GFP fusion construct in the mammalian cells [101]. These authors showed that a PMP travels via the ER to the peroxisomes and that *de novo* peroxisome formation contributed significantly to the total peroxisome population. However, recent studies in human fibroblasts lacking peroxisomes showed that Pex3 and Pex14 targeted to mitochondria, where they exited in vesicles [112]. On the contrary, Pex16 trafficked to the ER and was released in vesicles, which appeared to fuse with the mitochondria derived ones, thereby generating fully import competent, peroxisomes. These findings suggest roles of both the ER and mitochondria in *de novo* formation of peroxisomes in mammalian cells [112]. Also, in *S. cerevisiae* a Pex3 variant that artificially contained a mitochondrial targeting signal was shown to sort to mitochondria in Pex3-defecient cells followed by the de novo formation of import competent peroxisomes [113]. Taken together, these findings suggest that natural or artificial targeting of Pex3 to any intracellular membrane membrane may initiate peroxisome formation.

In summary, *de novo* formation of peroxisomes has only been observed in yeast mutants lacking peroxisomes and not in the WT cells. The prevalent mode of peroxisome proliferation is fission in WT yeast, however the fact that some peroxisomes may be formed via a *de novo* mechanism from other membranes cannot be ignored. A detailed study of PMP targeting, how PMPs are targeted to multiple membranes and how this is regulated would immensely improve our understanding regarding the role of ER and mitochondria in peroxisome formation.

Peroxisome growth

Peroxisomal growth requires the import of matrix and membrane proteins and, incorporation of lipids for their membrane expansion. One of the extraordinary features of peroxisomes, which differentiates it from mitochondria or chloroplast is the capacity to import fully folded,

oligomeric proteins [114]. However, this process is not fully understood, yet. Once the peroxisomes reach a certain size, peroxisome fission is initiated.

Peroxisomal matrix protein import

Peroxisomes do not contain their own DNA. Hence, all peroxisomal proteins are nuclear encoded and post-translationally imported into the peroxisomes. This process involves matrix protein targeting and an import machinery comprising of several protein complexes. These function to dock cargo-bound import receptors at the peroxisomal membrane, transfer cargo into peroxisomes and export receptors back to the cytosol. For cargo recognition and transport to peroxisomes, peroxisomal matrix proteins possess specific peroxisome targeting signals (PTSs). Most of the peroxisomal matrix proteins contain a C-terminal PTS1 signal, which formerly was specified as the tripeptide serine-lysine-leucine (SKL) or variants such as (S/A/C)-(K/R/H) – (L/A), at the end [115,116]. The latest prediction programmes that have been developed to identify PTS1 sequences in proteins make predictions based on C-terminal dodecamers as it turned out that additional adjacent amino acids are crucial for receptor-cargo interactions as well [117]. The PTS1 targeting signal is recognized by a receptor protein, Pex5, via its C-terminal tetratricopeptide repeat domain [117–119]. Recently two independent studies have identified a new PTS1 receptor in *S. cerevisiae* named as Pex9, which participates in the import of the PTS1 containing enzyme malate synthases (Mls1 and Mls2) and glutathione transferase (Gto1) when yeast cells are gown on oleate containing medium [120,121]. Pex9 follows the same import-cycle as Pex5 and interacts with the docking protein Pex14. It is not produced in glucose or ethanol grown cells. Existence of an another PTS1 receptor increases the efficiency of protein import into peroxisomes.

The second evolutionarily conserved peroxisomal targeting signal (PTS2) is present at the N-terminal of peroxisomal proteins. The consensus sequence consists of the nonapeptide (RK) - (LVIQ) – XX – (LVIHQ) – (LSGAK) -X – (HQ) – (LAF) [122]. Import of PTS2 containing proteins is mediated by the PTS2 receptor protein Pex7 and its co-receptors, which are Pex18 and Pex21 in *S. cerevisae* and Pex20 in other yeast and filamentous fungi [123–126]. In higher eukaryotes, the long isoform of Pex5 (Pex5L) assists Pex7 in the recognition and targeting of PTS2 proteins to the peroxisome [127]. The PTS2 mediated import pathways is absent in *Caenorhabditis elegans* [128], *Drosophila melanogaster* [129] and diatoms [130].

The import of matrix proteins involves several steps. After the binding of PTS1 or PTS2 proteins with their respective co-receptors to the cargo, the receptor-cargo complex docks on the peroxisome membrane by binding to the receptor docking complex (Pex13, Pex14 and Pex17) present at the peroxisomal membrane, followed by import of the cargo via a still speculative mechanism [131]. The current model suggests that cargo-loaded Pex5 forms a large transient import pore in the peroxisomal membrane for PTS1 protein import [132]. In *H. polymorpha* the release of cargo inside the peroxisome matrix depends on Pex8 [133]. In S. cerevisiae Pex8 was reported to link the docking and RING complexes [134], whereas in P. pastoris Pex3 was proposed to have this function [135]. The delivery of the cargo to the peroxisome lumen is followed by recycling of the receptor to the cytosol by the exportomer [136]. The Pex5 receptor is recycled into the cytosol by the recycling machinery, which comprises of RING complex

proteins Pex2, Pex10 and Pex12, the E2 ubiquitin-conjugating enzyme Pex4 along with Pex22 (anchoring protein) and the AAA-ATPases Pex1 and Pex6 with their anchoring protein Pex15. Mono-ubiquitination of Pex5 by Pex4 along with the RING complex proteins that function as E3-ligases, leads to its recycling into the cytosol for next round of import [137]. Ubiquitinated Pex5 is extracted from the membrane through the action of Pex1 and Pex6. On the contrary, polyubiquitination of Pex5 leads to its proteasomal degradation [138,139]. Pex1 and Pex6 form a heterohexameric complex with alternating subunits on the cytosolic side of peroxisomal membrane [140,141]. In *S. cerevisae*, Pex1 and Pex6 are recruited to the peroxisomal membrane by Pex15 [142] whereas Pex26 recruits Pex1 and Pex6 to the peroxisomes in mammals [143].

Interestingly, some matrix proteins do not contain PTS1 or PTS2 targeting signal. Studies in yeast, plants and mammals have reported that proteins lacking a PTS can be imported into peroxisomes by piggybacking together with a protein that does contain a PTS. For example, thiolase with truncated N-terminal PTS2 was shown to be imported to peroxisomes when the full length form was co-produced, suggesting that the two subunits interact before import into the peroxisomes [144]. Later, various findings have confirmed piggyback import pathway as a third mechanism of matrix protein targeting to peroxisomes. Like, the removal of the PTS1 from yeast Eci1, delta3-delta2-enoyl-CoA isomerase, does not affect its targeting to peroxisomes. It can be imported into peroxisomes as hetero-oligomer together with its paralog Dci1 [145]. In mammals, the Cu/Zn superoxide dismutase 1 (SOD1) can import into peroxisomes by piggybacking with its interaction partner 'copper chaperone of SOD1' [146]. A recent study using *A. thaliana* showed that the protein phosphatase holoenzyme 2A is imported to peroxisomes via piggybacking on a subunit containing putative PTS1 [147].

Sorting of PMPs

The PMP sorting pathway to peroxisomes differs from the matrix protein import pathway. Based on the involvement of Pex19, PMPs are divided into 2 classes. Class I PMPs are recognized by the soluble cytosolic protein Pex19, which serves as a receptor and recognizes PMPs via their mPTS sequence. Pex19-bound PMPs are then recruited to the peroxisomal membrane by Pex3 followed by cargo insertion into the peroxisome membrane by a yet unknown mechanism [148]. Pex19 was proposed to also behave as a chaperone in this event [149,150]. Recently, it was shown that farnesylation of Pex19 at its C-terminal domain triggers conformational changes, which facilitates the recognition of conserved aromatic or aliphatic side chains in PMPs [151]. This model is supported by the fact that Pex19 shows interaction with several PMPs and that certain PMPs are mistargeted in the absence of Pex19 or Pex3. Furthermore, it has been shown that the levels of PMPs such as the RING complex proteins were reduced in *pex19* deletion strains in comparison to the wild-type strain in yeast [152,153].

Conversely, class II PMPs are targeted to peroxisomes via an alternative Pex19-independent mechanism involving the ER. Some of the PMPs in yeast, plant and vertebrate cells, concentrate in specialized regions called the peroxisomal-ER (pER) [154]. These PMPs depend on Sec61 and GET complexes for their ER insertion [154,155]. Studies in *Yarrowia lipolytica* [156], mammals [101] and plants [157] have shown that Pex2 and Pex16 travel through the ER before being delivered to peroxisomes. Interestingly, studies in *H. polymorpha* and *S. cerevisae* [110,111], indicate the presence

of pre-peroxisomal vesicles in cells of a *pex3* mutant strain. These vesicles contain Pex13 and Pex14 [110]. It is evident that Pex13 and Pex14 can reach these structures independent of Pex3 and /or Pex19. A possible explanation could be that these proteins first sort to the ER, followed by the formation of the vesicles from the ER.

Most PMPs are shown to localise only to peroxisomes in WT cells except mammalian and plant Pex16 and *Pichia pastoris* Pex30 and Pex31 which localise to peroxisomes as well as the ER in WT cells [157,158]. Perhaps, it is possible that both routes, direct sorting of PMPs to peroxisomes or transit through ER, exist at the same time, with different PMPs employing different pathways to reach peroxisomes. The mechanism of when, where and how a PMP will be sorted and the factors that regulate the process are unknown and requires further research.

Uptake of membrane lipids

Incorporation of membrane lipids is important for growth of peroxisomes. Enzymes required for phospholipid biosynthesis are absent in yeast peroxisomes because of which peroxisomes rely on different sources for their membrane lipids. Most of the peroxisomal membrane lipids are known to be synthesized at the ER. So far, two pathways have been proposed for the transport of lipids to the peroxisomes. Several studies indicate vesicular trafficking of proteins to peroxisomes from other cellular compartments [102,159]. Another mechanism implies that lipids are supplied to peroxisomes via non-vesicular transport from the ER [160]. The peroxisome membrane also contain cardiolipin which is synthesized in the mitochondria suggesting another transport pathway existing between mitochondria and peroxisomes [161].

Non-vesicular lipid transfer takes place at Membrane Contact Sites (MCS), the regions where the membranes of two organelles are closely opposed [162]. The membranes from two intracellular compartments do not fuse at the MCS. Several specific proteins or lipids are enriched at these junctions and MCS affects the function of at least one of the organelles in the MCS [163]. Initial reports suggested their role in intracellular exchange of calcium and lipids. However, their function in intracellular signalling, metabolites trafficking, organelle transport, division and autophagy; has been reported recently [164]. Most of the identified MCS occur between the ER and a second organelle and may facilitate direct lipid transfer like for example, the ERMES (ER-mitochondria encounter structure) complex between ER and mitochondria [165]. Another MCS identified in yeast is the nucleus-vacuole junction (NVJ), which is formed between the vacuolar membrane and the outer nuclear membrane. These sites promote piecemeal autophagy of the nucleus (PMN), which involves degradation and recycling of non-essential parts of the nuclear envelope [166]. A contact site between mitochondria and vacuole was referred as vCLAMP (vacuole and mitochondria patch). These are important for lipid transport between the ER and mitochondria [167,168].

Recent data indicate the presence of contact sites between peroxisomes and different membranes to facilitate lipid transport between organelles. Contact sites between the ER and peroxisomes were identified as EPCONS, which includes a complex containing Pex30 together with reticulon family (Rtn1, Rtn2, Yop1) proteins [169,170]. A second one is a tether formed by the interaction between Pex3 and the inheritance protein Inp1, which brings ER membranes and peroxisome together. Although this contact site has been implicated in peroxisome retention, it

cannot be ruled out that it also contributes to lipid exchange between the ER and peroxisomes. The absence of this ER-peroxisome tether causes accumulation of peroxisomes in the daughter cells due to disturbance in peroxisome retention [171]. Recently, two independent studies have identified the first peroxisome-ER tethering complex, formed by the peroxisomal membrane protein ACBD5 (acyl-CoA binding domain containing 5 and the ER proteins VAPA and VAPB (vesicle-associated membrane protein-associated proteins A and B/C, in mammalian cells [172,173]. The knockdown of ACBD5 and VAPB resulted in reduced peroxisome-ER interaction and loss of ACBD5 or VAPB perturbs peroxisomes membrane expansion [172]. Hua et al., showed that the VAP-ACBD5 tether is necessary for the exchange of lipids between them as the cellular levels of two peroxisomal lipids, plasmalogens and cholesterol, was reduced in the absence of VAPs and ACBD5. Furthermore, another ACBD family protein, ACBD4, has also been shown to interact with the ER-protein VAPB to facilitate interaction between the peroxisomes and the ER [174].

In *S. cerevisae*, it was shown that peroxisomes are juxtaposed to sites in mitochondria where pyruvate dehydrogenase is localized, which might enhance acetyl-CoA transfer to mitochondria for energy production or the synthesis of certain lipids [175]. Both organelles are tethered via the interaction between Pex11 and Mdm34 which is a ERMES component suggesting a possible role of ER in peroxisome-mitochondria contacts [176]. It is likely that these contact regions may facilitate transport of acetyl-CoA between both organelles.

In addition, Pex34 and fuzzy onions homolg 1 (Fzo1), are responsible for the interaction between peroxisomes and mitochondria in yeast. Expansion of peroxisome-mitochondria contact facilitates the transport of β-oxidation product acetyle-CoA to mitochondria [177]. Peroxisomes may form several other MCSs with different cellular membranes. The molecular mechanisms of contact site formation and their function is an interesting and relatively unexplored aspect.

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