Peroxisomes: The current understanding

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Abstract

Peroxisomes are small extremely specialized organelles present in the cytoplasm of all eukaryotic cells. Peroxisomes originate from the endoplasmic reticulum and therefore are considered a specialized branch of the cell's secretory system. Peroxisomes are the host cellular compartment where many vital metabolic pathways take place, including: α and β-oxidation of fatty acids, ether lipid biosynthesis and peroxides detoxification. In the past 20 years there has been a significant growth in our understanding of the biology of eukaryotic peroxisomes. Research on peroxisomes biogenesis and function revealed that mutations in genes encoding four peroxisomes proteins are responsible for the development of severe genetic diseases known as Peroxisome Biogenesis Disorders. This review will cover the latest knowledge on peroxisome morphogenesis, assembly and cellular function with a special focus on the importance of peroxisome functions to normal human development and physiology. Moreover, the paper will discuss the prominent contributions that studies in model organisms have made to our current understanding of peroxisomes, with a special highlight on how research in *Drosophila* melanogaster has furthered our understanding of peroxisome dysfunction and how it leads to various disease states.

Introduction

Peroxisomes were firstly identified in 1960 by Christian DeDuve (De Duve and Baudhuin, 1966) who discovered a new group of organelles with sedimentation properties and protein content that differed from the other known cellular organelles. DeDuve named the neo-discovered organelles peroxisomes, because he observed the presence of hydrogen peroxide-generating oxidases in their matrix.

Since the discovery of DeDuvet, scientists have made large strides in our understanding of peroxisomes biology.

Peroxisomes are small organelles (0.1-1 μm) constituted of a *proteinaceous* matrix surrounded by a single membrane. Peroxisomes are present nearly in every eukaryotic cell. They originate from the Endoplasmic Reticulum (ER) by *de novo* synthesis, however mature peroxisomes can grow and divide (Tabak et al., 2013). The composition, number and size of peroxisomes can vary in different cell types and in the same cell according to its physiological status. The peroxisome matrix is host to multiple essential cellular pathways, including α-oxidation of branched fatty acids, β-oxidation of fatty acids, ether lipid biosynthesis and peroxide detoxification (Mast et al., 2010; Wanders and Waterham, 2006). Today we know that in mammals more than 50 proteins are involved in peroxisomal pathways and 34 of these are highly conserved proteins that localize exclusively in peroxisomes (Platta and Erdmann, 2007; Tower et al., 2011). To this group belong the Peroxins (Pex), a class of proteins involved in peroxisome biogenesis and maintenance (Shimozawa et al., 1999). Peroxisomal proteins are synthesized in the cytoplasm and transported to the peroxisomes through different mechanisms. One well-characterized mechanism targets peroxisomal proteins to the peroxisome via one

or two canonical targeting signals (PTS1 and PTS2). Proteins containing the tripeptide PTS1 signal in the carboxy-terminus of their sequences are bound by the Pex5 protein and translocate to the peroxisomal matrix by means not yet fully understood (Gould et al., 1987). Alternatively, proteins containing the characterized peptide canonic PTS2 signal at their amino-terminus are recognized by the Pex7 receptor protein in the cytoplasm and moved to peroxisomes (Swinkels et al., 1991). On the other hand, proteins present in the peroxisomal membrane are shuttled to the membrane by their interaction with Pex3 and/or Pex19 via a membrane protein-targeting signal (mPTS) (Fang et al., 2004). The identification of PTS signals in *Saccharomyces cerevisiae* has aided computer–based searches for orthologous proteins in mammals that also contain a PTS1 or a PTS2.

The identification of a large class of human genetic diseases known as peroxisome biogenesis disorders (PBDs) provided new insights into the biological functions of peroxisomes. In 1973, Sydney Goldfisher (Goldfisher 1973) reported the absence of peroxisomes in kidney and liver cells of patients affected by the so-called Zellweger syndrome (ZS), a syndrome previously observed in children to cause craniofacial dysmorphism and malformations in the brain, eyes, liver and kidney. This observation suggested peroxisomes might be the cause for the development of the disease. The connection became more evident later on, when it was discovered that ZS patients lack some Pex enzymes. PBDs are now classified as an heterogeneous group of autosomal recessive diseases that originate from mutations in genes responsible for peroxisome biogenesis and maintenance (Braverman et al., 2013). Of the 34 conserved Pex genes known to be involved in peroxisome biogenesis, 13 of them have been found mutated in different PBDs (Steinberg et al., 2004). Researchers encountered many difficulties in assigning a particular genetic complementation group to a defined PBD phenotype because different complementation groups share the same clinical phenotypes. Furthermore, most of the defects in enzymes involved in different steps of the peroxisome biogenic pathways, result in the loss of the organelles. Consequently, it remains unclear how the loss of peroxisomes cause the pathologies associated with PBDs. Recent evidence identified the accumulation of very long chain fatty acids and low levels in ether lipids as potential causes for the insurgence of the PBD state (Steinberg et al., 2006). Advances in sequencing technologies allow researchers to catalog and annotate a range of mutations in every PBD disorder. In addition, studies in model organisms have helped to identify and characterize peroxisomal genes (Titorenko and Rachubinski, 2001a, b) in the hope to associate specific genotypes with defined PBDs defects.

Despite advances in our understanding of peroxisome biogenesis and maintenance, many aspects of peroxisome biology and dysfunction are still unclear.
Although unicellular models such as yeasts and mammalian cells are valuable to identifying proteins central to peroxisome biogenesis, they do not appear to be suitable models to unravel the mechanisms that cause PBDs in humans. Therefore the use of multicellular organisms is necessary to advance our understanding on peroxisome biology and PBDs.

This review summarized the latest knowledge about the peroxisome biogenesis, structure and functions with a special focus on the organelle functions in humans and their defects linked to the insurgence of PBDs. Moreover, The paper reported the latest research progress from studies carried out in the well-established model system *Drosophila* melanogaster. The results obtained in these studies highlight the importance of this model to unravel the role of peroxisomes in the development of multicellular

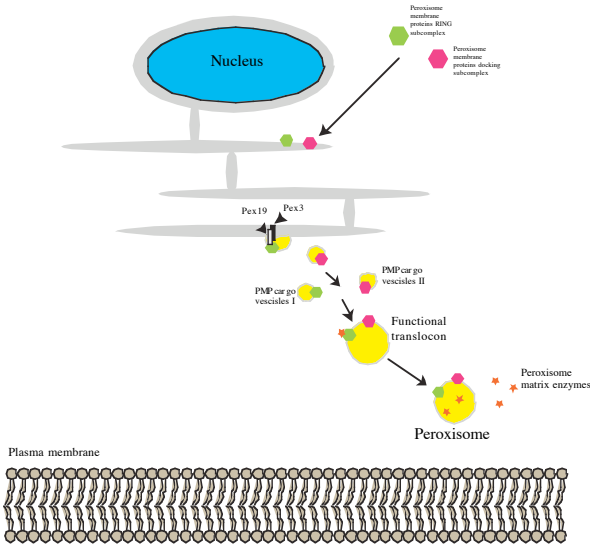

Fig.1- Diagram of the formation of new peroxisomes. Peroxisomes originates from the Endoplasmic reticulum. Peroxisomes membrane proteins are recruited to the ER and assemble in various subcomplexes at peroxisomal vesicles exit sites. Pex3 and Pex19 are responsible for the recruitment of the PMPs at the pre-peroxisome formation site. The neo-formed peroxisomal vesicles can be distinguished in subgroups based on their unique PMP cargo. A subgroup is thought to carry the docking complex for the import of peroxisomal enzymes (yellow) while the ubiquitin ligase complex is carried by a different vesicles pool (fuchsia and green).The vesicles bud off from the ER and heterotypically fuse leading to the assembly of the functional peroxisomal translocon. The assembly of the two different vesicle pools is essential to bring together all the components necessary for the import of matrix proteins. The import of the matrix proteins is essential to complete the maturation process that makes the peroxisomes metabolically active. Extracted and modified from (Tabak et al., 2013).

organisms and the molecular mechanisms leading to the PBDs.

Peroxisomes biogenesis

Many years elapsed between the discovery of peroxisomes and the first understanding of their biogenesis. This was due in part to discordances in data on peroxisome biogenesis between research groups. Only recently, was it apparent that peroxisomes are highly versatile organelles. It is this remarkably plastic nature of peroxisomes that accounted for the discordance in the findings reported by different studies, which were conducted in different cells and under different environmental pressures.

Most of the studies that elucidated the mechanisms of peroxisome biogenesis came from investigations in the yeast *Saccharomyces* cerevisiae and *Yarrowia* lipolytica. It is fairly well established now that peroxisomes originate from ER in every cell and organism (Hoepfner et al., 2005; Kim et al., 2006; Tabak et al., 2013; Tam et al., 2005; van der Zand et al., 2012). The proteins involved in the biogenetic process are a particular subfamily of the PEX genes called peroxisomal membrane protein (PMPs). In their microscopy studies in yeast cells, Hoepfner and his colleagues (Hoepfner et al., 2005) established that the PMP protein PEX3 accumulates at the perinuclear ER and supports the formation of vesicles that subsequently disconnect from the ER. The localization of Pex3 with the ER is not specific in yeast but it was also observed in mammals. It was then discovered that other PMPs (at least 15) travel from the ER to the neo-forming peroxisome-assembling site and start to specialize the organelle. Meanwhile, other peroxisomal proteins migrate from the cytoplasm to the ER (Hegde and Keenan, 2011) and then to the neo-forming peroxisome. (Schuldiner et al., 2008; van der Zand et al., 2012). Pex3 and Pex19 are responsible for the recruitment of the PMPs at the pre-peroxisome formation site. Pex3 interacts with Pex19; Pex19 recruits other PMPs binding to their mPTS sequence signals (membrane Peroxisomal targeting sequence) (Gotte et al., 1998; Rottensteiner et al., 2004). Therefore, PMPs are selected and inserted in the peroxisomal vesicles before they leave ER, through their mPTS, which works as a sorting signals and both Pex3 and Pex19 together are responsible for the insertion

of PMPs in the membrane and for the sorting/exit of the vesicles from ER (Tabak et al., 2013; Yonekawa et al., 2011). The PMPs that form the active translocation machinery (also known as the peroxisome traslocon) for the import of matrix enzymes into the peroxisome are sorted in the ER and target to two separated class of pre-peroxisomal vesicles (or carriers). One group of vesicles carries the PMPs of the so-called docking complex (Pex13, Pex14 and Pex17) that is essential for the binding of the proteins to be imported in the peroxisomal matrix (Fig.1). The second group of vesicles carries the PMPs of the RING (really interesting new gene) finger complex (PEX2, PEX10 and PEX12), that are ubiquitin ligase E3 like proteins involved in the recycling of the receptor delegate for the import of the peroxisomal enzymes (see below)(Kerppola, 2008; van der Zand et al., 2012). The vesicles belonging to each of the two diverse types will then fuse in the cytoplasm in order to bring the translocon parts together and postpone the import of enzymes until the final moment of maturation. Filling the matrix with enzymes finalizes the formation of metabolically active peroxisomes (Fig.1).

The heterotypic fusion strategy adopted by the cell could function in this way to protect the ER from the import of peroxisomal enzymes that could disturb the ER environment with misplaced enzymatic actions (Titorenko and Rachubinski, 2000, 2001a).

Peroxisome enzymes are synthesized in the cytoplasm and imported into the maturing vesicles (Elgersma et al., 1993; Erdmann et al., 1989; Tan et al., 1995; Tsukamoto et al., 1990). Newly synthesized enzymes get imported to the peroxisome through their target signals: PTS1 and PTS2 (Brocard and Hartig, 2006; Lazarow, 2006). Each of these signals is recognized by a specific receptor protein (or cargo), Pex5p for PTS1 and Pex7 (Marzioch et al., 1994), together with the co-chaperons Pex18p/Pex21p (Purdue et al., 1998), bind to PTS2 (Brocard et al., 1994). The cargo-loaded protein contacts the docking complex of the translocon (Pex13p, Pex14p and Pex17p). How the cargo is carried through the membrane and how the receptor is recycled remains unclear. The most accepted model supports the theory that the cargo proteins can form with the co-chaperons a transient pore that allows the passage of the transported protein. The transient pore is then disassembled by ubiquitination of the cargo by the Ring finger complex (Gouveia et al., 2003). How the cargo receptors are deubiquitinated and recycled, is still an open question. However, *in vivo* and *in vitro* studies in the rat liver have demonstrated that Pex5 (Gouveia et al., 2000), is monoubiquitinated by Pex12 in its cysteine residue, and it is polyubiquitinated by Pex2 and Pex10 at the lysine residual (Platta et al., 2009; Platta et al., 2007). The proteins AAA atpases Pex1 and Pex6 take then care of the dislocation of Pex5 out the membrane. A similar mechanism has been reported for the cargo duo Pex7/Pex18 (Grunau et al., 2009; Schafer et al., 2004) but is not as clear as for Pex5 transport (Fig.2).

To maintain the cellular peroxisome population, neo-synthesized peroxisome can derive by the ER biogenic route or also by fission

Mutations in a large number of proteins that have been linked to peroxisome fission affect peroxisome numbers and/or size. The proteins involved in the fission are divided in three categories the Pex11 group, the DRP group and the Pex30 group. The Pex11 group proteins are membrane proteins that coordinate peroxisome proliferation (Erdmann and Blobel, 1995; Li and Gould, 2002). In mammals there are 3 isoforms for Pex11. Pex11a expression is inducible and triggers peroxisomal proliferation, whereas Pex11b and Pex11g are constitutively expressed and regulate peroxisomal division (Koch and Brocard, 2012; Li et al., 2002; Schrader et al., 1998). In yeast *S.* cerevisiae, there is one Pex11p, but several other proteins with amino acid similarities, as Pex25p and Pex 27p, and involved in peroxisome fission at the ER (Huber et al., 2012; Tam et al., 2003).

The other group of proteins is a dynamin related family. Dynamins are soluble proteins involved in such processes as clathrin- and caveolae-

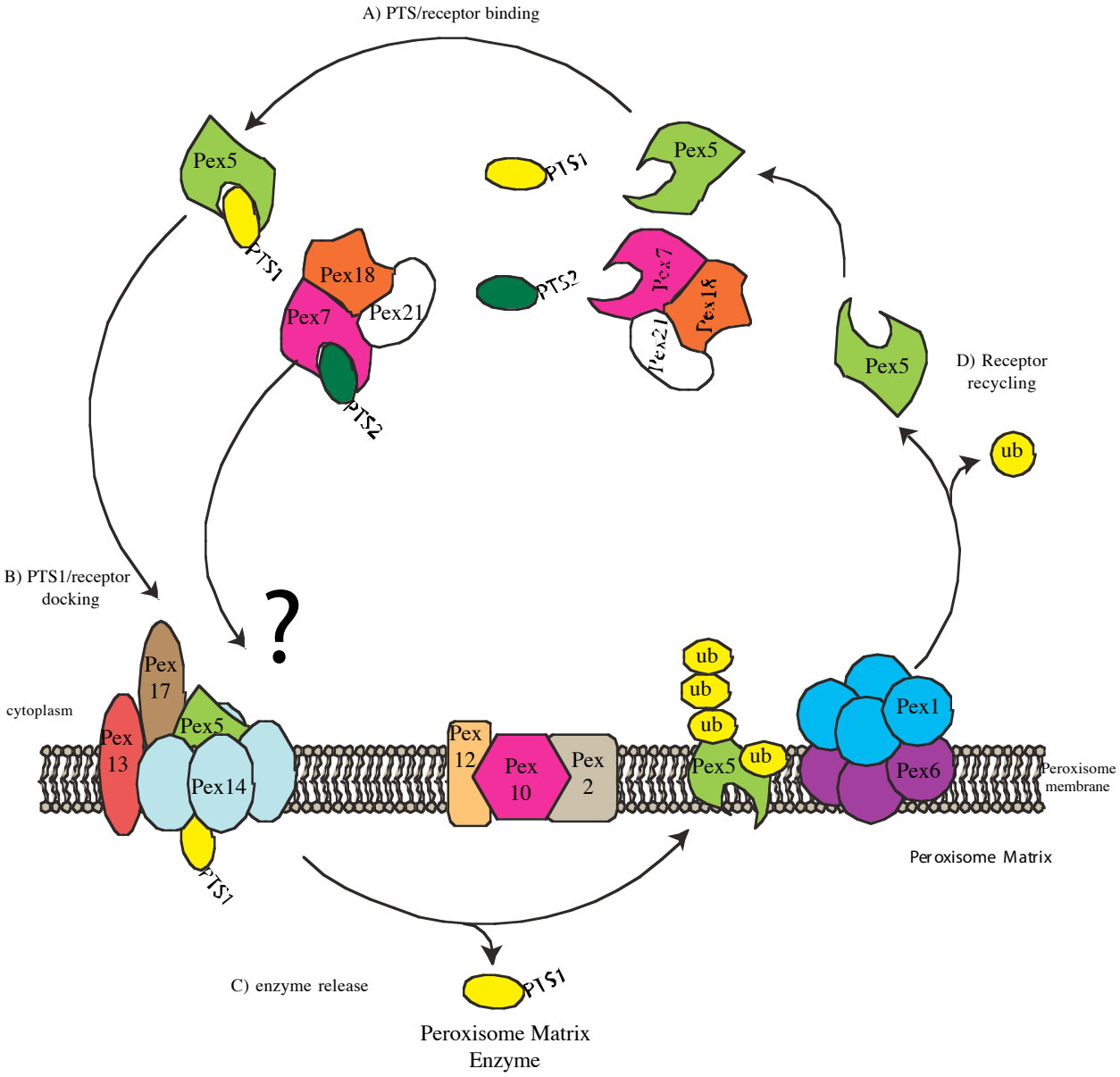

Fig.2-Model of peroxisome matrix protein PTS-import. The protein Pex5 is the cytosolic receptor that transport the peroxisome target signal 1 (PTS1) peroxisomal proteins into the peroxisome matrix. A) Pex5 binds to the PTS1 on the target protein in the cytoplasm. B) the complex target-receptor dpcks at the peroxisome membrane by interacting with Pex13, Pex14 and Pex17. Pex5 and Pex14 together form a dynamic membrane pore that allows the transport of the complex ligand/receptor into the peroxisome matrix. C) Pex5 is monoubiquitinated by Pex12 in its cysteine residue, and it is polyubiquitinated by Pex2 and Pex10 at the lysine residual in order to promote its recycling. D) The complex Pex1 and Pex6 push the receptor out of the membrane and the cycle restarts. Pex7 together with the co-chaperons Pex18p/Pex21p bind to PTS2 . The cargo-loaded protein contacts the docking complex of the translocon (Pex13p, Pex14p and Pex17p). How the cargo is carried through the membrane and how the receptor is recycled remains unclear. Extracted and modified from (Braverman et al., 2013).

-mediated endocytosis (Hinshaw, 2000). In yeast, defects in the DRP proteins Vps1p and Dnm1p severely affect peroxisome number and size (Kuravi et al., 2006).

The Pex30 PMP protein family members (Vizeacoumar et al., 2004) have been seen interacting with a complex of the ER reticulon group (Vizeacoumar et al., 2006). Reticulon proteins support tubulation of the ER (Voeltz et al., 2006), and the interaction with Pex30 indicates that there must be contacts between the ER subdomain and peroxisomes. Consequently, this model speculates that there are two locations at which proteins affect the size and/or number of peroxisome: the ER and the peroxisomal syncytium, which forms upon fusion of ER-derived vesicles.

Many evidences support the hypothesis that peroxisomes originate preferentially through fission in response to fast changes in physiological conditions that demand a rapid alteration of peroxisome number or before cell division.

Upon cell division peroxisomes need to be portioned among the daughter cells. The mechanisms of peroxisomes inheritance have been mainly dissected in yeast. At each round of cell division, peroxisomes follow a defined sequence of events that results in their equitable distribution between daughter and mother cells at cytokinesis. In not dividing cells, peroxisomes are scattered over the entire cortex. As soon as cell division starts, peroxisomes start detaching from their original cell position and travel towards the nascent budding site. The recruitment of peroxisomes from the mother cell cortex to the bud continues until an equal number of peroxisome in the mother cell and the bud is approximately equal. Peroxisome division that is needed to maintain peroxisome number at cell division occurs only for fission and it is strictly coordinated with the cell cycle. Rachubinski and his group largely contributed to dissect the mechanisms of peroxisome-dynamics during cell cycle in yeast. This topic is beyond the aim of this review and I refer the reader to another font (Fagarasanu et al., 2010).

Peroxisomal biochemistry

Peroxisomes are involved in a variety of metabolic pathways that require the presence of a large number of proteins (more than 50 in mammals). Most enzymatic activities are unique to peroxisomes but some are shared with other organelles like mitochondria and cytosol (Ferdinandusse et al., 2000; Islinger et al., 2009; Luo et al., 2008). Most of the catabolic and anabolic synthetic pathways that occur in peroxisomes are involved in intermediary lipid metabolism, like the β-oxidation and the α-oxidation of Very long chain fatty acids (VLCFA) and the synthesis of ether-phospholipids. The other main peroxisomal reactions reported in mammals are amino acid catabolism, glyoxylate metabolism, polyamine oxidation and metabolism of reactive oxygen and nitrogen species (Fig.3). Importantly, biochemistry reactions that occur in peroxisomes in one cell type it might not be peroxisome-specific in different cell types, thus recalling the concept that peroxisomes are highly versatile organelles. For example, the well-characterized fatty acids (FAs) β-oxidation occurs only in peroxisome in yeasts and plants, while in mammals occurs in peroxisomes and mitochondria.

The FAs β-oxidation reaction in peroxisomes, in contrast to reaction in the mitochondrial, metabolizes only VLCFas (>C22). This reaction is usually not completed in peroxisomes but serves to produce shorter FAs (C16) that are then transported to the mitochondria where the reaction is completed.

VLCFAs destined for b-oxidation in peroxisomes enter the reaction as acyl coA esters, since the only FAs that can enter directly into the β-oxidation are the saturated un-branched and 2-metyl-branched FAs. Other FAs such as the mono-and polyunsaturated FAs, 3-methyl,

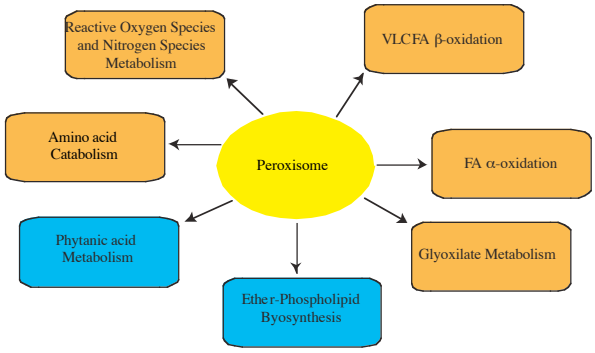

Fig.3-Overview depicting the main peroxisomal pathways. In the orange box are indicated the reactions that take place in peroxisomes of all eukaryotic cells. In the blue box are reported the peroxisomal pathways that have been described only in mammalian cells to date.

branched FAs and 2-hydroxy FAs need to undergo remodeling before they become substrates for peroxisomal β-oxidation. FAs with methyl–groups at the carbon 3 position like phytanic acid, a dietary very long chain branched FAs, must first undergo α-oxidation to produce the corresponding FAs with the methyl group in position 2 before they can go through b-oxidation. FAs α-oxidation is confined to peroxisomes and only accepts acyl-CoA esters as substrates. The phytanic acid needs to be converted to phytanoil-CoA by a Very long chain acyl-CoA synthetase enzyme (Watkins et al., 1996). Through a series of reactions that occur in the lumen of the peroxisome phytanoil-CoA is then converted to pristanic acid (Wanders et al., 2003), which is finally catabolized through β-oxidation.

Although the β-oxidation is considered a catabolic pathway, the peroxisomal β-oxidationin mammals, is also required for anabolic pathways, such as the production of poly-saturated omega-3 fatty acids, critical for CNS (central nervous system) function and primary bile acids (Kase et al., 1985; Su et al., 2001). Moreover, peroxisome β-oxidation also contributes to the regulation of complex lipids like pro-inflammatory molecules (Wanders and Waterham, 2006).

During the oxidation of fatty acids, the first reaction is performed by an oxidase that produces reactive oxygen species (hydrogen peroxide) that are then catabolized by the peroxisome catalase. Indeed, one of peroxisome's main metabolic pathways includes the metabolism of oxygen, reactive oxygen species (ROS) and reactive nitrogen species.
Peroxisomes have a number of oxidases that reduce oxygen to hydrogen peroxide. (Schrader and Fahimi, 2004) The hydrogen peroxide produced by this reduction can be disposed by three different classes of enzymes in the peroxisome: catalases, glutathione peroxidases and peroxiredoxin V (PMP20) (Fig.3).
Peroxisomes also produce superoxide anions. Superoxide anions are converted by superoxide dismutase (SOD). Copper/Zinc-SOD (Keller et al., 1991) and Manganese-SOD have also been found in peroxisomes. Recent evidence shows that peroxisomes contain inducible nitric oxide (NO) synthase that would be a source of peroxynitrite species, highly reactive species that are catabolized by peroxideredoxin V in peroxisomes (Dubuisson et al., 2004).

Peroxisomes are the site for the biosynthesis of ether-phospholipids (plasmanyl-phospholipids and plasmenyl-phospholipids or plasmalogens). The physiological role of the ether-phospholipids is unclear but they seem to be involved in membrane dynamics, cholesterol transport oxidative stress and FAs metabolism. The key step of the synthesis of ether-phospholipids required the enzymes Dihydroxyacetone phosphate Acyltransferase (DHAPAT), Acyl dihydroxyacetone phosphate synthase and Alkyl dihydroxyacetone phosphate synthase (ADHAPS) which are well-established peroxisomal enzymes (Wanders and Waterham, 2006). Deficiencies of DHAPAT and Alkyl-DHAP synthase in humans are associated with severe clinical abnormalities and early death, indicating that ether-phospholipids are essential for early development.
In humans, peroxisomes are the compartment also for the glyoxylate metabolism. The enzyme

alanine:glyoxylate aminotransferase (AGT) is exclusively expressed in liver peroxisomes and converts glyoxylate generated in peroxisomes into glycine using alanine as a primary amino group donor. This prevents the conversion of glyoxylate into the toxic metabolite oxalate, which can be catalyzed by various dehydrogenases and oxidases including lactate dehydrogenase in peroxisomes (Baumgart et al., 1996).

The continual identification of more peroxisomal proteins has further illuminated the metabolic function of peroxisomes. In particular, these discoveries revealed that enzymatic reactions once thought to occur in the cytoplasm actually are peroxisomal functions. An example of these is the catabolism of amino acids. Mammalian peroxisomes contain D-amino acid oxidase, which oxidase the D-isomers of neutral and basic amino acids, and D-aspartate (Van Veldhoven et al., 1991) oxidase that oxidase acidic amino acids.

For a more detailed analysis of the biochemistry of peroxisomes, I refer the reader to a more specific font (Wanders and Waterham, 2006).

Peroxisomal Biogenesis Disorders

The occurrence of defects in peroxisomal biogenetic pathways results in the insurgence of a group of inherited diseases in humans known as the peroxisome biogenesis disorders (PBDs) (Wanders et al., 1987a; Wanders et al., 1987b).
PBDs are autosomic recessive diseases caused by mutations in one or more genes involved in peroxisome biogenesis and maintenance. Approximately 1 in 50,000 children are born every year with defects in genes that block the formation and function of peroxisomes. These mutations can result in the total absence of peroxisomes or the impairment of metabolic pathways in peroxisomes. The absence of peroxisomes cause a large spectrum of metabolic disorders due to the accumulation of metabolites normally channeled by peroxisomes e.g. VLCFAs, phytanic acid, DHCA (dihydroxycholestanoic acid), THCA (trihydroxycholestanoic acid), or to a shortage of peroxisomal products e.g. plasmalogens, cholic and chenodeoxycholic acid, or to the presence of toxic products e.g. reactive oxygen species and glyoxylate.
PDBs represent a very heterogeneous group of severe autosomal recessive diseases, including the Zellweger syndrome (Zs), rhizomelic condrodysplasia punctate (RCDP), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD). The spectra of the phenotypes span from metabolic defects to developmental abnormalities that produce distinct dysmorphic features.
In general, most patients with PBDs present neural malformations, craniofacial dysmorphisms, visual defects, growth and mental retardation.
Since the disorders are today recognized as a clinical spectrum the PDBs are therefore classified as Zellweger spectrum disorders.
One of the most severe examples of Zellweger spectrum disorders is the cerebro-hepato-renal syndrome, Zellwenger syndrome (ZS). ZS patients are characterized by the complete absence of peroxisomes. The biochemical defect detected in ZS patients is linked to failure in VLCFAs β-oxidation since the patients present accumulations in Fas. Patients present multiple problems as muscular hypotonia, visual defects (as glaucoma and optic atrophy), sensorineural deafness, characteristic craniofacial dysmorphism, seizers, liver dysfunctions, renal cysts and brain developmental defects, ultimately resulting in death in early childhood.
The Zellweger spectrum diseases can develop in consequence of mutations in 14 different PEX genes (Wanders and Waterham, 2005; Weller et al., 2003) in different complementation groups. Mutation in Pex1 enzyme accounts for 60% of the Zs spectrum diseases.
The Rhizomelic chondrodysplasia punctate (RCDP) is another ZS disease where the symptoms have been attributed mainly to mutations in the peroxin Pex7. Pex7 defects

impair PTS2 protein import thereby causing distinct RCDP phenotypes, but not affecting the number or morphology of peroxisomes.
Mutations in peroxisomal enzymes required for plasmalogen synthesis (glyceronephosphate acyltransfarase and/or akylglycerone phosphate synthase) have also been found in RCDP patients. RCDP patients, indeed do not show defects in the β-oxidation of fatty acids as ZS patients, but present defects in plasmalogen synthesis and/or α–oxidation of fatty acids, as demonstrated by the accumulation of phytanic acid over time. RCDP patients present symptoms in the neonatal period. The main aspects of the disease are a peculiar skeletal dysplasia, heart diseases, renal malformations, seizure, profound psychomotor delay and growth failures. The neural defects in RCDP are different from those present in ZS patients. In ZS patients, there are defects in neural migration where RCDP present a reduced number of neurons and cerebellar degeneration is observed.

In addition to the ZS diseases that are caused by alterations in multiple peroxins, there are other peroxisomes disorders caused by mutation in a single peroxisome enzyme. These single-enzyme mutation pathologies are more difficult to classify because every peroxisomal enzyme deficiency is associated with several phenotypes. Remarkably, defects in enzymes within the same metabolic pathway may result in different phenotypes, therefore the diseases have been subdivided depending from which peroxisomal function is impaired (Table 1).
In the past 20 years, enormous progresses have been made in understanding the biology of peroxisomes and to dissect the molecular defects that lead to the insurgence of PBDs. The use of mouse model for PBDs has already given great contribution to the study of the disease pathologies. Furthermore, pharmacological therapies for PDB patients are today under development. For instance, 4-phenylbutyrate and related compounds activate peroxisome proliferation in cell line from ZS patients (Wei et al., 2000).

The increase in peroxisome number in the cells seems to raise the absolute level of functionality of peroxisome per cell thus the development of more compounds that can induce human peroxisome proliferation, it might be beneficial to a number of PBD patients. For more information on the pharmacological interventions for PBD, I direct the reader to a recent review on the topic (Braverman et al., 2013).

Peroxisomes Studies in model systems
The last decade has been remarkably important in the understanding of peroxisome biogenesis and its disorders. Studies in model systems have been essential in the identification of peroxisomal genes, the dissection of peroxisomes biogenesis and functions and predominately to understand the connections between PBDs and metabolic cellular defects.
Many peroxisome genes (PEX) have been identified and very well characterized at cell biology levels in the yeast *S*. cerevisiae. Despite the contribution that yeast has given in understanding the peroxisome biochemistry and cell biology (Fagarasanu et al., 2010), we still have much to learn particularly about the peroxisomal dysfunctions that cause the insurgence of the severe PBDs. The yeast system still stands as a great model to continue to identify novel peroxisomal specific enzymes and characterize their cellular functions, however, the model organism presents some limits. For instance, most peroxisomal enzymes in yeast fulfill functions that in mammals are performed by multifunctional proteins. The use of mammalian cell lines has helped to overcome this problem nevertheless they are limited in utility to identify the effects of these gene products at a system level. Hence, many studies aimed to understand the peroxisome functions and dysfunctions during development have been

Groups	Peroxisomal disorder	Mutant gene	Clinical signs
Group 1 (Peroxisomes biogenesis disorders)	Zellweger syndrome, Neonatal Adrenoleukodystrophy, Infantile Refsum disease	*Pex1, 2, 3, 5, 6,10,12,13, 14, 16,19,26*	Craniofacial dysmorphism, Seizure, Hypotonia, Neural migration disorders, Liver dysfunction, Calcium oxalate renal stones, Cataracts, Retinal abnormalities, Sensorineural deafness, Psychomotor retardation, Peripheral Neuropathy.
	Rhizomelic chondrodysplasia punctate (RCDP) type 1	*Pex7*	Disproportional short stature, Facial abnormalities, Ocular aberrations, Mental retardation, Chondrodysplasia punctate, Decreased neurons number, Congenital heart disease, Renal malformation.
Group 2 (single peroxisomal enzyme deficiencies)	Refsum Disease	*PAHX/PHYH*	Loss of vision, Cerebellar ataxia, Cardiac problems
	Rhizomelic chondrodysplasia punctate (RCDP) type 2	*GNPAT*	Mental retardation, Severe growth defects, Early death
	Rhizomelic chondrodysplasia punctate (RCDP) type 3	*AGPS*	Mental retardation, Severe growth defects, Early death
	Acyl-CoA oxidase deficiency (ACOX1 deficiency)	*ACOX1*	Hypotonia, seizures, deafness, retinopathy, neurological abnomalities
	Acatalasaemia	*CAT*	Increased tendency to develop oral gangrene
	Hyperoxaluria type 1	*AGXT*	Progressive loss of kidney function
	X-linked adrenoleykodystrophy (X-ALD)	*ABCD1*	Development is normal until six years of age, followed by rapid deterioration and death

Table.1-Genetics and clinical feature of peroxisomes disorders in humans

carried out in mice. The increased use of mouse models for peroxisome research is crucial to clarify the pathophysiologies connected to peroxisome defects and to help develop experimental treatments for PBDs (Weller et al., 2003).

Despite progressed achieved in this systems, the mouse is a very expensive and risky model organism to employ for the study of a specific protein in a multicellular organism. Therefore, the necessity to conduct studies in a lower risk multicellular model is warranted. The genetic model organism *Drosophila* melanogaster is emerging as a promising alternative multicellular model in peroxismomes research. *Drosophila* demands low research costs and offers a unique availability of sophisticated array for genetics and molecular tools for analysis of genes function.

In this last section of the review, I will report an overview of the recent contributions of *Drosophila* to our understanding of peroxisomal biology.

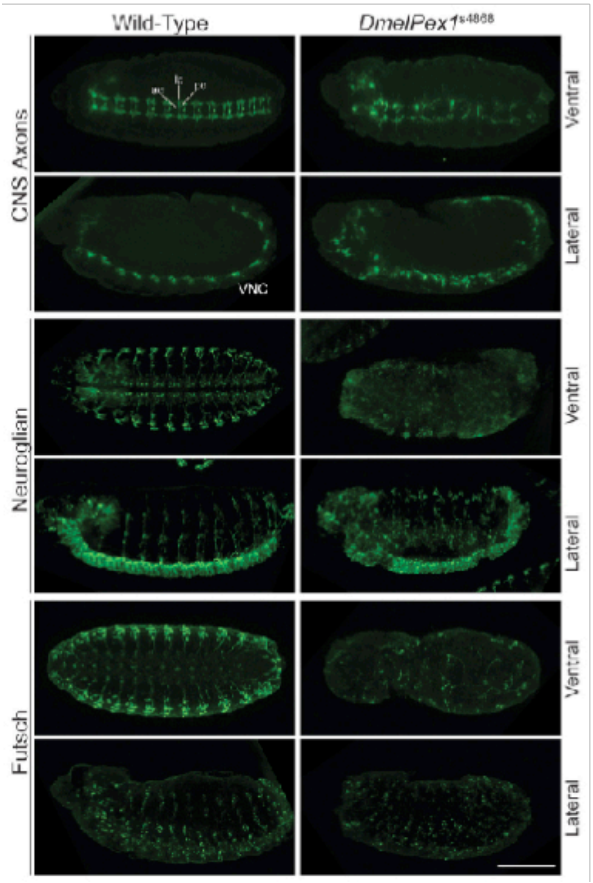

Fig.4- The developmental pattern of CNS and PNS is altered in *Pex1* homozygous mutant embryos. Wild-type and *Pex1* homozygous embryos (stage 15 of Drosophila embryos development) were analyzed by immunofluorescent microscopy using monoclonal antibodies BP102 (anti-CNS axons), BP104 (anti-Neuroglian; recognizing CNS and PNS neurons), and 22C10 (anti-Futsch, recognizing neuron and axon subsets of the CNS and PNS). Anterior is to the right in all the images. In lateral views, dorsal is up for BP102 and 22C10 and down for BP104, ac, anterior commissure, lc, longitudinal connective; pc, posterior commissure; VNC, ventral nerve cord. Scale bar represents 100 mm. (Extracted from (Mast et al., 2011)

Drosophila as model system for Peroxisomal Biogenesis diseases studies

The fruit fly, *Drosophila* melanogaster, is a complex multi-cellular organism that has given outstanding contributions in genetics research for the past 100 years.

Over 75% of human genes have homologs identified in flies and therefore *Drosophila* melanogaster has been widely used as model system for the study of many human diseases including neurodegenerative diseases, mitochondrial diseases and metabolic syndromes (Arquier and Leopold, 2007; Bilen and Bonini, 2005; Sanchez-Martinez et al., 2006).

The first studies related to peroxisomes in fly can be dated back to the discovery of the gene *rosy*, involved in the biogenesis of eye pigment (Bridges, 1944). *rosy* encodes for a xanthine dehydrogenase enzyme that localizes to peroxisomes of malpighian tubules (equivalent of the human kidney) and gut cells (Beard and Holtzman, 1987).

Surprisingly, until recently, very few studies about peroxisomes had been carried out in *Drosophila* and even less focused on the effect of peroxisome function on early development.

Studies of human peroxisomes biogenesis disorders in *Drosophila* have been only recently undertaken.

Lately, advances in sequences technologies have allowed the identification of 15 predicted peroxins genes in *Drosophila* genome and 13 homologs of known human Pex genes. Moreover, different research groups have shown that flies carrying mutations in peroxins genes manifest some of the PDB clinical phenotypes including early death, VLCFA accumulation, neural and locomotors defects.

In 2011, Mast and colleagues presented one of the first detailed studies of the effect of peroxisomes dysfunction on development (Mast et al., 2011). The group dissected the effects of mutation in the homolog of the peroxin *Pex1* in fly on *Drosophila* embryonic development. *Pex1* homozygous mutant embryos exhibited growth defects and premature death at larval stage (equivalent of human childhood). Locomotor defects in developing *Drosophila* were also observed, modeling some of the abnormalities observed in ZS patients. The authors presented a detailed analysis of the central and peripheral nervous system in *Pex1* mutants aimed to understand the reason of the locomotor defects in mutants and also to investigate whether the mutant embryos showed neural aberrations as reported in PBD patients. Immunofluorescence studies, using antibodies against CNS axons (BP1020) and against CNS and peripheral nervous system (PNS) neurons (anti-Neuroglian and anti-Futsch) in embryos showed malformations of the ventral nerve cord (VNC), breaks in connective of the VNC, disruption, disorganization, loss and hypoplasia of PNS and VNC neurons and/or neuritis (Fig. 4). Further analysis of the glial cells, PNS-glia-support cells and midline glia showed dramatic lost of glia organization in mutant embryos compared to wild type. All together their data demonstrated the essential role for *Pex1* in the development of fly nervous system and assessed the validity of *Drosophila* as model for studying systemic effects of PBDs on the growth and development of multicellular organisms (Mast et al., 2011).

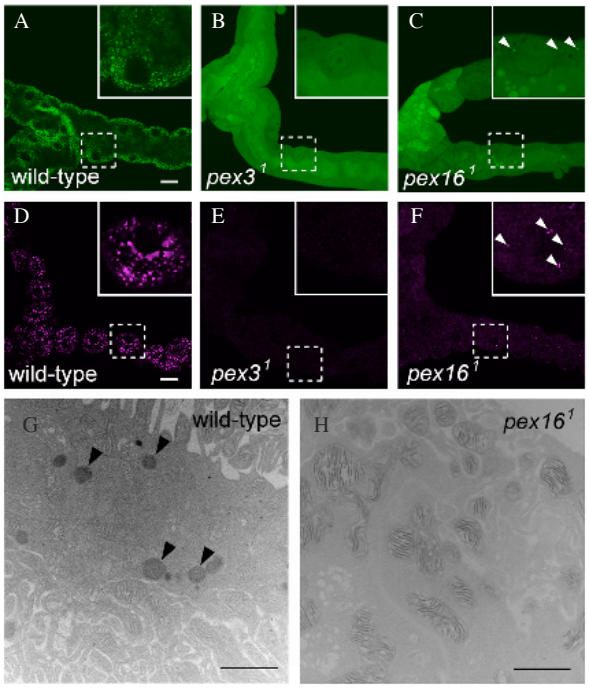

Fig.5-*Pex3* and *Pex16* homozygous mutants lack normal peroxisomes. (A-F) Peroxisomes detected *in vivo.* Malpighian tubules dissected from wild-type larvae (A, D) *Pex3* homozygous mutant (B, E) and *Pex16* homozygous mutant (C, F), ubiquitously expressing a chimeric enhanced green fluorescent protein (EGFP) that presents a C-terminal PTS1 signal and an enhanced cyan fluorescent protein (ECFP) with a peroxisomal membrane targeted signals to detect peroxisomes granules. Scale bars in A and D represents 10 mm. (G-H) Electron microscopy imaging of adult malpighian tubule sections stained with 3,3'-Diaminobenzidine (DAB) in wild-type (G) and *Pex16* homozygous mutant flies. Arrowheads in G indicated peroxisomes. Scale bar in G is 1 μm. (Extracted from (Nakayama et al., 2011)

In the same year (2011), Nakayama and colleagues (Nakayama et al., 2011) analyzed the phenotypes of flies respectively mutant for the homologs of human peroxins *Pex3* and *Pex16*. Pex3 and Pex16 are reported together with Pex19 to be essential proteins for *de novo* biogenesis of peroxisomes (Tabak et al., 2013). Mutations in both *Pex3* and *Pex6* have been found in ZS patients. The group reported that *Drosophila* carrying *Pex3* or *Pex16* mutations present resembling ZS syndrome symptoms. Homozygous mutant for *Pex3* showed developmental arrest and die at larval stage (corresponding of childhood death in humans) while *Pex16* mutants can reach the adult stage, although the adults are smaller (growth defects) and live shorter (reduced lifespan) compared to wild type animals.

Analysis aimed to verify the effect of lack of Pex3 or Pex16 on peroxisomes biogenesis were then carried out *in vivo* in animal tissue. The group expressed a chimeric enhanced green fluorescent protein (EGFP) that presents a C-terminal PTS1 signal and an enhanced cyan fluorescent protein (ECFP) with a peroxisomal membrane targeted signals to detect peroxisomes granules. When expressed in malpighian tubules cells (*Drosophila* equivalent of human kidney) of wild type animals, the fusion proteins localized to peroxisomes as it was evident from the presence of a punctate stain that resembles peroxisome granules staining (Fig. 5A, D). On the contrary, when the tagged proteins were expressed in malpighian tubules cells of *Pex3* homozygous animals, the peroxisomes granules staining was absent, indicating that *Pex3* mutants are not able to form peroxisomes (Fig. 5 B, E), as reported in cells of ZS patients with mutation in *Pex3*. Surprisingly, when the tagged proteins were expressed in malpighian tubules cells of homozygous mutants for *Pex16*, few large peroxisomal like-granules staining were observed (Fig. 5 C, F). This suggested that few peroxisomes are still formed in *Pex16* mutants in *Drosophila* (Fig.5 G-H) (Nakayama et al., 2011). These same phenotypes were observed in yeast *Yarrowia* lipolytic *pex16p* mutants(Eitzen et al., 1997). However, Pex3 and Pex16 are known to be both essential for the presence of peroxisomes in human cells corroborating the hypothesis that the requirement of Pex16 in peroxisomes biosynthesis is different in different species (Honsho et al., 1998; Shimozawa et al., 2000). On the other hand, *Pex16* homozygous mutant in *Drosophila* shared other common defects with the PBDs patients that present *Pex16* mutations. A characteristic observed in PBDs patients is the accumulation of VLCFAs in plasma. Pex16 homozygous animals presented 2-10 folds greater VLCFAs contents in their hemolymph (corresponding human plasma), thus the fatty acid metabolism is altered in *Pex16* flies mutant. Moreover *Pex16* homozygous adults showed locomotors defects and defects in the development of neuron system. Interestingly, the neural defects did not become worst with age, harboring the idea that they are consequence of a developmental disorder. Another phenotype of the *Pex16* mutants was male sterility caused by the absence of development of the early spermatocytes (early stage male gametes) in mature elongated germ cells. This characteristic is not observed in PBDs patients because their lives end before they reach the sexual maturation, however the germ cells developmental arrest phenotype observed in *Pex16* mutants is consistent with those previously observed in other *Drosophila Pex* mutants. For instance, the work reported by Chen and colleagues demonstrated that the peroxins genes *Pex2* and *Pex10* (involved in peroxisome proteins import in mammals and yeast) are essential for male fertility in *Drosophila.* Homozygous mutants for *Pex2* or *Pex10* alone present defects in spermatocytes growth and cytokinesins. The phenotype seems dependent by VLCFA accumulation. In fact, the authors demonstrated that the mutant for *Pex2* and *Pex10* accumulated high levels of VLCFA and treatments that reduce VLCFA levels in mutant flies can rescue the fertility defects. The accumulation of VLFAC is indeed one of the main

causes of developmental defects in *Pex* mutants in fly, as in human PDBs patients (Chen et al., 2010).

Recently, McNew laboratory have published an inventory of predicted peroxisome proteins and pathways in *Drosophila* (Faust et al., 2012). The group presents a genome wide analysis obtained by bioinformatics studies aimed to identify peroxisomal proteins in the fly genomes. All the predicted peroxisomal proteins were clustered in pathways and compared with the corresponding homologous in other organisms. Parts of the results obtained by bioinformatics analysis have been confirmed *in vivo* by fluorescent confocal microscopy in fly cells. This study provides a platform to many other investigators for future studies on peroxisomes in fly.

The results achieved in the reported peroxisome studies in *Drosophila* have confirmed the fruit fly as a suitable model for further investigations of peroxisomes biology and of the molecular consequences of peroxisome lost and dysfunctions at system level. However, although fundamental molecular pathways are highly conserved between fly and humans, anatomic divergence between the fruit fly and humans is apparent, which may be not sufficient to recapitulate some morphological features of peroxisomes diseases. Therefore, one has to bear in mind that the differences between mammals and invertebrates represent potential drawbacks in modeling diseases.

Conclusions

The progress made over the past years in understanding the biochemistry and biogenesis of peroxisomes is largely owed to the contributions of peroxisome research performed in yeast. The recognition that Zellweger spectrum disorders derive from peroxisome dysfunctions is a realization of the important contributions peroxisomes pathways provide at the organism level. Despites all these achievements, many of the fine details of the metabolism and biogenesis of peroxisomes remain to be unraveled. The isolation of more peroxisome-specific proteins is an important step to fill the gaps in our understanding of peroxisomes functions. Interestingly, peroxisome damage caused during isolation from cellular homogenates has impeded the development of *in vitro* studies of peroxisomes, which were crucial in the study of mitochondria, chloroplast and ER biogenesis. Therefore, the development of techniques that allow the examination of peroxisomal protein biochemical function *in vitro* (e.g. how proteins are imported into peroxisomes) would help define the mechanisms of the organelles biogenesis.

As reported previously, mutations in genes involved in peroxisome biogenesis and metabolites transport are accounted as the main cause for the developmental defects observed in PBDs. There is currently no cure for PBDs and the treatments only reduce the severity of the symptoms. Improvements in the understanding of peroxisome biogenesis are essential to our knowledge of the PBDs diseases and the development of a cure.

Screening in multicellular model organism as *Drosophila* melanogaster might help to fill the puzzle of peroxisome metabolic biogenesis functions and advance our understanding in the field.

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