The return of the peroxisome

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Summary

Of the classical compartments of eukaryotic cells, peroxisomes were the last to be discovered. They are small, single-membrane-bound vesicles involved in cellular metabolism, most notably the β-oxidation of fatty acids. Characterization of their properties and behavior has progressed rather slowly. However, during the past few years, peroxisomes have entered the limelight as a result of several breakthroughs. These include the observations that they are not autonomously multiplying organelles but are derived from the endoplasmic reticulum, and that partitioning of peroxisomes to progeny cells is an active and well-controlled process. In addition, we are discovering more and more proteins that are not only dedicated to peroxisomes but also serve other organelles.

Key words: Peroxisomes, Peroxisome biogenesis, Endoplasmic reticulum, Organelle, Maintenance, Protein import

Introduction

The concept that peroxisomes constitute a unique member of the organelle family took some time to mature. The first enzymes found in mammalian peroxisomes were H_2O_2-producing peroxidases, hence the name peroxisomes (De Duve, 1996), and catalase. However, similar organelles from other organisms sometimes show remarkably different specializations and were classified under different names. The so-called microbody family also includes glyoxysomes in plants (which contain the glyoxylate cycle enzymes to convert lipid into carbohydrate) and glycosomes in trypanosomatids (which contain part of the glycolysis pathway). The variation in enzymatic content depends not only on the species but also on the cell type and environmental conditions. A feature common to members of the microbody family is the ability to degrade fatty acids (reviewed by Van den Bosch et al., 1992; Purdue and Lazarow, 2001; Eckert and Erdmann, 2003). Moreover, enzymes imported into microbodies share similar trafficking signals: the peroxisomal targeting signals PTS1 and PTS2 (Subramani, 1998).

In the 1990s, studies began to examine peroxisome formation and maintenance by using genetic approaches. Model organisms including certain fungi and mammalian (Chinese hamster ovary) cells were screened for mutants that display defects in biogenesis, such as partial or complete loss of peroxisomes. Particularly in fungi, such screens are facilitated by the fact that the need for peroxisome function depends on the external conditions. For instance, *Saccharomyces cerevisiae* grown on glucose can dispense with peroxisomes; indeed, only when offered a fatty acid as the sole carbon source are peroxisomes required for growth, because they are the exclusive site for fatty acid degradation in this organism (Erdmann et al., 1989).

The combined efforts of several groups have identified some 32 *PEX* genes that contribute to biogenesis or maintenance of these organelles. Some of the proteins produced have a role in protein import. The functions of others can only be guessed at on the basis of their location within the membrane or matrix, their interactions with other Pex proteins, or the phenotypes that result from their deletion and/or overexpression.

These studies provided a framework to understand an enigma presented by several peroxisome-related diseases: the peroxisome biogenesis disorders (PBDs) (Gould and Valle, 2000). A defect in a gene can of course lead to a single enzyme deficiency, but in some extraordinary cases the complete peroxisome population disappears from the cell. We now understand the cause of such phenotypes: when the affected protein is involved in peroxisome biogenesis or maintenance, a severe pleiotropic effect is the result. Lack of protein import, for example, affects the targeting of up to 100 enzymes, which consequently remain in the cytosol, where they cannot function or are degraded.

Peroxisome formation

Early studies on peroxisome formation came to opposing conclusions. Extensive morphological investigations using electron microscopy led to the proposal that peroxisomes are formed from the endoplasmic reticulum (ER) (Novikoff and Novikoff, 1972). This was difficult to reconcile with biochemical data that showed peroxisomal enzymes are synthesized on free polyribosomes and imported post-translationally into the organelle (Rachubinski et al., 1984). Such work formed the basis for the idea that peroxisomes are autonomous organelles that multiply by growth and division, like mitochondria and chloroplasts (Lazarow and Fujiki, 1985). This concept received strong support following the subsequent identification of the PTS1 and PTS2 import signals, and the discovery that peroxisomes posses their own protein-import machinery. However, one question remained difficult to answer: in certain mutants that lack peroxisomes, the organelles reappear once the wild-type gene is introduced (Subramani, 1998). This property is hard to reconcile with the idea that peroxisomes are autonomous organelles. Where do these peroxisomes come from?

Several clues pointed to the ER as a contributor to peroxisome formation. In 1997, Rachubinski and co-workers showed that two peroxisomal membrane proteins, Pex2p and Pex16p, in *Yarrowia lipolytica* are glycosylated, suggesting that they pass through the ER en route to peroxisomes (Titorenko et al., 1997). In mouse dendritic cells (cells of the immune system), peroxisomal membrane proteins were subsequently observed in specialized regions of the ER, in intermediate compartments (lamellae) and in mature peroxisomes (Fig. 1) (Geuze et al., 2003). Matrix proteins (enzymes) were only seen in spherical/ovoid-shaped mature peroxisomes, whereas the membrane proteins Pex13p and PMP70 were found both on lamellae and peroxisomes. This indicated that peroxisome biogenesis might indeed start in the ER and end in the formation of mature peroxisomes. Electron tomography subsequently demonstrated that membrane continuities link the ER with lamellae, lamellae with the peroxisomal reticulum, and the peroxisomal reticulum with mature peroxisomes (Tabak et al., 2003). These pre-compartmental lamellar structures are prominent in dendritic cells for unknown reasons, but are also present in a less dramatic form in hepatoma cells (H.G., unpublished).

We recently visualized the reappearance of peroxisomes in peroxisome-free *S. cerevisiae* mutants by fluorescence microscopy in living cells (Hoepfner et al., 2005). To do this, we and others (see below) used the membrane-anchored protein Pex3p as a reporter. Pex3p, together with the mostly cytosolic and farnesylated protein Pex19p, facilitates incorporation of integral membrane proteins into the peroxisomal membrane (Fang et al., 2004; Rottensteiner et al., 2004) (Fig. 2), which suggests that the two must have an early role in peroxisome formation. Indeed, budding yeast pex3Δ or pex19Δ mutants show no trace of residual peroxisomes (Hettema et al., 2000). In pulse-chase-like experiments, newly synthesized yellow fluorescent protein (YFP)-tagged Pex3p or Pex19p can be tracked in the corresponding pex3Δ or pex19Δ mutants, using cyan fluorescent protein (CFP)-tagged Sec63 (Sec63-CFP) to mark the ER or CFP-PTS1 to monitor peroxisomal import (Fig. 3). We found that Pex3p-YFP first targets to and distributes over the ER and then concentrates in one or two 'dots' in, or at, the ER. Later, the connection with the ER is lost, and cells become able to import proteins into these dot-like structures. Finally, the cell gains a full complement of peroxisomes. Without Pex19p, Pex3p remains all over the ER and no dot-like structures appear. Pex19p therefore appears to be required for the formation of Pex3p-containing structures.

Similarly, some Pex19p becomes associated with the ER-connected dot-like structures and follows the same maturation pathway; the rest remains cytosolic. Thus, the initial concentration of Pex19p into dots at the ER supports the notion that the earliest events in peroxisome formation occur at the ER. In addition, a dynamic equilibrium might exist between the cytosolic and organelle-bound pools, which would be in line with the suggested chaperone-like function of Pex19p (see Fig. 2). In wild-type cells, the same series of events take place.

Recently, Rachubinski and co-workers have made similar observations using a truncated, green fluorescent protein

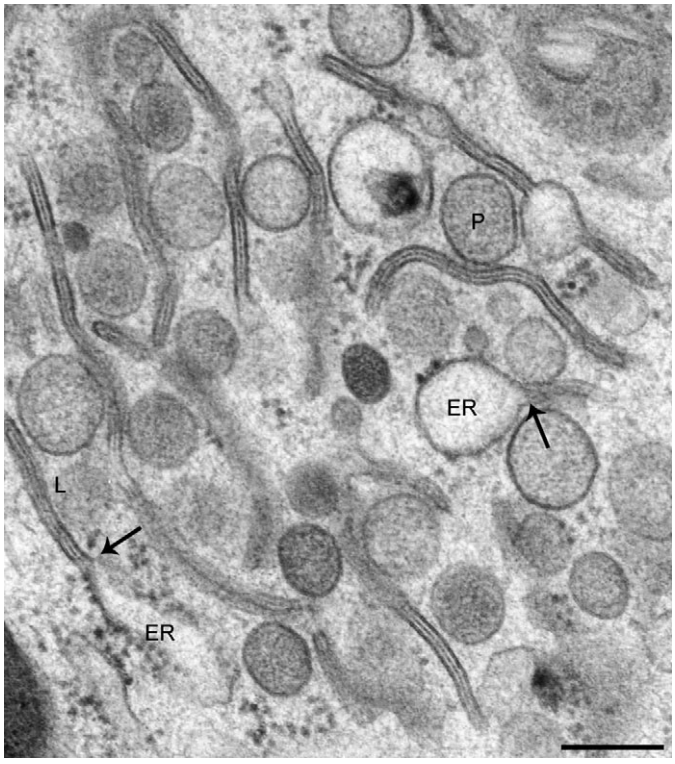

Fig. 1. Birth of peroxisomes in a mouse dendritic cell. Ultra-thin section of a high-pressure frozen D1 cell showing clusters of electron-dense peroxisomes (P) that are often positioned adjacent to a lamella (L). Arrows indicate areas of membrane continuity between the endoplasmic reticulum (ER) and the lamellae (Geuze et al., 2003; Tabak et al., 2003). Bar, 200 nm.

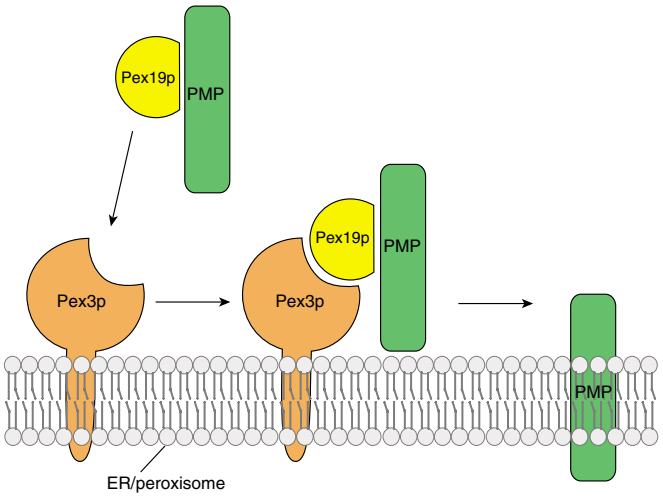

Fig. 2. Model for the roles of Pex3p and Pex19p in the insertion of proteins into the peroxisomal membrane. According to the model, the insertion of Pex3p into the ER membrane and the subsequent recruitment of Pex19p to the membrane leads to the formation of a pre-peroxisomal organelle. At this stage, cytosolic Pex19p can also engage in peroxisomal membrane protein (PMP) import by binding peroxisomal membrane proteins and targeting these to the pre-peroxisomal membrane. By an unknown process, the PMP cargo is released from the membrane-associated Pex19p and is inserted into the membrane. Once PMP insertion is complete, matrix proteins begin to be imported, resulting in the functional maturation of the pre-peroxisome into a peroxisome.

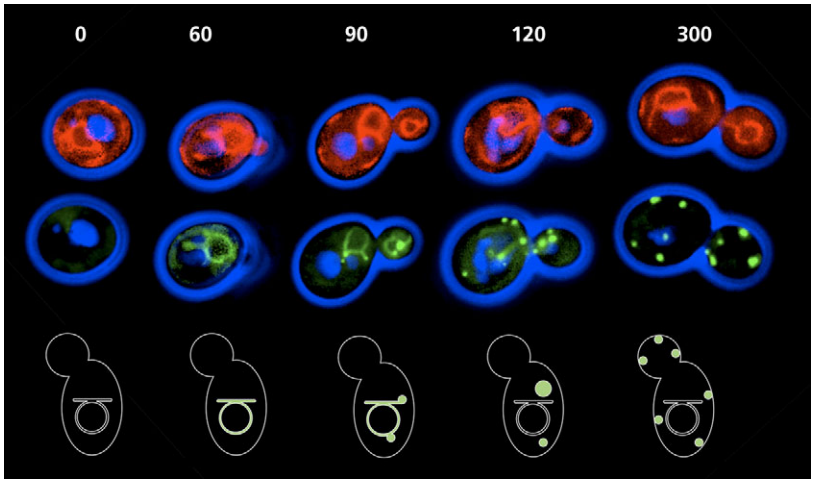

Fig. 3. Peroxisome formation in *S. cerevisiae*. The top two rows show a time course of peroxisome formation induced by expression of Pex3-YFP (green) (based on Hoepfner et al., 2005); the ER is visualized by constitutive expression of Sec63-CFP (red); blue false colour shows the phase contrast. Schematics below display the events that are taking place. At the onset of the induction experiment, no Pex3-YFP signal is present (0). 60 minutes later, the first detectable weak Pex3-YFP signal localizes to structures containing the perinuclear ER marker Sec63-CFP (60). At 90 minutes after induction, Pex3-YFP starts to concentrate into dots, which are frequently localized at the periphery of the ER (90). At 120 minutes after induction, the Pex3-YFP is localized exclusively to dot-like structures that are significantly brighter and no longer overlap with the Sec63-CFP signal (120). Finally, 5-10 individual Pex3-YFP dots per cell are discernible and show no apparent ER colocalization but instead mostly localize to the cell cortex (300).

(GFP)-tagged version of Pex3p (Tam et al., 2005). This 40-residue N-terminal fragment targets to one or two subdomains of the ER, but it cannot support the formation of peroxisomes. When full-length Pex3p is co-expressed, truncated Pex3p-GFP moves from the small punctate structures to import-competent peroxisomes. This process is dependent on the contribution of Pex19p and Pex14p. The results of biochemical experiments are in line with these microscopical observations (Kragt et al., 2005a). Kragt et al. attached an ER signal peptide followed by a short, glycosylatable peptide to Pex3p. This construct targets to the ER, as demonstrated by glycosylation of the modified Pex3p. However, probably because of this modification, Pex3p remains trapped in the ER. If the peptide is now exchanged for a non-glycosylatable peptide, Pex3p ends up in peroxisomes. Kragt et al. therefore concluded that Pex3p travels to peroxisomes through the ER.

The results discussed above solve the long-standing riddle of whether or not peroxisomes are autonomous organelles. They also explain how new peroxisomes might acquire the lipids they need for their membranes: these come from the ER, the major site of phospholipid biosynthesis.

Partitioning of peroxisomes during cell division

Even though new peroxisomes can form from the ER, faithful segregation of these, like other, organelles during cell division is necessary. For multi-copy organelles, the traditional view has been that stochastic principles suffice. However, it is now becoming clear that strict rules govern the inheritance of non-nuclear organelles, which require many accessory factors, such as cytoskeletal proteins, motor proteins and specific linker proteins. Certainly, in the case of autonomously multiplying organelles, a suite of proteins are required for organellar fission to maintain their numbers (Okamoto and Shaw, 2005).

Peroxisomes turn out to be no exception to these rules. *S. cerevisiae* is an attractive model for studies of peroxisome partitioning because its polar bud growth necessitates extra care to make sure that each bud receives a full complement of organelles. Real-time imaging of yeast with peroxisomes labeled with CFP-PTS1 over several division cycles indicated that peroxisomes are equally distributed between mother and daughter cells (Hoepfner et al., 2001). During partitioning, part of the peroxisome population remains relatively statically associated with the cortex of the mother cell, whereas the rest moves in a more dynamic way towards and into the bud. Deleting the gene encoding the dynamin-like protein Vps1p (see below) results in a single, giant peroxisome per cell. Surprisingly, the single peroxisome is faithfully distributed over the next generations of cells, mimicking the precision of nuclear division. In common with the transport of mitochondria, vacuoles and secretory vesicles along the actin cytoskeleton in yeast, the myosin motor Myo2p carries peroxisomes along actin cables into the bud.

Recently, a new participant in this process was described (Fagarasanu et al., 2005). Inp1p is a peroxisome-associated protein identified in a genome-wide screen assigning subcellular locations to yeast open reading frames (Huh et al., 2003). Deletion of *INP1* or its overexpression has dramatic effects on peroxisome partitioning. In its absence, almost all the peroxisomes move into the bud. However, upon its overexpression, they all remain in the mother cell close to the cortex. Overexpressed *INP1* is associated with both peroxisomes and the cortex. Fagarasanu et al. suggest that it tethers some of the peroxisomes to the mother cell cortex to immobilize them and prevent them from entering the bud. To leave some peroxisomes free to move to the bud, Inp1p levels must be critically controlled. Indeed, Fagarasanu et al. find that they fluctuate during the cell cycle.

It is clear that additional proteins that remain to be identified are involved in peroxisome partitioning. Cortical protein(s) must bind to Inp1p, for example, and a (membrane) protein probably anchors Myo2p to peroxisomes. The involvement of the actin cytoskeleton may be particular to *S. cerevisiae*. In mammalian cells, peroxisomes move along microtubules, propelled by dynein or kinesin motor proteins. Whether these are responsible for peroxisome partitioning during mammalian cell division is not yet clear.

Fission-like processes

To maintain peroxisomes, one can envisage that fission-like processes are required at two stages: (1) to uncouple peroxisomal pre-compartments from the ER; and (2) to vary the number of peroxisomes per cell depending on external conditions. Attempts to implicate COP (for ‘coat protein’) vesicle coat components in peroxisome biogenesis have failed

thus far (South et al., 2001; Voorn-Brouwer et al., 2001). However, recently, Emp24p, a COPII-like protein, was found to colocalize with relatively young peroxisomes (Marelli et al., 2004). This new observation should stimulate investigation of this matter with more direct assays that use the formation of 'dot-like' structures pinching off from the ER as a read-out. A consequence of involvement of the standard Sec trafficking machinery would be that the small budded vesicles produced need to coalesce into larger peroxisomes by homotypic fusion (Schekman, 2005).

Other candidates for proteins involved in peroxisome fission are dynamin-like proteins: Vps1p in yeast and DLP1 in mammals. Deleting the gene in yeast or silencing it in mammalian cells results in formation of elongated, tubular peroxisomes that have occasional constrictions (Hoepfner et al., 2001; Koch et al., 2003; Koch et al., 2004; Li and Gould, 2003), an indication that the last step in a fission process can no longer take place. Note that this phenotype suggests that Vps1p/DLP1 does not contribute to the severing step at the ER. Surprisingly, the single 'sausage-shaped' organelle in the yeast vps1Δ mutant faithfully partitions between mother and bud cell (Hoefner et al., 2001). Are there still other proteins that mediate fission or is this organelle mechanically torn in two by Myo2p motor-pulling forces? Although this last possibility would be less precise, it would pose no serious problem since cells can regenerate peroxisomes from the ER.

Both Vps1p and DLP1 are mostly cytosolic proteins, but DLP1 has been demonstrated to colocalize with peroxisomes (Koch et al., 2003; Koch et al., 2004; Li and Gould, 2003). How might the proteins be recruited to their respective targets? Koch et al. have made the remarkable observation that the mitochondrial fission protein Fis1p is involved (Koch et al., 2005). Fis1p is a single-membrane-span protein located in the outer membrane of mitochondria, where it interacts with DLP1. But Fis1p has now also been found in peroxisomes. In conjunction with DLP1, it thus appears to support the fission not only of mitochondria, but also of peroxisomes.

New insights into import of proteins into peroxisomes

Although peroxisomes no longer appear to be autonomous organelles, they can nevertheless import their matrix proteins. A property that sets this machinery apart from most of the other import systems is the capacity to import (partially) folded proteins. Genetic screens have identified many of the proteins involved but functional insight into its workings has been hampered by a lack of a reconstituted in vitro system. Recently, Azevedo and co-workers have made important progress in this direction, allowing us to probe the initial steps of the process in action. A key observation was that 15% of the total Pex5p (the PTS1 receptor) is present in the peroxisomal fraction and behaves as an integral membrane protein that is associated with other known members of the protein import complex (Gouveia et al., 2000; Reguenga et al., 2001). A protease-sensitivity assay has allowed Azevedo and co-workers to demonstrate conformational and/or topological changes in Pex5p related to its role in protein import. They have shown that entry of Pex5p into the membrane is cargo dependent but ATP independent, whereas return of Pex5p to the soluble phase does require ATP (Gouveia et al., 2003; Oliveira et al., 2003).

Azevedo and co-workers propose that cargo proteins are transported across the peroxisomal membrane by the PTS1 receptor itself (reviewed by Azevedo et al., 2004). To do this, Pex5p must behave both as a hydrophilic cytosolic protein and, temporarily, as a transmembrane protein. Erdmann and Schliebs have drawn an elegant analogy with pore-forming toxins (Erdmann and Schliebs, 2005). These also enter the membrane, self-associate and form a pore that mediates transport of various products, depending on the toxin involved. Erdmann and co-workers have reconstituted the Pex5p cycle in vitro, showing that return of Pex5p from the membrane into the soluble phase depends on Pex1p, Pex6p and Pex15p (Platta et al., 2005).

Pex15p (or Pex26 in mammalian cells) is an integral peroxisomal membrane protein that interacts with the soluble AAA ATPases Pex1p and Pex6p (Birschmann et al., 2003). Other proteins related to AAA ATPases include *N*-ethylmaleimide-sensitive fusion protein (NSF) and Cdc48/p97. NSF functions in *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE)-mediated membrane fusion, whereas Cdc48/p97 extracts proteins from the ER that are destined for ubiquitin-dependent degradation by the proteasome (Jarosch et al., 2002). This analogy between extraction of misfolded proteins from the ER and extraction of Pex5p from the peroxisomal membrane goes even further, because several groups have shown that Pex5p can be ubiquitylated (Platta et al., 2004; Kiel et al., 2005a; Kiel et al., 2005b; Kragt et al., 2005b). This might require Pex4p, a protein that resembles ubiquitin-conjugating enzymes. Pex4p binds to the membrane through Pex22p and the RING-finger membrane proteins Pex2p, Pex10p and Pex12p, which in turn resemble ubiquitin ligases (Platta et al., 2005). Both mono- and polyubiquitylation have been reported, and polyubiquitylated Pex5p is degraded by the proteasome. How and whether ubiquitylation is involved in the normal receptor-cycling process remains a matter for further investigation.

Household proteins involved in peroxisome formation and maintenance

The possible contributions of genes essential under all growth conditions to peroxisome biology can now be examined by approaches including DNA microarrays, two-hybrid studies, proteomics, mass spectrometry, subcellular location screens and large-scale co-immunoprecipitation of protein complexes. DNA microarray studies have allowed correlation of expression of genes with conditions affecting the performance of peroxisomes or the number of organelles per cell. In particular, genes encoding enzymes show dramatic changes in expression levels under these conditions, which is in contrast to the *PEX* genes (Koerkamp et al., 2002).

Proteomic analysis combined with mass spectrometry is also providing interesting clues. For instance, the complete protein composition of highly purified peroxisomes has been established (Kikuchi et al., 2004). An example of a newly discovered peroxisomal protein, whose location was validated by immunocytochemistry, is an ATP-dependent protease with an AAA domain, which is a member of the Lon family of proteins. Remarkably, proteins that are localized in other compartments have also been found, such as certain ER proteins and Rabs. It of course remains difficult to exclude the possibility that these proteins simply contaminated the purified peroxisome fraction. However, this problem has been

circumvented in the elegant approach developed by Marelli et al. (Marelli et al., 2004). They have enriched for peroxisomes by using density gradient centrifugation and followed the protein composition of the fractions along the gradient by mass spectrometry. Comparison with the profiles of known organelle-specific marker proteins provides leads to the identification of new proteins involved in peroxisome function(s). Again, proteins functionally linked to other compartments have been found such as the small GTPase Rho1 and the COPII-like Emp24p (Marelli et al., 2004). Moreover, the list of proteins serving multiple organelles includes the already mentioned dynamin-like protein Vps1p/DLP1, Myo2p and Fis1p. In this way, new players have appeared in the limelight, and some of these have been shown to be essential actors.

A whole new range of questions to answer

Here, we have highlighted several new developments in the peroxisome field, including advances in our understanding of their partitioning during cell division, the role of the PTS1 receptor in import of proteins into the organelle and the formation of new peroxisomes from the ER. In each of these areas, important and interesting questions remain.

The controlled and regulated partitioning of peroxisomes upon cell division depends on the concerted action of numerous proteins, of which only a few have been identified. Our knowledge is still descriptive and studies of the mechanistic aspects have not yet started. The implication of Fis1p in peroxisome fission opens the possibility to explore whether peroxisome fission and fusion processes are as intricate and exquisite as they seem to be in mitochondria. Similarly, studies of the reversible cycling of Pex5p from the cytosol, through a transmembrane state, back to the cytosol, with the possible involvement of ubiquitylation, provide a basis for further study of the mechanistic aspects, for the first time in a fully reconstituted in vitro system. One wonders whether the handicap that peroxisomes are leaky after isolation is perhaps due to Pex5p itself, which might leave the peroxisomal import machinery in an ‘open’ conformation when peroxisomes are taken from their cellular context.

The demonstration that peroxisomes derive from the ER topples the long-held opinion that they are autonomously multiplying organelles. It forces us to view their evolutionary origin differently but also allows us to formulate and address more direct and simple questions. A few peroxisomal membrane proteins, such as Pex3p, enter the ER, but how this is achieved is unknown. Previous attempts to implicate the Sec61p translocon in peroxisome biogenesis have failed but warrant renewed attention. How Pex3p sorts from the perinuclear ER and accumulates into a specialized domain of the ER, how ER-resident proteins are prevented from entering this compartment and how this pre-compartment is severed from the ER are questions for future study. Do small vesicles bud from the ER, driven by COPI, COPII or clathrin-like components, and subsequently coalesce into larger mature peroxisomes or are larger parts of the ER pinched off? New tools and improved morphological analysis using electron microscopy will allow us to reinvestigate these matters.

The peroxisome field has been enriched over the past few years with completely different lines of investigation. This might stimulate researchers from other fields to notice these fascinating organelles.

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