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DISSERTATION

Titel der Dissertation

„ER-to-Peroxisome tethering and Membrane Rearrangement during Peroxisome Maintenance“

Verfasser

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If we knew what it was we were doing,
it would not be called research, would it?

Albert Einstein

ER-to-Peroxisome tethering and Membrane Rearrangement during Peroxisome Maintenance

For you…

Time to say Thank You…

The work I am presenting herein is a mere compilation of what I have contributed to during the last years. Although the breakthroughs, the highlights have always been the driving force that stimulated further commitment, unsuccessful experiments might comprise up to 95% of the total time spent. The remainder however, carried me through the bad times. Like never before, I learnt to cope with these hassles and to focus on the really important things – teamwork. In a team, one, two or even a load of disappointing events do not throw you off balance. In a team, the interesting world of science becomes even more fascinating. And only in a team, experimental results and published work makes sense because you know that there are others who will build upon it.

My very special thanks go to Cécile Brocard who supported me throughout my PhD despite difficult circumstances. I can frankly admit that I would not be the person I am, if I had not performed my PhD in her lab. In fact, she taught me how to manage my own projects independently, properly set up experiments, and importantly, interpret and present the data. She encouraged me to think in new ways and explore new terrains, simply to do it my way. Besides, I learned much on a personal level about motivation, enduring difficult situations and the importance of teamwork in the lab.

I want to thank Sophie Melchior, Christine David and Wolfgang Benetka, the "old" guys. We did not only spend time drinking coffee or complaining about non-working experiments, we also shared our private lives and became friends. Not to forget about the "younger" guys: Thomas Heil, Alexandra Lärnsack, Mathias Hochgerner. Their personalities breathed some fresh air into the lab and I very much enjoyed the time with them. Anja Huber, Veerle de Wever, Gisela Dechat and Kornelija Pranjic, colleagues from other labs, also made up our little community. Here, I like to explicitly mention Andreas Hartig. I will not forget our endless discussions about scientific and non-scientific issues and the helping hand he offered in countless instances. Also, Friedrich - Fritz - Kragler was always willing to help and open for discussions. I want to acknowledge our administrative staff, Erna Huber and Harald Nierlich as well as our "floor-mother" Silvia Tömö – always friendly and accurate. On top, there are many other people who made the 5th floor lively, too many to mention all their names. Thanks to all of you!

I am aware that science demands a lot of dedication and I did not spend as much time with family and friends as I liked. Thank you for your understanding and support and for sustaining my moans, especially my love Beatrice and my grandmother.

Although I am looking towards new experiences and a new chapter of life, I do not want to forget my time at the University. Science has gradually changed during the last years, not to its best, and I truly hope that it finds the way back to its origin. Science should be a pure quest for the truth, not corrupted by politics, nepotism or intrigues.

I cordially wish all my friends and colleagues the motto of the great visionary Steve Jobs *"Stay hungry, stay foolish"* – I certainly will…

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1 Peroxisomes – versatile organelles

1.1 The Microbody Family

The characteristic of eukaryotic in contrast to prokaryotic cells is their compartmentalization into membrane-bound substructures. These organelles segregate metabolic pathways to tailored microenvironments thereby optimizing metabolite processing and adding spatial control over metabolic functions.

In 1954, a new structure, the microbody, was described in mammalian cells as small, round-shaped, single membrane-bound organelle (Rhodin, 1954). It was only in 1966 that de Duve and Baudhuin carried out the biochemical characterization (De Duve and Baudhuin, 1966) and described the microbody as hydrogen peroxide generating and degrading organelle, which led to the name "Peroxisome".

In fact, peroxisomes are present in every eukaryotic cell, from lower single cell eukaryotes to multicellular organisms. Peroxisomes are generally involved in the metabolism of lipids and also participate in a variety of other metabolic pathways ranging from hormone production to amino acid biosynthesis. Peroxisomes are versatile organelles and adapt their size, shape, number and even their protein content depending on the organism and tissue, and according to environmental conditions (Veenhuis and van der Klei, 2002). Indeed, specialized peroxisomes exist, that harbor exclusive metabolic pathways and hence, these peroxisomes are often named differently based on their major metabolic function (Hayashi et al., 2000; Pracharoenwattana and Smith, 2008). In plants, some peroxisomes contain enzymes of the glyoxylate cycle and are called glyoxysomes (Hayashi et al., 2000). Trypanosomes break down glucose in specialized peroxisomes, which are then called glycosomes (Michels et al., 2006). Further, peroxisomes often perform unique functions: In the firefly *Photinus pyralis*, peroxisomes harbor the light-generating enzyme luciferase (Keller et al., 1987), in the yeast *Penicillium chrysogenum* they take part in the biosynthesis of penicillin (Meijer et al., 2010), and in filamentous fungi, the function of Woronin bodies, peroxisome-derived organelles, is important for sealing septal pores (Jedd and Chua, 2000).

Overall, metabolic pathways are seldom confined to one organelle. For instance, photorespiration requires chloroplasts, peroxisomes and mitochondria to collaborate. Hence, efficient communication and cooperation between organelles is required for metabolite exchange, acquisition of structural components or for adaptation to new conditions. Especially, the multi-facetted roles of peroxisomes demand connectivity and cross-talk with other organelles. Indeed,

peroxisomes strongly depend on the endoplasmic reticulum (ER) and they share proteins and metabolites with mitochondria to properly maintain the organellar network as well as a functional metabolism in the cell (Mullen and Trelease, 2006; Neuspiel et al., 2008; Schrader and Yoon, 2007; Theodoulou et al., 2011).

1.1.1 The metabolism of peroxisomes

Peroxisomes participate in a variety of metabolic pathways, some of which exist in all organisms, others represent species-specific functions. Metabolic functions include α- and β-oxidation, fatty acid elongation, saturation and δ_4-desaturation, glycerol catabolism, ether lipid and isoprenoid biosynthesis, polyamine breakdown, lysine metabolism, purine and nicotinamid-adenin-dinucleotide (NAD) salvage pathway and ketogenesis (Ashmarina et al., 1999; de Vet et al., 1998; Hayashi et al., 2000; Kovacs and Krisans, 2003; Mimouni et al., 1991; van den Bosch et al., 1992; Van Roermund et al., 1998; Wanders and Waterham, 2006a; Zaar et al., 1986). Strikingly, peroxisomes seldom harbor the complete set of enzymes of a certain metabolic pathway. For instance, while all steps of the β-oxidation occur in peroxisomes, only part of the biosynthesis of higher isoprenoids takes place in the peroxisomal matrix.

In contrast to other organelles, peroxisomes are capable of rapidly adjusting their protein content and adapt to new environments thereby influencing the entire cellular metabolism. The glycosomes of *Trypanosoma brucei*, which is living in the mammalian blood stream, contains almost exclusively glycolytic enzymes. This glycosomal population undergoes massive changes during differentiation into its parasite's form living in the insect midgut which no longer utilizes glucose as main carbon source (Herman et al., 2008). Also in animals, the peroxisomal population may differ in the various tissues with regard to their number and enzymatic content, e.g., in hepatocytes, peroxisomes contain the enzyme bile acid-coenzyme A: amino acid N-acetyltransferase which is cytosolic in fibroblasts from the same organism (Pellicoro et al., 2007). Overall, in whichever tissue or organism, lipid metabolism constitutes the main function of peroxisomes.

1.1.1.1 Peroxisomal lipid metabolism

Cells process a plethora of lipids which derive either from the diet or are specifically synthesized in the cell. Lipids from the diet or adipose tissue are first modified to set free the fatty

acids in a process which can differ substantially. While most fatty acids are saturated with an even number of carbon atoms (14 to 26), some contain an uneven number of carbon atoms, some are mono- or polyunsaturated and some are even branched. All these fatty acids are eventually oxidized into C2-units (acetyl-CoA), which can be used for either energy generation under various metabolic conditions (tricarboxylic acid cycle (TCA-cycle), ketogenesis) or as building block for macromolecules (e.g., isoprenoids, prostaglandins).

1.1.1.1.1 The peroxisomal lipid catabolism

The majority of fatty acids is catabolized in a process called β-oxidation (Figure 1). Here, every second carbon atom is sequentially oxidized and fused to coenzyme A yielding acetyl-CoA. In yeasts and plants, the β-oxidation takes place exclusively in peroxisomes, whereas in mammals, only very long chain fatty acids (VLCFA) including polyunsaturated dicarboxylic fatty acids are processed in peroxisomes (Nguyen et al., 2008; Wanders et al., 2010). Indeed, fatty acids containing up to 18 carbon atoms are oxidized in mitochondria in which the β-oxidation directly feeds the obtained energy equivalents into ATP generation.

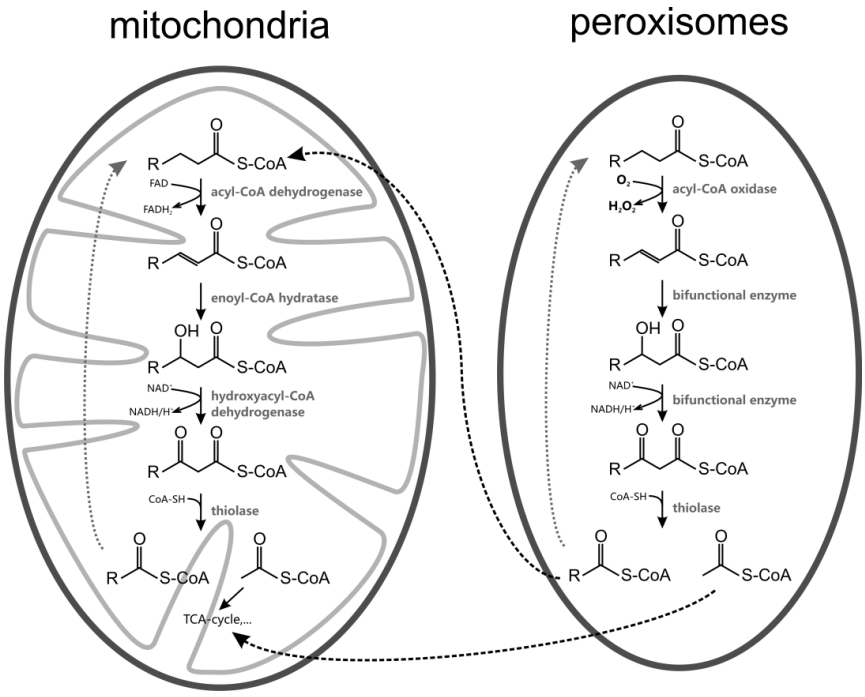

Figure 1: Comparison of the β-oxidation in peroxisomes and mitochondria in mammals. VLCFA are oxidized in peroxisomes identical to mitochondria except for the first oxidation. In mitochondria, the fatty acid is dehydrogenized using the cofactor FAD^+, which subsequently reduces the Coenzyme Q for ATP generation. In peroxisomes, the hydrogen atoms are eventually passed onto O_2 creating H_2O_2. This reactive oxygen species is degraded by catalase, thus this first peroxisomal oxidation results only in thermal energy. Once the fatty acids are shortened to about 18 carbon atoms, they escape the peroxisomal compartment and are actively transported via carnitine into mitochondria for further break-down. The peroxisomal acetyl-CoA is also used in the citric acid cycle (TCA cycle).

The major difference between mitochondrial and peroxisomal β-oxidation lies in the recycling of the oxidizing agents used. In mitochondria, the first oxidation is achieved with the help of the co-factor FAD^+, which recycles via feeding the hydrogen atoms to Coenzyme Q of the respiratory chain for ATP generation. The peroxisomal acyl-CoA oxidase also contains a flavin ring which however, is exposed to the solvent and transfers its hydrogen atoms onto elemental oxygen, O_2, creating hydrogen peroxide, H_2O_2 (Nakajima et al., 2002). To prevent damage induced by this reactive oxygen species, H_2O_2 is degraded by peroxisomal catalase (ROS, see chapter 1.1.1.2 and Figure 1). Thus, this first peroxisomal oxidation does not preserve energy. In mammals, shortened acyl-CoA molecules are then actively transported to mitochondria for further break-down.

Branched and *cis*-unsaturated fatty acids or those with uneven-numbered carbon atoms cannot be directly processed via β-oxidation and need additional enzymes or separate pathways to finally obtain molecules that can be fully oxidized. The proper decomposition of these fatty acids is not only a metabolic rationale to gain energy but is also vital to prevent the accumulation of substances or metabolites such as phytanic acid whose accumulation would be toxic (Wanders et al., 2010). Phytanic acid or phytol are prominent components of our diet as products of the chlorophyll metabolism. They are processed via α-oxidation in peroxisomes, which interestingly takes place in the cytosol in rodents (Figure 2, Singh et al., 1993).

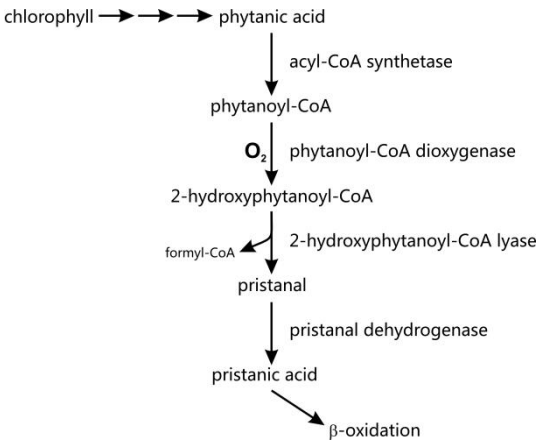

Figure 2: Peroxisomal α-oxidation. A schematic representation of the key steps in peroxisomal α-oxidation. Phytanic acid, a catabolite of chlorophyll, is methylated at the Cβ position, inhibiting direct oxidation at this atom. To process this fatty acid via β-oxidation, it is first oxidized at the Cα-atom using O_2 and shortened by one carbon atom. The obtained pristanic acid is now amenable for β-oxidation and further oxidized in peroxisomes and then in mitochondria.

The oxidation of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA is carried out using elemental oxygen, O_2. While phytanoyl-CoA is hydroxylated, 2-oxoglutarate accepts the other oxygen atom and is decarboxylated into succinate. Thus, unlike in the β-oxidation, this reaction produces CO_2, and not ROS (Jansen and Wanders, 2006; Wanders and Komen, 2007). Other enzymes involved in the peroxisomal lipid catabolism include the 2,4-dienoyl-CoA reductase, the 3,2-trans-enoyl-CoA isomerase which process unsaturated fatty acids prior to β-oxidation and the malonyl-CoA decarboxylase (Gurvitz et al., 1998; He et al., 1995; Sacksteder et al., 1999).

1.1.1.1.2 The peroxisomal lipid anabolism

The major products of peroxisome lipid anabolism are ether lipids, including plasmalogens. These lipids are generated *de novo* in peroxisomes from glycerol and acyl-CoA. Acyl-CoA is first coupled to dihydroxyacetonephoshpate (DHAP) via an ester bond by dihydroxyacetonephosphate acyl-transferase (DHAPAT). The fatty acid is then replaced with a long chain fatty alcohol by alkyldihydroxyacetonephosphate synthase (ADHAPS). Further steps include the reduction to 1-alkyl glycerol-3-phosphate, the transfer of a second fatty acid to obtain 1-alkyl-2-acyl-glycerol-3-phosphate, removal of the phosphate group and coupling to either ethanolamine or choline followed by a facultative desaturation (Hajra, 1995; Wanders, 2004b). Both, DHAPAT and ADHAPS are exclusively located in peroxisomes, while the other steps of the ether lipid biosynthesis are carried out in the cytosol and the ER.

Isoprenoids are also synthesized in peroxisomes. These anabolites are the precursors of a range of substances, such as cholesterol, sterol hormones, heme or bile acids. While isoprenoids can be synthesized completely in the ER, their biosynthesis can also be carried out peroxisomes up to farnesyl pyrophosphate (Kovacs and Krisans, 2003; Kovacs et al., 2002) and all further steps take place in the ER. Importantly, the conjugation of bile acids to taurine or glycine occurs exclusively in peroxisomes of mammalian hepatocytes (Ferdinandusse et al., 2009; He et al., 2003; Kase and Bjorkhem, 1989; Pellicoro et al., 2007).

Finally, the peroxisomal β-oxidation exerts also anabolic function as it constitutes a trimming mechanism in the formation of certain lipids. For instance, the fatty acid C22:6ω-3 is generated from linoleic acid via C24:6ω-3 using several elongation and desaturation steps. The final oxidation to obtain C22:6ω-3 is performed in peroxisomes (Sprecher et al., 1995).

1.1.1.2 Peroxisomal detoxification

Peroxisomes are often considered as cellular detoxifiers. Indeed, they contain manifold of enzymes that scavenge emerging reactive oxygen or nitrogen species (ROS, RNS). ROS typically contain either an oxygen radical as such, or a peroxide species which sporadically disintegrates homolytically yielding oxygen radicals. Singlet-oxygen is also a highly potent ROS. RNS encompass molecules of the NO_x type, most notably peroxynitrate $ONOO^-$, and (di)nitrogen dioxide, $^{\cdot}NO_2$ and N_2O_3. If set free, both ROS and RNS react with DNA, proteins and lipids thereby damaging them. Despite the efforts of the cells to repair or exchange harmed molecules, damage induced by ROS and RNS can accumulate and implications for cancer and ageing have been proposed (Benz and Yau, 2008; Dugan and Quick, 2005; Vigneron and Vousden, 2010; Waris and Ahsan, 2006; Ziech et al., 2011). However, under controlled conditions ROS and RNS or precursors thereof are often used as signaling molecules (Brune et al., 2003; D'Autreaux and Toledano, 2007; Kalyanaraman, 2004).

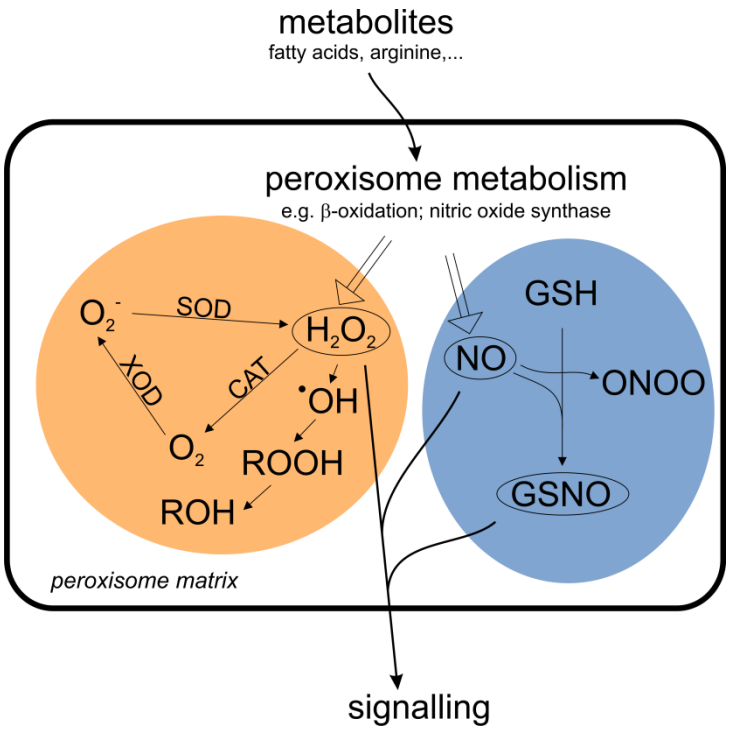

Figure 3: Peroxisomal *DE*toxification mechanisms – ROS and RNS. Peroxisomal metabolism is a source for ROS. Fatty acid oxidation creates H_2O_2 and xanthin oxidase (XOD) generates O_2^-. These species are degraded by dedicated enzymes, such as catalase (CAT) or superoxide dismutase (SOD). If H_2O_2 homeolytically disintegrates, hydroxyl radicals are generated that preferably react with other hydroxyl groups, again leading to peroxide species which can be enzymatically reduced. Alternatively, H_2O_2 or other ROS can react with nitric oxide, and build the very reactive peroxynitrate radical that destroys any kind of molecule including lipids and proteins. However, under normal conditions, NO and also its glutathion (GSH) derivative (GSNO) are used in signaling.

While peroxisomes contain ROS-scavenging enzymes such as catalase or the peroxiredoxins (e.g., PMP22), they are also major players in the generation of ROS and RNS (Figure 3, Angermuller et al., 2009; Antonenkov et al., 2009a; Bonekamp et al., 2009; Del Rio, 2011; Schonfeld et al., 2009; Schrader and Fahimi, 2004). The exact contribution of peroxisomes versus mitochondria with regard to redox balance remained unclear. However, a detailed study on the intraperoxisomal redox balance and the crosstalk with mitochondria shed new light on this topic (Ivashchenko et al., 2011). The authors showed by using redox-sensitive fluorescence marker proteins that the redox state inside peroxisomes is strongly influenced by the environment. Interestingly, mitochondria were strongly affected by ROS generated in peroxisomes of catalase-deficient cells. These findings place peroxisomes as ROS-signaling platform to a level equal to mitochondria. Indeed, correlations between production of peroxisomal ROS and ageing, inflammation and immune response have already been reported (Dixit et al., 2010; Koepke et al., 2007; Koepke et al., 2008; Terlecky et al., 2006; Titorenko and Terlecky, 2011).

Various other important ROS-unrelated detoxification processes occur in peroxisomes. Excessive retinal is removed by the dehydrogenase/ reductase SDR family member 4 present in peroxisomes in several organisms (Lei et al., 2003; Usami et al., 2003). Furthermore, localization studies on a thiol metalloendopeptidase, insulin degrading enzyme (IDE), showed its presence in peroxisomes (Kuo et al., 1994; Morita et al., 2000). This protein cleaves small molecules such as insulin or glucagon, but is also capable of degrading Aβ peptides, the accumulation of which is known as critical cause of Alzheimer's disease (Chesneau et al., 2000; Fernandez-Gamba et al., 2009; Qiu et al., 1998; Valera Mora et al., 2003). Indeed, in some mammalian organisms, peroxisomes were shown to protect against neurodegenerative diseases (Kou et al., 2011; Santos et al., 2005). Recently, the activity of IDE has been correlated to the cellular redox state outlining again the primordial role of peroxisomes as cellular detoxifiers (Cordes et al., 2011).

1.1.1.3 Peroxisomes in yeasts

Each *Saccharomyces cerevisiae* yeast cell typically contains 4-8 peroxisomes of 0.2-0.5µm diameter. In contrast to higher eukaryotes, yeasts do not entirely rely on the presence of peroxisomes. While peroxisomes are essential for plants and mammals, yeasts are able to grow without peroxisomes if supplemented with sugars as carbon source. This is due to the fact that the β-oxidation takes place exclusively in peroxisomes, and that yeasts do not rely on fatty acid oxidation when sugars are available. However, if grown on fatty acids as sole carbon source, such as oleic acid,

peroxisomes become essential which is also reflected by an increase in peroxisome number. Obviously, this makes yeasts an excellent model organism to study peroxisomes. Multiple yeast species have been used to analyze peroxisomal function and formation, including *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Pichia pastoris* (Erdmann et al., 1989; Liu et al., 1992; Veenhuis et al., 1979). While several metabolic functions are common to most species (e.g., β-oxidation of all fatty acids), some specialized functions are only present in the respective yeast species. For example, methanol oxidation takes place exclusively in peroxisomes in *P. pastoris* or *H. polymorpha* (van der Klei et al., 2006). Similar to the first oxidative step in the β-oxidation, peroxisomes use elemental oxygen to produce formaldehyde from methanol which is subsequently fully oxidized in the cytosol via formic acid.

A metabolic pathway common to all yeast species is the glyoxylate cycle. Herein, C4 carbohydrate molecules are synthesized from C2-units enabling yeast cells to grow on alkane carbon sources. The glyoxylate cycle is initially identical to the TCA-cycle. Then, instead of the decarboxylation of isocitrate, it is split by isocitrate lyase into succinate and glyoxylate, the latter being subsequently condensed with acetyl-CoA through malate-synthase yielding malate. Finally, malate is oxidized to oxaloacetate by malate-dehydrogenase. While important steps occur doubtlessly in the peroxisomal matrix, isocitrate lyase and aconitase are present in the cytosol in yeast (Kunze et al., 2006). Indeed, the inherently necessary transport of glyoxylate metabolites through the peroxisomal membrane has been established *in vitro* using electrophysiological measurements confirming that the enzymes of the glyoxylate cycle are not exclusively present in peroxisomes (Antonenkov et al., 2009b).

Filamentous fungi grow as syncytium providing rapid growth and fast signaling. However, upon lesion of the plasma membrane, the septal pore has to be plugged to prevent cell death. Woronin bodies, hexagonal structures related to peroxisomes, serve to seal these septal pores in the plasma membrane in response to wounding and possibly also function in growth and development (Jedd, 2011; Jedd and Chua, 2000).

1.1.1.4 Peroxisomes in plants

Peroxisomes are indispensable for plant development and growth. Previously classified as leaf peroxisomes, gerontosomes, glyoxysomes and unspecialized peroxisomes, the differences in matrix protein content are only subtle, and hence it was proposed to refer to the term peroxisome only (Pracharoenwattana and Smith, 2008). These peroxisomes perform a variety of metabolic

functions depending on tissue and developmental stage (Hayashi and Nishimura, 2006). Similar to yeasts, plant peroxisomes are the only site of β-oxidation for all fatty acids. Furthermore, important steps of the glyoxylate cycle (see section 1.1.1.3) take place in peroxisomes (Beevers, 1969; Eastmond and Graham, 2001).

Photorespiration requires the function of three organelles, chloroplasts, mitochondria and peroxisomes, to cope with relatively high O_2 concentrations. Herein, the enzyme RUBISCO adds oxygen instead of CO_2 to ribulose-1,5-bisphosphate which is cleaved into phosphoglycolate and 3-phosphoglycerate. While the latter can easily enter the Calvin-cycle and is converted back to ribulose-1,5-bisphosphate, phosphoglycolate shuttles to peroxisomes and subsequently mitochondria before it reenters the chloroplasts. In peroxisomes, glycolate becomes oxidized using O_2 to glyoxylate which is then transaminated to glycine. The latter is then transported to mitochondria and modified into serine which is afterwards converted into glycerate in peroxisomes and finally transported back into chloroplasts. The oxidation to obtain glyoxylate produces H_2O_2 thereby influences the redox state of peroxisomes which again, might play a role in signaling (Foyer et al., 2009).

Plant peroxisomes participate significantly in the biosynthesis of jasmonic acid and auxins, important hormones for plant growth, development and stress response (Delker et al., 2006; Vanneste and Friml, 2009). Furthermore, polyamine and urate degradation as well as sulfite oxidation were reported to take place in peroxisomes (Hansch and Mendel, 2005; Tavladoraki et al., 2006; Todd et al., 2006). Finally, peroxisomal functions in photomorphogenesis and plant-pathogen defense have also been shown (Bednarek et al., 2009; Hu et al., 2002; Lipka et al., 2005).

1.1.1.5 Peroxisomes in mammals

Mammalian peroxisomes feature many metabolic pathways previously described including etherlipid biosynthesis (section 1.1.1.1.2), detoxification (section 1.1.1.2) and polyamine breakdown (Seiler, 2004). However, some differences exist: Peroxisomes are responsible for the β-oxidation of only VLCFA (see section 1.1.1.1.1) and they lack the glyoxylate cycle. Although absent in humans, rodent peroxisomes contain urate oxidase to convert uric acid to allantoin (Motojima et al., 1988; Wu et al., 1989). Furthermore, glycolysis and related reactions are strictly cytosolic. Thus, the main metabolic contribution of peroxisomes lies in lipid metabolism and ROS homeostasis.

1.2 Peroxisomal Diseases

An organelle that exerts such a plethora of metabolic functions is prone to be involved in diseases due to mutations in genes essential for i) a single pathway or ii) the formation of the whole organelle (Fidaleo, 2010; Gould and Valle, 2000; Steinberg et al., 2006; Wanders, 2004a; Wanders, 2004b; Wanders and Komen, 2007; Wanders and Waterham, 2005; Wanders and Waterham, 2006b; Wei et al., 2000; Wierzbicki, 2007). Indeed, over 20 diseases have been correlated with peroxisomal dysfunction (Table 1).

Disease	**Protein involved**	**Molecular pathway affected**
single enzyme deficiencies		
X-linked adrenoleukodystrophy	ALDP	β-oxidation
Acyl-CoA oxidase deficiency	ACOX	β-oxidation
D-bifunctional protein deficiency	DBP	β-oxidation
2-Methylacyl-CoA racemase deficiency	AMACR	β-oxidation
Sterol carrier protein X deficiency	SCPx	β-oxidation
Rhizomelic chondrodysplasia punctata type II	DHAPAT	ether phospholipid biosynthesis
Rhizomelic chondrodysplasia punctata type III	ADHAPS	ether phospholipid biosynthesis
Acatalasaemia	CAT	H_2O_2-katabolism
Hyperoxaluria type I	AGT	glyoxylate detoxification
Refsum disease	PHYH/PAHX	α-oxidation
Amyotrophic lateral sclerosis ALS type I	SOD1	ROS detoxification
Malonic aciduria	MLYCD	odd-chain fatty acid oxidation
Sjorgen-Larsson syndrome	ALDH3A2	α-oxidation
Xanthinuria type I/ II	XDH	purine salvage pathway
Glutaric aciduria type III	GAO	amino acid catabolism
peroxisome biogenesis disorders (PBDs)		
Zellweger syndrome *	PEX1/2/3/5/6/10/12/13/14/16/19/26	peroxisome formation/ protein import
Neonatal adrenoleukodystrophy *	PEX1/5/6/10/12/13/26	peroxisome formation/ protein import
Infantile Refsum disease *	PEX1/2/6/12/26	matrix protein import
Rhizomelic chondrodysplasis punctata type I	PEX7	PTS2-dependent matrix protein import

Table 1: Overview of selected peroxisomal diseases. Single enzyme deficiencies and peroxisome biogenesis disorders are listed together with the protein and the molecular pathway affected. Note that different mutations in the same or different proteins can result in the same disease. * Zellweger spectrum diseases

1.2.1 Single enzyme deficiencies

Most peroxisomal disorders are recessive and inheritable. They originate from mutations in single genes coding for enzymes involved in one of the peroxisomal metabolic activities (Table 1). Onset and progression vary, but usually the phenotypes become visible in early childhood. As indicated in Table 1, patients fail to perform mostly α- or β-oxidation properly, thus accumulating phytanic acid, VLCFA or metabolites thereof in various tissues. Phenotypes vary greatly, and include neurological abnormalities, growth and developmental retardation, retinopathies and liver pathologies. The expected lifespan lies between early child- to adulthood, depending on severeness and time of diagnosis.

The most prominent disease is X-linked adrenoleukodystrophy (X-ALD, 1:20,000 in caucasian population, Bezman et al., 2001). Patients carry a mutation in the ABCD1 transporter protein which is involved in VLCFA transport across the peroxisomal membrane, and are thus unable to perform β-oxidation properly (Fourcade et al., 2009; Hettema and Tabak, 2000; Kemp et al., 2011). VLCFA are thought to accumulate in the membranes of brain and kidney and are adversely affecting their function (Khan et al., 2010). Currently six phenotypic variants have been described, the two prevalent being childhood cerebral ALD and adrenomyeloneuropathy (Wanders, 2004b).

While in ALD a catabolic process is impaired, in rhizomelic chondrodysplasia punctata (RCDP), the biosynthesis of ether lipids is dysfunctional due to mutations in either DHAPAT (type II) or ADHAPS (type III). Usually, plasmalogens make up 30% of the lipid content in the heart muscle, 20% in the brain and 70% in myelin sheaths (Farooqui and Horrocks, 2001). Thus, RCDP patients display demyelinated nervous tissue resulting in severe mental retardation, dwarfism, disproportional growth and spasticity.

Only a limited range of therapies are amenable to slow down progression of the peroxisomal single enzyme deficiencies. Besides medication against secondary effects provoked by the diseases and dietary therapies, mostly bone marrow or organ transplantation is an effective treatment for some diseases such as X-ALD (Hitomi et al., 2003). The addition of certain lipids (Lorenzo's oil) to balance the body's lipid metabolism slowed down progression in many cases but since random studies and controls are missing this strategy is controversially discussed (Moser et al., 2007; Shapiro et al., 2000). Basically, only gene therapy could provide permanent cure for these diseases. Recently, Patrick Aubourg and colleagues reported a breakthrough in the case of X-ALD by using allogeneic hematopoietic cell transplantation (Cartier et al., 2009).

1.2.2 Peroxisome biogenesis disorders and Peroxins

With a remarkable high incidence rate of 1 in 50,000, individuals display phenotypes similar to the diseases described previously (section 1.2.1). However, no metabolic peroxisomal enzyme is involved. Rather, peroxisomes as such are not assembled properly. Here, proteins are mutated that are responsible for the formation of peroxisomes. Hence, a malfunction in these important mechanisms leads to mislocalization of peroxisomal proteins and thus to massive metabolic pathologies.

These diseases are called peroxisome biogenesis disorders (PBD) and encompass the Zellweger spectrum diseases (Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease) as well as RCDP type I (see Table 1, Fidaleo, 2010; Gould and Valle, 2000; Steinberg et al., 2006; Wanders and Waterham, 2005).

Zellweger patients display severe cerebrohepatorenal symptoms, low muscle tone, facial abnormalities and eye defects and usually die within the first years of age. The milder Zellweger spectrum diseases show less grave phenotypes and some motor-functional development. Similar to patients suffering from RCDP, these individuals might live a few years or even reach early adulthood. However, therapies are limited to dietary supplementation and eventually organ transplantation or gene therapy.

Screening for mutants reflecting these PBDs in model organisms have been performed to identify the factors involved in the formation of peroxisomes (Elgersma et al., 1993; Erdmann et al., 1989; Gould et al., 1992; Liu et al., 1992; Nuttley et al., 1993; Subramani, 2002; Titorenko et al., 1993; Tsukamoto et al., 1990). These proteins are called peroxins (PEX) encoded by the PEX genes and execute vital functions in peroxisome proliferation and protein import (Distel et al., 1996). Today, over 30 peroxins are known (Table 2, (Kiel et al., 2006; Vizeacoumar et al., 2004). Not all of them are present in every organism and the molecular function of most peroxins is still a matter of debate.

A deeper understanding of how peroxisomes are formed and maintained, and of how peroxins contribute on a molecular level is essential for proper diagnosis of PBDs at early age and effective therapies.

Gene	Functional orthologs	Identified in			Function in biogenesis	Characteristics	Molecular function	first described in
		Sc	*Yl*	*Hs*				
PEX1		+	+	+	Matrix protein import	AAA-type ATPase	ATP-dependent dislocation of Pex5	(Erdmann et al., 1991)
PEX2		+	+	+	Matrix protein import	RING-finger		(Erdmann and Kunau, 1992)
PEX3		+	+	+	PMP-targeting; *de novo* formation		Membrane anchor of Pex19	(Höhfeld et al., 1992)
PEX4		+	-	-	Matrix protein import	Ubc	Mono-ubiquitination of Pex5	(Wiebel and Kunau, 1992)
PEX5		+	+	+	Matrix protein import	WxxxF-motifs; TPR; ubiquitinated	PTS1-receptor	(Van der Leij et al., 1992)
PEX6		+	+	+	Matrix protein import	AAA-type ATPase	ATP-dependent dislocation of Pex5	(Voorn-Brouwer et al., 1993)
PEX7		+	+	+	Matrix protein import	WD40- domain	PTS2-receptor	(Marzioch et al., 1994)
PEX8		+	+	-	Matrix protein import	coiled-coil domain, leu-zipper	Connection of docking- and RING- complex; cargo release (?)	(Waterham et al., 1994)
PEX9		*Eliminated, wrong ORF*			Matrix protein import		*ORF of YlPex9 misidentified; corresponds to Pex26*	
PEX10		+	+	+	Matrix protein import	RING-finger		(Erdmann and Kunau, 1992)
PEX11	PEX25/27**?**	+	+	+	Proliferation		**Elongation of peroxisomes**	(Erdmann and Blobel, 1995)
PEX12		+	+	+	Matrix protein import	RING-finger		(Kalish et al., 1996)
PEX13		+	-	+	Matrix protein import	SH3- domain	Member of docking complex	(Erdmann and Blobel, 1996)
PEX14		+	+	+	Matrix protein import	PxxP- motif, phosphorylated	Member of docking complex	(Komori et al., 1997)
PEX15	PEX26	+	-	-	Matrix protein import	Phosphorylated	Membrane anchor of Pex6	(Elgersma et al., 1997)
PEX16		-	+	+	PMP-targeting; *de novo* formation			(Honsho et al., 1998)
PEX17		+	-	-	Matrix protein import		Member of docking complex	(Huhse et al., 1998)
PEX18	PEX20	+	-	-	Matrix protein import	WxxxF-motifs, ubiquitinated	PTS2-co-receptor in *Sc*	(Purdue et al., 1998)
PEX19		+	+	+	PMP-targeting; *de novo* formation	CAAX-box, farnesylated	PMP class I receptor and chaperone	(Kammerer et al., 1997)
PEX20	PEX18/PEX21	-	+	-	Matrix protein import	WxxxF-motifs, ubiquitinated	PTS2-co-receptor in most fungi	(Titorenko et al., 1998)
PEX21	PEX20	+	-	-	Matrix protein import	WxxxF-motifs, ubiquitinated (?)	PTS2-co-receptor in *Sc*	(Purdue et al., 1998)
PEX22		+	-	-	Matrix protein import		Membrane anchor of Pex4	(Koller et al., 1999)
PEX23	PEX30/31/32	-	+	-	Proliferation	DysF	Growth regulation in *Yl*	(Brown et al., 2000)
PEX24	PEX28/29	-	+	-	Proliferation		Separation of peroxisomes in *Yl*	(Tam and Rachubinski, 2002)
PEX25	PEX11	+	-	-	Proliferation		Elongation of peroxisomes	(Smith et al., 2002)
PEX26	PEX15	+	-	+	Matrix protein import		Membrane anchor of Pex6 in *Hs*	(Matsumoto et al., 2003)
PEX27	PEX11	+	-	-	Proliferation		Elongation of peroxisomes	(Rottensteiner et al., 2003)
PEX28	PEX24	+	-	-	Proliferation		Separation of peroxisomes in *Sc*	(Vizeacoumar et al., 2003)
PEX29	PEX24	+	-	-	Proliferation		Separation of peroxisomes in *Sc*	(Vizeacoumar et al., 2003)
PEX30	PEX23	+	-	-	Proliferation	DysF	Growth regulation in *Sc*; **ER tethering ?**	(Vizeacoumar et al., 2004)
PEX31	PEX23	+	-	-	Proliferation	DysF	Growth regulation in *Sc*	(Vizeacoumar et al., 2004)
PEX32	PEX23	+	-	-	Proliferation	DysF	Growth regulation in *Sc*	(Vizeacoumar et al., 2004)
PEX33	PEX17 **?**	-	-	-	Matrix protein import		Member of docking complex in *N. crassa*	(Managadze et al., 2010)
PEX34		+	-	-	Proliferation		link to fission machinery **?**	(Tower et al., 2011)

Table 2: The Peroxins. Functions investigated in this study are marked in red. AAA: ATPase associated with diverse cellular activities; CAAX-box: farnesylation motif; DysF: Dysferlin domain; PXXP: class II SH3 interacting motif; RING: really interesting new gene; SH3: Src homology 3; TPR: tetratricopeptide repeat; Ubc: ubiquitin-conjugating enzyme; WD40: 40 amino acid long domain containing conserved Trp-Asp; adapted from Koch J., 2008, Diploma thesis, "Functional Analysis of Protein of the PEX11-Family in Human Cells and their Role in Peroxisome Proliferation".

2 Formation of Peroxisomes

Peroxisome number and function are maintained throughout cellular life. The life cycle of a peroxisome can be conceptually structured into membrane and matrix protein import, proliferation, inheritance and degradation. The proteins involved are called peroxins (Table 2). Peroxisomes multiply by growth and division and can be generated *de novo* from the ER. Matrix and membrane proteins are continuously replenished to maintain the peroxisome pool in a mature functional state.

2.1 Import of matrix and membrane proteins

Peroxisomes do not contain DNA and thus every protein has to be imported or transported to the peroxisomal membrane and matrix. Some peroxisomal proteins are synthesized on free polyribosomes and thus peroxisomes were believed to import every protein posttranslationally (Fujiki and Lazarow, 1985; Fujiki et al., 1984; Lazarow et al., 1982; Rachubinski et al., 1984). Most peroxins are involved in matrix protein import and a peroxisomal protein import complex has been characterized that is called importomer (Brown and Baker, 2008; Lanyon-Hogg et al., 2010; Ma and Subramani, 2009; Rucktaschel et al., 2010). For membrane proteins, import pathways remain dubious and no detailed import mechanism has been established, so far (Brown and Baker, 2003; Fujiki et al., 2006).

2.1.1 Matrix protein import

The import of matrix proteins is one of the best studied processes within the field of peroxisome research. Figure 4 provides an overview of the current knowledge. Proteins destined for the peroxisomal matrix carry one of two peroxisomal targeting signals, PTS1 or PTS2, which are specifically recognized in the cytosol by receptor proteins, PEX5 and PEX7, respectively (Brocard et al., 1994; McCollum et al., 1993; Rehling et al., 1996; Zhang and Lazarow, 1995). The PTS1, which is the targeting signal of the majority of peroxisomal matrix proteins, has originally been described as the consensus tripeptide (S/A/C)-(K/R/H)-(L/M) at the extreme C-terminus of the cargo protein (Gould et al., 1989). However, detailed studies revealed that the region upstream of this tripeptide is also crucial for protein sorting, as it contributes significantly to the interaction with the tetratricopeptide region (TPR) of the receptor protein, PEX5 (Brocard and Hartig, 2006; Brocard et al.,

1994; McCollum et al., 1993; Neuberger et al., 2004; Neuberger et al., 2003a; Neuberger et al., 2003b).

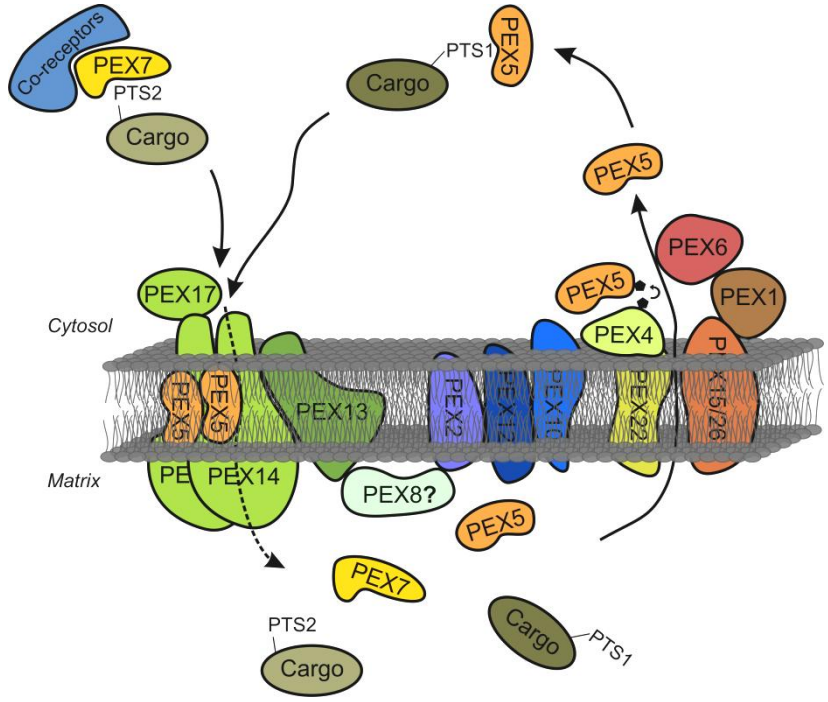

Figure 4: Peroxisomal matrix protein import. The cargo protein is recognized by a cytosolic receptor protein either by its PTS1 signal (PEX5) or via the PTS2 signal (PEX7). PEX5 interacts with the importomer (PEX13/14) and is transported together with the cargo into the peroxisomal matrix, where the cargo is released. The PEX7-PTS2-cargo complex needs additional coreceptors to be transported. PEX5 is exported back into the cytoplasm involving the RING finger complex (PEX2, PEX10, PEX12, (Chang et al., 1999; Leon et al., 2006) as well as PEX1 and PEX6 for another round of import (monoubiquitinylation via PEX4). Alternatively, PEX5 is polyubiquitinylated and marked for degradation (not shown).

The cargo-receptor complex is docked onto the peroxisomal import machinery, a multi-enzyme complex comprising PEX13 and PEX14, and translocated into the peroxisomal matrix (Albertini et al., 1997; Brocard et al., 1997; Dammai and Subramani, 2001; Komori et al., 1997). PTS2-PEX7 complexes need to interact with PEX5 in order to bind to the import machinery (Stein et al., 2002). Here, species-specific co-receptors exist, such as Pex18p, Pex21p in *S.cerevisiae*, or a long splice variant of PEX5, PEX5L, in mammals (Einwachter et al., 2001; Stein et al., 2002). In plant, PEX7 can directly interact with PEX5 (Nito et al., 2002). Upon release of the cargo, the receptor is shuttled back into the cytosol also involving the RING-finger proteins PEX2, PEX10 and PEX12 (Dammai and Subramani, 2001; Dodt and Gould, 1996; Nair et al., 2004). Here, ubiquitination is essential for the release of the receptor PEX5 from the membrane and also plays a decisive role in the fate of PEX5 (Platta et al., 2007). For recycling, PEX5 is monoubiquinated and released in the cytosol (Alencastre

et al., 2009; Kragt et al., 2005b; Platta et al., 2009; Williams et al., 2008). Upon polyubiquitination, PEX5 is marked for degradation and broken down by the proteasome (Kiel et al., 2005; Leon and Subramani, 2007).

Already a long time ago it was shown that peroxisomes are capable of importing fully folded, even oligomeric proteins (McNew and Goodman, 1994). Indeed, Walton et al. showed that large protein complexes such as IgGs genetically engineered to contain a PTS1 and even PTS-coated gold particles of up to 9nm in diameter were able to enter peroxisomes (Walton et al., 1995). This amazing property of the peroxisomal translocation machinery explained some hitherto unsolved problems; for instance, catalase can be imported as fully folded protein already loaded with its cofactor. Hence, de- and refolding mechanisms including chaperones and a separate import or biosynthesis of the cofactor are not required. Additionally, a PTS-independent mode of import via piggyback translocation was reported allowing the import of protein complexes also comprised of proteins without a PTS (Islinger et al., 2009). However, unlike the nuclear pore complex, peroxisomes do not leak higher molecular weight matrix content into the cytosol (Antonenkov et al., 2005; Antonenkov et al., 2004; Verleur and Wanders, 1993; Wolvetang et al., 1990), and thus the translocation pore must be tightly sealed, only transiently open for the import and then accurately adapt to its cargo. It has not been fully clarified which peroxins are involved in the pore formation, however, electrophysiological measurements on reconstituted proteoliposomes with membrane protein complexes isolated from various yeast strains point to the involvement of the receptor PEX5 together with PEX14 (Meinecke et al., 2010).

2.1.2 Membrane protein import

The import of peroxisomal membrane proteins (PMPs) has not been completely characterized, so far. Evidently, the whole importomer consists of PMPs and thus, PMPs have to be present to maintain peroxisomes functional. PMPs have been categorized into two classes: Class I is believed to directly target to peroxisomes in a PEX19-dependent manner, whereas class II proteins first enter the ER and are subsequently transported to the peroxisomal membrane PEX19-independent (Figure 5, Fang et al., 2004; Jones et al., 2004). Although targeting signals for class I PMPs, the membrane PTS (mPTS), were reported, no consensus sequence has been found (Dyer et al., 1996; Honsho and Fujiki, 2001; Pause et al., 2000; Rottensteiner et al., 2004; Wang et al., 2001). It seems that multiple regions including a PEX19 binding site and at least one transmembrane domain are necessary for proper import. Proteins necessary for the import of class I PMPs include PEX3,

PEX19, and in some organisms PEX16. PEX19 is a soluble protein thought to act as chaperone-like receptor for PMPs in the cytosol. The cargo-loaded PEX19 is then docked to the peroxisomal membrane by interaction with PEX3 or PEX16 and the cargo is somehow inserted into the membrane (Hettema et al., 2000; Purdue and Lazarow, 1995; South and Gould, 1999). PEX3 and PEX16 however, the only class II PMPs reported, are also involved in the *de novo* biogenesis of peroxisomes from the ER. Consequently, dysfunctions of these factors result in the loss of peroxisomes and hence, cells bearing a mutated version of one of these proteins are not amenable to study PMP import.

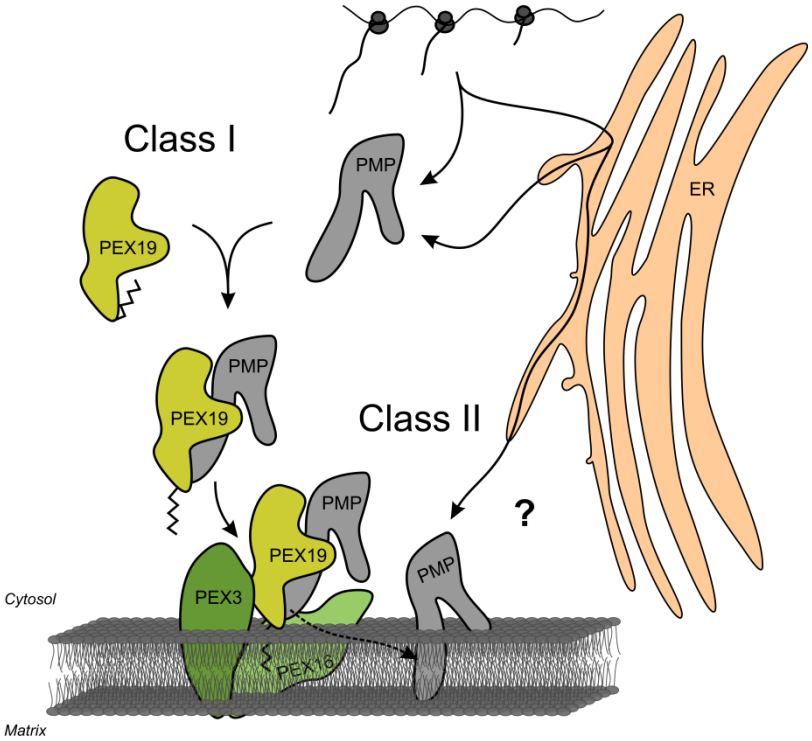

Figure 5: Membrane protein sorting. Class I PMPs are translated on free ribosomes in the cytosol and recognized by the soluble form of PEX19 which could act as chaperone. The cargo-loaded PEX19 is then recruited to peroxisomes through PEX3 and/or PEX16 and additionally anchored via its farnesyl group (Rucktaschel et al., 2009), while the PMP is inserted into the membrane via an unknown mechanism. Some PMPs including class II proteins sort via the ER to peroxisomes. Here, some proteins also require the function of PEX19 and PEX3 (e.g., PEX15), others might directly sort to pre-existing peroxisomes via direct membrane contact.

The classification of membrane proteins in class I and II has been challenged repeatedly. The class II protein PEX3 could also be delivered in a PEX19-dependent manner to mature peroxisomes behaving as class I PMP (Matsuzaki and Fujiki, 2008). Moreover, cell-free synthesized PEX19 was targeted to peroxisomes in a cell fraction of CHO cells, and delivered *in vitro* synthesized PEX26 and PEX16 to enriched peroxisomes, both integrating into the peroxisomal membrane. Hereby, a ternary

complex of PEX3, PEX19 and the cargo was reported *in vitro* (Matsuzono and Fujiki, 2006). Following the reintroduction of several GFP-tagged *GalS*-driven PMPs in the respective mutant yeast strains demonstrated the ability of a total of 16 PMPs to traffic via the ER to peroxisomes suggesting ER sorting as mode of delivery for many if not all PMPs (van der Zand et al., 2010).

It still remains unclear whether in wild type conditions all PMPs sort via the ER to peroxisomes. While some PMPs could integrate in the membrane of mature peroxisomes even *in vitro*, no adequate import factors have been described. Structural studies on PEX3/ PEX19 fragments characterized the interaction site between these two proteins and showed that the cytosolic part of PEX3 consists of a helix bundle (Sato et al., 2010; Schmidt et al., 2010). This structural insight creates doubts as to whether and how these proteins can indeed facilitate membrane protein import from the cytosol to the peroxisomal membrane. Thus, PMP transport in a PEX3/ PEX19-dependent manner from another membrane is more likely than direct incorporation from the cytosol. Herein, a classical vesicular traffic would involve a minimal fusion machinery at the peroxisomal membrane encompassing SNARE proteins, which were not reported in several in-depth peroxisomal proteomic approaches (Arai et al., 2008; Islinger et al., 2006; Marelli et al., 2004; Reumann et al., 2009; Saleem et al., 2008; Wiese et al., 2007). Alternatively, membrane contact sites could provide the environment for direct transfer of PMPs between membranes of different organelles, but has not been analyzed, so far. Overall, the ER plays a pivotal role for PMP import and thus is tightly connected to peroxisome biogenesis.

2.2 Peroxisome Proliferation

Similar to mitochondria, peroxisomes can proliferate via growth and division from pre-existing peroxisomes (Lazarow and Fujiki, 1985; Motley et al., 2008). This requires the growth of the organelle, its elongation and constriction followed by a membrane scission step. Indeed, a membrane fission machinery was identified at peroxisomes consisting of hFis1, Mff and DRP1, the latter being a dynamin-related protein performing the scission event (Gandre-Babbe and van der Bliek, 2008; Koch et al., 2004; Koch et al., 2003; Koch et al., 2005; Li and Gould, 2003; Otera et al., 2010). This fission machinery is well-known from mitochondrial division and seems to be shared between the two organelles (Schrader and Fahimi, 2006; Scott and Youle, 2010).

Interestingly, while the fission machinery is hijacked from mitochondria and all factors required for peroxisome formation including peroxins are purely eukaryotic, most peroxisomal enzymes have prokaryotic origin (Gabaldon et al., 2006). Phylogenetic studies in fact exclude a

prokaryotic ancestor of peroxisomes (de Duve, 2007; Gabaldon et al., 2006; Schluter et al., 2006). It looks as if peroxisomes evolved from the ER compartmentalizing key metabolic processes. Here, some well-evolved features could be adopted, such as the mitochondrial fission machinery; however, specialized proteins had to be established that coordinate peroxisome formation and also direct the fission machinery selectively to peroxisomes.

In mammals and plants, the PEX11 protein family was shown to recruit the fission machinery to peroxisomes (Kobayashi et al., 2007; Lingard et al., 2008). Indeed, the lack of the PEX11 proteins in mutant yeasts results in fewer and enlarged peroxisomes per cell, whereas its overexpression leads to more, smaller peroxisomes (Erdmann and Blobel, 1995).

2.2.1 The PEX11 proteins and the peroxisomal fission machinery

The finding that PEX11 directly influences the peroxisome number and shape stimulated many studies and eventually led to the discovery of the peroxisomal fission machinery. PEX11 proteins were identified in most eukaryotic organisms and also the fission machinery seems to consist of similar proteins in yeast, plant and human (Fujimoto et al., 2009; Motley et al., 2008; Nagotu et al., 2008; Zhang and Hu, 2009; Zhang and Hu, 2010; Zhang and Hu, 2008). Interestingly, most organisms contain more than one PEX11 protein (Abe and Fujiki, 1998; Abe et al., 1998; Lingard and Trelease, 2006; Marshall et al., 1995; Orth et al., 2007; Rottensteiner et al., 2003; Schrader et al., 1998; Smith et al., 2002; Tam et al., 2003; Tanaka et al., 2003). Besides the established link between members of the PEX11 protein families and the fission machinery, the molecular function of the PEX11 proteins remains unclear. Only, enlargement of the peroxisomal membrane were reported upon over-expression of some PEX11 family members (Schrader et al., 1998; Tanaka et al., 2003). Therefore, it was believed that the PEX11 protein could be involved in the growth of the peroxisomal membrane prior to fission. Although growth of the organellar membrane is crucial for proliferation, an isotropic omnidirectional growth cannot lead to fission because the membrane has to meet some physical requirements concerning shape and diameter before the fission machinery can assemble and act.

The molecular mechanism by which DRP1 (Dnm1p in yeasts) executes the scission is still a matter of debate. Recent findings, combining biophysical experiments, structural studies and *in vitro* as well as *in vivo* assays, propose that dynamin dimerizes, and upon GTP hydrolysis form spirals around the membrane exerting tension and deforming the membrane to its extreme. These spirals were shown to fit exactly mitochondrial constriction sites with a diameter in the 100nm range

(Ingerman et al., 2005). Then, upon release of GDP the spiral collapses, releasing the tension in the membrane, which spontaneously retracts and thereby divides (Bashkirov et al., 2008; Chappie et al., 2010; Gao et al., 2010; Ghosh et al., 2006; Low and Lowe, 2006; Low and Lowe, 2010; Low et al., 2009; Pucadyil and Schmid, 2008). Indeed, membrane fission and fusion are closely related phenomena, only the mechanism by which the membrane is deformed differs. For assembling at membrane constrictions, the DRPs need membrane elongation factors to pre-curve the membrane and shape it as the DRPs alone cannot form constrictions on organelles having several micrometers in diameter (Ramachandran, 2011; Roux et al., 2010). In our work (Section 6), we characterize the PEX11 protein family as membrane elongation factors that actively protrude the membrane and regulate the fission machinery (Koch and Brocard, 2011a; Koch and Brocard, 2011b, *in revision*; Koch et al., 2010).

Besides these proteins, the cytoskeleton which is responsible for the transport of peroxisomes was shown to also be actively involved in peroxisomal proliferation (Brocard et al., 2005; Fagarasanu et al., 2006; Jourdain et al., 2008; Mathur et al., 2002; Nguyen et al., 2006; Wiemer et al., 1997).

Still, the intriguing question of lipid recruitment remains. A proliferating organelle needs lipids to provide sufficient membrane environment during and after division. Since peroxisomes cannot generate phospholipids *de novo*, organellar contact, especially with the ER might be involved. Studies in yeast mutants deficient in phosphatidylethanolamine biosynthesis suggested membrane contact between peroxisomes and the ER, mitochondria and lipid droplets (Rosenberger et al., 2009). Indeed, a non-vesicular lipid transfer from the ER to peroxisomes has been proposed making the ER the central stage for peroxisome proliferation (Raychaudhuri and Prinz, 2008).

2.2.2 Peroxisomes and the ER

The ER plays a unique, unquestioned role in lipid biosynthesis and protein sorting – important for every organelle. The specialization into membrane-bound compartments requires controlled crosstalk between the organelles, especially between the ER and other organelles. This is effectively achieved via vesicles (COPI, COPII,…), non-vesicular signaling (Ca^{2+} release) or direct membrane contact (ERMES, PM-ER MCS, Duden, 2003; Elbaz and Schuldiner, 2011; Hajnoczky et al., 2000). In fact, the mitochondria and the ER maintain exchange sites for the transport of proteins and lipids (Giorgi et al., 2009; Kornmann and Walter, 2010). These require certain membrane shapes for both, the ER and mitochondria. In fact, the ER forms a network throughout the cell with distinct

architectures: the perinuclear ER, sheets often decorated with ribosomes, smooth tubules and a cortical ER in yeasts tightly fitting to the cell´s borders (Lynes and Simmen, 2011; Park and Blackstone, 2010; Pendin et al., 2011). Indeed, dedicated proteins maintain the different shapes of the ER. Reticulon proteins insert into the outer leaflet of the tubular ER membrane and through their hairpin-like shaped transmembrane regions keep the ER tubule in shape (Voeltz et al., 2006). Atlastin proteins (Sey1p in yeast) were shown to be involved in ER tubule fusion to generate the interconnected network structure (Rismanchi et al., 2008). The different architectures restrict biological processes to certain areas, e.g., fission of vesicles is likely to occur at regions of high membrane curvature, present in the tubular ER (Friedman and Voeltz, 2011; Pendin et al., 2011).

The ER contributes significantly to peroxisome maintenance. Apart from speculations on PMP and lipid transfer from the ER to peroxisomes, it has been shown that peroxisomes can form *de novo* from the ER. *pex3Δ* mutant yeast cells lacking peroxisomal structures could reform peroxisomes upon reintroduction of the missing gene (Geuze et al., 2003; Haan et al., 2006; Kragt et al., 2005a). Similar results were obtained in mammalian cells (Toro et al., 2007; Toro et al., 2009). In fact, PEX3 sorts to specific regions of the ER, thereby marking sites at which budding of peroxisomes will occur (Hoepfner et al., 2005; Tam et al., 2005). In contrast to the fission of pre-existing peroxisomes, *de novo* formation was shown to be Dnm1p-independent in yeast (Motley and Hettema, 2007). Recently, two *in vitro* assays for peroxisome biogenesis were reported (Agrawal et al., 2011; Lam et al., 2010). Both approaches followed the budding of PEX3 vesicles co-packaged with another membrane protein, PEX15 or PEX11. The budding did not depend on a functional COPII system (Lam et al., 2010). Interestingly, even in the absence of PEX3, vesicles containing PEX11 were observed questioning the role of PEX3 as sole initiator of peroxisome formation from the ER. PEX3 could rather act as control station for peroxisome fate. PEX3 definitely is required for the generation of functional, mature peroxisomes and furthermore, has been suggested to also act in peroxisome inheritance and its absence seems to be required for peroxisome degradation (section 2.2.3, Bellu et al., 2002; Munck et al., 2009). The specific interaction with other proteins or its absence seems to mark peroxisomes and classify them according to certain criteria as young, mature, inheritable or old peroxisomes. The underlying molecular principles remain to be discovered.

The two processes by which peroxisomes can proliferate seem to be independent. Studies using photoactivatable GFP and pulse-chase experiments differentiated between *de novo* formation and growth/ division in living mammalian and yeast cells, and reported that while yeasts mostly rely on fission of pre-existing peroxisomes, *de novo* formation is prevalent in mammalia (Kim et al., 2006; Motley and Hettema, 2007). Interestingly, although yeast peroxisomes multiply by growth and fission, Pex3p was distributed via the ER to existing peroxisomes (Motley and Hettema, 2007). In fact,

the two routes of peroxisome biogenesis seem to be effectively linked and regulated. Why should one pathway predominate over the other without regulation? How could PMP sorting and lipid transfer be possible without the participation of the ER in the growth and division? Already in the 1970s, close proximity of peroxisomes to the ER was observed in electron microscopy studies (Novikoff and Novikoff, 1972; Tabak et al., 2003). The molecular link between peroxisomes and the ER however, has not been established, so far. From our work in yeast cells, we propose the Pex30 protein family as regulators of peroxisome formation and suggest an ER-to-peroxisome tethering (David et al., 2011, *in revision*).

2.2.3 Inheritance and degradation of peroxisomes

During cell division, peroxisomes are inherited to the daughter cells. While in mammals, the large number of peroxisomes per cell and the symmetrical cytokinesis allows for equal distribution of peroxisomes, in budding yeast specific transporters are necessary. Two proteins, Inp1p and Inp2p, are regulating peroxisome inheritance in *S. cerevisiae*. Inp1p is anchoring peroxisomes at the cortex of the mother cell, whereas Inp2p attaches peroxisomes onto Myo2p, a myosin motor protein to transport these peroxisomes into the daughter cell (Fagarasanu et al., 2006; Fagarasanu et al., 2010; Fagarasanu et al., 2005; Saraya et al., 2010). The antagonistic action of Inp1p and Inp2p controls inheritance for the peroxisome population. However, whether preferably younger peroxisomes are inherited remains unclear.

Elderly, excessive or malfunctional peroxisomes are selectively degraded through an autophagic process called pexophagy (Iwata et al., 2006; Kiel, 2010; Oku and Sakai, 2010; Sakai et al., 2006). Two distinct modes are employed, micro- and macropexophagy. In micropexophagy, peroxisomes or accumulations thereof are engulfed in the vacuole, whereas in macropexophagy individual peroxisomes are sequestered by membrane layers forming the pexophagosome which subsequently fuses with the vacuole. It is still a matter of debate how peroxisomes are marked for degradation. PEX14 was shown to be solely required for peroxisomes degradation (Zutphen et al., 2008), and the removal of PEX3 was reported to initiate peroxisome breakdown (Bellu et al., 2002).

3 Contributions and Concepts

Cells maintain their number of peroxisomes through proliferation, inheritance and degradation. Herein, two modes of proliferation exist, *de novo* generation from the ER, and multiplication from pre-existing peroxisomes. These two pathways seem to be intimately linked, however, a mechanism for their coordination and cooperation has not been found, so far. Several proteins are involved in peroxisome proliferation, among which are proteins of the PEX11 and PEX30 family. We investigated these protein families to establish mechanisms for their molecular function.

Deletion of either a PEX11 or a PEX30 protein results in deregulated peroxisome proliferation. While the deletion of PEX11 correlates with a decreased number of peroxisomes (Erdmann and Blobel, 1995; Rottensteiner et al., 2003), alterations or the absence of PEX30 leads to hyper-proliferation (Vizeacoumar et al., 2004; Vizeacoumar et al., 2003). Although initially suggested as peroxisomal protein in *S. cerevisiae*, the localization of Pex30p in *P. pastoris* was shown to be partly peroxisomes, partly ER (Vizeacoumar et al., 2003; Yan et al., 2008). Our studies on Pex30p in *S. cerevisiae* now clearly show that most Pex30p resides in the ER. Indeed, Pex30p is present in high molecular weight complexes together with resident cortical ER proteins, the reticulons, Rtn1p, Rtn2p and Yop1p, and transiently interacts with Sey1p as well as all subunits of the COPI coaotmer (David et al., 2011, *in revision*). Pex30p contains a KKXX ER-retrieval motif, and seems to shuttle between peroxisomes and the ER. Although Arf proteins, known players in coatomer formation, have already been implicated in peroxisome proliferation, little is known about their contribution (Anthonio et al., 2009; Anton et al., 2000; Lay et al., 2006; Passreiter et al., 1998). Interestingly, yeast cells deficient for selected Arf proteins contain peroxisomes, and similar to *pex30*Δ cells, their number is increased suggesting the involvement of Arf proteins in the regulation of peroxisome maintenance (Anthonio et al., 2009).

The reticulon homology proteins (RHPs) present in Pex30p complexes significantly contribute to peroxisome maintenance. They act upstream of Pex30p and seem to provide the proper ER-architecture to facilitate peroxisome formation. Deletion of the RHPs leads to a dramatic increase in the number of peroxisomes per cell and peroxisomes appear clustered (David et al., 2011, *in revision*). Indeed, upon induction of peroxisome proliferation, Pex30p accumulates at ER-subdomains and tethers peroxisomes. We propose these subdomains to represent ER-to-peroxisome contact sites (EPCONS), similar to other inter-organellar contact sites. Membrane contact between ER and peroxisomes would enable lipid exchange and PMP transport. Moreover, the EPCONS are in good agreement with previous observations reporting ER substructures continuous with peroxisomes (Geuze et al., 2003). Thus, it is tempting to speculate that EPCONS also represent ER exit sites specific

for peroxisomes used during *de novo* formation allowing for tight spatiotemporal control between *de novo* formation and multiplication of peroxisomes.

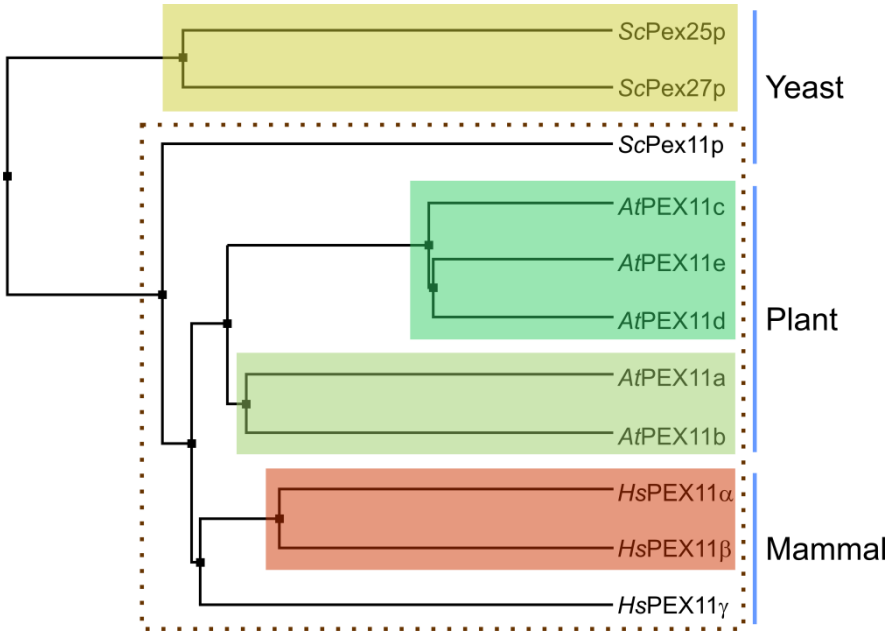

Figure 6: Phylogenetic tree of the PEX11 proteins from *S.cerevisiae, A. thaliana and H. sapiens*. Close homologues are boxed. PEX11 proteins evolved from a common ancestor, however, the homologues Pex25p and Pex27p, split up early in evolution from the other PEX11 proteins (dotted box). This already points at a function for Pex25p and Pex27p distinct from other PEX11 proteins. The tree has been calculated via: www.ebi.ac.uk/Tools/es/cgi-bin/tcoffee.

Among other factors important for peroxisome formation from the ER, Pex25p plays a crucial role in peroxisome reintroduction (Huber et al., 2011; Saraya et al., 2011). Interestingly, Pex25p is a member of the PEX11 protein family in yeast, consisting of Pex11p, Pex25p and Pex27p (Erdmann and Blobel, 1995; Rottensteiner et al., 2003; Smith et al., 2002), yet it has a role distinct from the other PEX11 proteins. Although their deletion phenotype is similar as yeast cells lacking either of these proteins contain fewer peroxisomes, only Pex25p rescues triple mutant *pex11*Δ*pex25*Δ*pex27*Δ cells (Rottensteiner et al., 2003). In fact, all PEX11 proteins seem to have evolved from a common ancestor, Pex25p and Pex27p however, split up early in evolution (Figure 6). Thus, PEX11 proteins of higher eukaryotes are more closely related to *Sc*Pex11p, than *Sc*Pex25p or *Sc*Pex27p. This reflects that while in yeasts each PEX11 protein exerts different roles, in higher eukaryotes, all PEX11 proteins seem to function cooperatively in the same pathway to promote peroxisome proliferation.

Plant and mammalian cells contain five and three PEX11 proteins, respectively. In humans, the three proteins, PEX11α, PEX11β and PEX11γ, are differently expressed in every tissue, but only

PEX11α is inducible (Abe and Fujiki, 1998; Abe et al., 1998; Li et al., 2002a; Li et al., 2002b; Li and Gould, 2002; Schrader et al., 1998; Tanaka et al., 2003). All these proteins represent membrane elongation factors that remodel the peroxisomal membrane prior to fission (Delille et al., 2010; Koch and Brocard, 2011a; Koch and Brocard, 2011b, *in revision*; Koch et al., 2010; Opalinski et al., 2010). PEX11 proteins contain an amphipathic α-helix, a common domain of membrane elongation factors, such as the BAR domain proteins (Frost et al., 2009; Koch and Brocard, 2011b, *in revision*; Opalinski et al., 2010). The amphipathic α-helix inserts into one leaflet of the lipid bilayer thereby increasing the surface area of one layer with respect to the other hence promoting membrane curvature. However, membranes are three-dimensional objects and only a single amphipathic helix cannot induce enough curvature to protrude membrane extensions. Only a spatiotemporally controlled action of many PEX11 proteins can substantially curve the membrane. Moreover, the role of lipids and their interaction with membrane proteins should not be neglected. All membrane proteins, especially membrane curvature factors have an affinity for certain phospholipids whose biophysical properties could facilitate the reshaping of the membrane.

In fact, PEX11 proteins in human were localized all around the peroxisomal membrane, even when peroxisomal membrane extension had formed (Delille et al., 2010; Koch et al., 2010). Other membrane proteins showed a differential localization across the peroxisomal membrane (Delille et al., 2010). While most PEX11 proteins are thought to contain an amphipathic α-helix at their N-terminus, PEX11γ spans its helix in its C-terminal region between to membranous segments (Koch and Brocard, 2011b, *in revision*; Opalinski et al., 2010). It is hard to envision that this anchored amphipathic helix is only inserted into the membrane when needed. Unlike soluble membrane curvature factors, PEX11γ is an integral membrane protein that is always present in the peroxisomal membrane. During proliferation, PEX11 proteins specifically protrude the peroxisomal membrane at one distinct site (Figure 7). How can PEX11 that virtually through its presence in the membrane constantly influences membrane curvature be distributed over the whole membrane and induce membrane protrusion at one specific site? The presence of PEX11 proteins throughout the - non-protruded - membrane suggests that two PEX11 species exist, the one at the site of membrane outgrowth, the other along the rest of the membrane. Even if all PEX11 proteins insert their amphipathic α-helix into the membrane; as long as these proteins are equally distributed no membrane outgrowth will occur. Upon assembling a critical number of PEX11 proteins at one site in a special geometry, the membrane would be sculpt outwards. Subsequently, the PEX11 proteins could assemble the fission machinery and trigger scission. Hence, interactions between members of

the PEX11 family and the fission machinery are likely to take place within the lipid bilayer and regulate peroxisome multiplication (Koch and Brocard, 2011b, *in revision*).

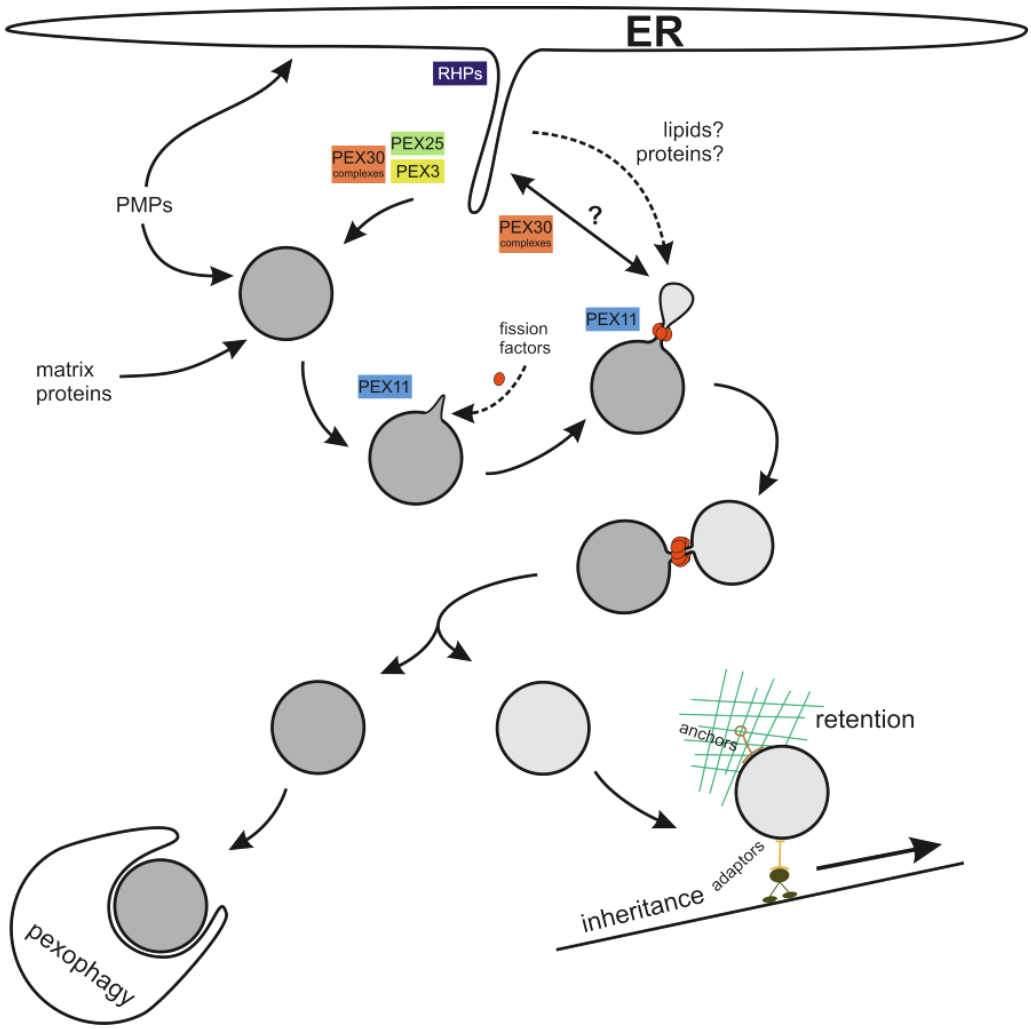

Figure 7: Proposed model for peroxisome proliferation. Peroxisomes are formed *de novo* from the ER at specialized ER exit sites requiring Pex3p and Pex25p (in yeast) or PEX16 (in higher eukaryotes). PEX30 proteins regulate this process, and the reticulon homology proteins (RHPs) provide the proper ER architecture for enrichment of the peroxisome biogenesis machinery at specific regions of the ER membrane. During growth membrane and matrix proteins are imported. For the multiplication of pre-existing peroxisomes, PEX11 proteins protrude the peroxisomal membrane at one distinct site. Here, matrix proteins are retained such that new material has to be imported to the newly formed protrusion inflating a new daughter peroxisome. During growth, peroxisomes transiently attach to the ER, mediated by PEX30 proteins, for the uptake of lipids and membrane proteins. These membrane contact sites could be identical to the ER exit sites allowing coordination between *de novo* formation and multiplication of peroxisomes. Older and possibly damaged peroxisomes are specifically degraded while others are inherited to the daughter cell upon cell division.

3.1 Peroxisome Proliferation revisited

Altogether, we propose a model for peroxisome proliferation in which the ER plays a central role in providing lipids and membrane proteins for both, *de novo* biogenesis and multiplication of pre-existing peroxisomes. The transfer of material is achieved through membrane contact sites (EPCONS) where cortical ER resident proteins provide the proper architecture and members of the PEX30 family are required for the tethering of peroxisomes to the ER. In fact, these membrane contact sites could be ER exit sites during *de novo* biogenesis of peroxisomes. Pex30p and the RHPs would then be important for the regulation and coordination of peroxisome proliferation. Indeed, further experiments using biogenesis and inheritance mutant yeast cells showed that deletions of RHPs or Pex30p increase the efficiency of yeast cells to form peroxisomes *de novo* (David et al., 2011).

The PEX11 protein family is mainly required for the coordinated DRP-dependent fission of pre-existing peroxisomes. Here, the coordinated interplay between members of the PEX11 protein family and the fission machinery controls peroxisome proliferation. Peroxisomal membrane outgrowth prior to fission allows for *i)* segregation of matrix proteins, *ii)* controlled delivery of membrane proteins and *iii)* direct assembly of the fission machinery, especially of DRP1 which can form spirals around the protruded membrane (Figure 7). Although seemingly independent from the ER, during the growth of the peroxisome, lipids and PMPs are likely to be delivered. In fact, ER tubules were shown to contact and effectively wrap around mitochondrial constriction sites possibly to administer proteins and lipid or even mediate physical constriction (Friedman et al., 2011). In a similar way, the ER could actively participate in peroxisome multiplication. Overall, the two modes of peroxisome proliferation seem to be tightly linked through the ER.

4 Outlook

Contact between the ER and peroxisomes has been proposed in the 70s, and with our work, we found proteins that are involved in this tethering. However, the architecture of the membrane contact sites remains to be investigated. Electron-microscopic studies will shed light on the nature of these contact sites and might even allow for differentiation between pre-existing peroxisomes that dock onto the ER and newly formed peroxisomes that bud off. Once established, the flow of material, including lipids and proteins could be recorded and thus pinpoint the role of the ER in peroxisome maintenance.

Upon membrane contact of two organelles and even during vesicular trafficking, proteins besides the cargo are co-transferred which have to be retrieved to their original membrane. A well-established Golgi-to-ER retrieval machinery is the COPI retrograde transport (Beck et al., 2009; Pinot et al., 2010). Pex30p interacts with all subunits of the coatomer suggesting such a retrieval mechanism to the ER membrane. Although in principle it might be that small portions of Pex30p enter the Golgi and thus have to be transported back to the ER, it is more likely that retrieval occurs between peroxisomes and the ER. Arf proteins, factors necessary for COPI vesiculation, were shown to participate in the formation of peroxisomes, their morphology and protein sorting (Anthonio et al., 2009; Anton et al., 2000; Passreiter et al., 1998). Interestingly, some PEX11 proteins of plant and human carry a KKXX motif. Also, Pex30p is the only yeast peroxin that carries such a signal. Understanding the contribution of retrograde vesicular trafficking in coordination with direct membrane contact to peroxisome maintenance would complete the picture of ER-peroxisome crosstalk and clarify the involvement of COPI proteins.

Peroxisomes segregate their matrix protein content during division and even membrane proteins seem to be differentially localized over the peroxisomal membrane (Delille et al., 2010; Koch and Brocard, 2011a; Koch et al., 2010). Indeed, this could represent a quality control mechanism to accumulate old and possibly damaged matrix proteins in the mother peroxisome. The selective inheritance of membrane proteins to the daughter peroxisome could mark it as younger peroxisome and thus prevent degradation, e.g., through PEX3 transfer to the new membrane. In contrast, the repeated loss of these marker proteins from the mother peroxisome below a certain threshold would trigger degradation of this organelle. Indeed, the loss of PEX3 was shown to be essential for initiation of pexophagy (Bellu et al., 2002). However, Pex3p was shown to sort to existing peroxisomes in yeast (Motley and Hettema, 2007) and the proposed quality control mechanism would require that those age-indicating proteins are not constantly delivered. A detailed analysis of these processes including

a thorough monitoring of individual peroxisomes and their PMPs over time would contribute to understand selectivity in peroxisome maintenance.

Eventually, most factors that are crucial for the assembly of functional peroxisomes have already been found. The deeper knowledge about the molecular mechanisms of many of these factors however, requires more subtle criteria as readout parameter than the simple absence or presence of peroxisomes. For inheritance proteins, the distribution of peroxisomes between dividing cells served as parameter (Fagarasanu et al., 2006; Fagarasanu et al., 2005). We used the shape of peroxisomes (juxtaposed elongated peroxisomes, JEPs) and also the motion of peroxisomes to study the effect of PEX11 and PEX30 proteins. Based on these criteria, new screens could be performed to identify hitherto unknown proteins that might also influence peroxisome maintenance.

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6 Original work

6.1 A Dynamic Tethering Complex Coordinates Peroxisome Maintenance through ER-to-Peroxisome Contact Sites

Revised version *In Revision*, Journal of Cell Biology

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Author contributions:

I contributed to the experimental setup concerning microscopy and dynamics, and carried out most microscopy work as well as the statistical analysis of peroxisome number per cell. I performed the FRAP experiments and analysis of peroxisome trajectories. For all these experiments, I analyzed and interpreted the data and arranged the figures. Moreover, I also participated in writing the manuscript.

A Dynamic Tethering Complex Coordinates Peroxisome Maintenance through ER-to-Peroxisome Contact Sites

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ABSTRACT

Peroxisome biogenesis initiates at the endoplasmic reticulum and maturation allows the formation of metabolically active organelles. Yet, peroxisomes can also proliferate through growth and division. Here, we demonstrate that peroxisome proliferation and function rely on the integrity of cortical ER tubules. Using SILAC-based quantitative proteomics, we established a network of stable as well as transient interactions around the membrane protein Pex30p. Through association with merely ER membrane proteins, in particular with proteins containing a reticulon homology domain and with other peroxins Pex30p designates peroxisomes contact sites at ER subdomains. We show that Pex30p traffics through the ER, segregates in punctae to which peroxisomes specifically attach and we ascertain its transient interaction with all subunits of the coatomer complex suggesting the involvement of COPI vesicle-mediated transport. We establish that Pex30p tethers peroxisomes to the cortical ER and propose that Pex30p complexes act as hub to regulate the formation of peroxisomes in time and space and that maintenance of the ER tubular architecture through the reticulon homology proteins Rtn1p, Rtn2p and Yop1p is essential to regulate this process.

INTRODUCTION

All nucleated cells contain essential round-shaped organelles called peroxisomes, whose function is mainly associated with lipid metabolism (Wanders and Waterham, 2006). Depending on the cellular requirements, the size, number and protein content of these single membrane-bound organelles can greatly vary. While peroxisomes are dispensable for unicellular species such as yeast, they are essential for the proper development of multicellular organisms (Fan et al., 2005; Faust et al., 2010). In human, mutations in *PEX* genes lead to defects in peroxisome function or formation and are associated with the development of lethal pathologies (Steinberg et al., 2006). These *PEX* genes code for proteins, called peroxins, which are involved in peroxisome assembly and maintenance (Distel et al., 1996).

Two major routes seem to lead to peroxisome formation, namely, de novo biogenesis and growth/ division of pre-existing peroxisomes. The division pathway operates with proteins of the Pex11 family and requires fission factors shared with mitochondria (Koch and Brocard, 2011). Studies in yeast and mammalian cells revealed that through the action of the protein Pex3p peroxisome precursors could also originate from the ER and via import of membrane and matrix proteins, mature into fully functional organelles (Hoepfner et al., 2005; Toro et al., 2009). Furthermore, several peroxisomal membrane proteins were shown to migrate to peroxisomes via the ER (Hoepfner et al., 2005; Kim et al., 2006; van der Zand et al., 2010). The molecular mechanism underlying the biogenic pathway of peroxisome formation has not been clarified, so far. Recent data based on cell-free vesicle-budding reactions, however, demonstrated that several resident peroxisomal proteins traffic from the ER to peroxisomes in a COPII vesicle-independent manner (Lam et al., 2010). These observations point to the existence of vesicular events to mediate the transport of peroxisomal membrane proteins from the ER. In fact, analysis of secretory mutant yeast cells already suggested that part of the ER-associated secretory machinery is involved in peroxisome biogenesis (Perry et al., 2009).

The *de novo* biogenesis of peroxisomes and the growth/ division pathway are usually seen as independent routes; however, these events may be coordinated and, thus, intimately linked. Indeed, peroxisomes need to acquire membrane components to proliferate and it has been proposed that their

binding to the cell cortex or to the cytoskeleton allows their partitioning and segregation during cell division (Fagarasanu et al., 2006; Fagarasanu et al., 2005; Hoepfner et al., 2001).

Among the proteins required for assembly of peroxisomes the membrane proteins Pex23p and Pex24p play an essential role in *Y. lipolytica* (Brown et al., 2000; Tam and Rachubinski, 2002). Homologues of these two proteins in *S. cerevisiae* are Pex30p, Pex31p and Pex32p, and, Pex28p, Pex29p. In the latter yeast species, these proteins seem to negatively control peroxisomal size and number (Vizeacoumar et al., 2004; Vizeacoumar et al., 2003). However, the molecular function of these proteins and the mechanism by which they act are still unknown. Interestingly, Pex30p was suggested to regulate peroxisome proliferation to varying extent. While the lack of Pex30p in *S. cerevisiae* leads to an increase in the number of normal-sized peroxisomes (Vizeacoumar et al., 2004), in *P. pastoris* its absence correlates with the appearance of fewer and clustered peroxisomes (Yan et al., 2008). Although peroxisomes are highly versatile organelles, under given conditions their total number per cell remains fairly constant owing to the delicate balance of proliferation, inheritance and degradation (Fagarasanu et al., 2007; Manjithaya et al., 2010). The question is what are the molecular mechanisms responsible for the spatiotemporal organization of these events?

Here, we present data obtained from a dual approach based on quantitative proteomics using stable isotope labeling with amino acids in cell culture (SILAC; Mann, 2006; Ong et al., 2002) and live-cell imaging revealing the interaction network around Pex30p and its function in the organization of ER-to-peroxisome membrane associations. We report the existence of a macromolecular membrane protein complex that acts as hub for the regulation of peroxisome proliferation and movement. Our data establish a physical link between the ER and peroxisomes. As an initially cortical ER protein and through its interaction with proteins containing a reticulon homology domain, Pex30p is shown in this work to designate contact sites on ER tubules and act as tethering factor for peroxisomes. In addition, we show that Pex30p specifically traffics through the ER and interacts with components of the coatomer complex suggesting a route via COPI vesicle-mediated transport. Our data reveal a central role for ER-to-peroxisomes contact sites and the tethering factor Pex30p in coordinating peroxisome maintenance.

RESULTS

Evidence for the role of Pex30p in the regulation of peroxisome biogenesis

Members of the Pex11 protein family were previously shown to also influence the number of peroxisomes (Erdmann and Blobel, 1995; Rottensteiner et al., 2003). To test whether Pex30p acts in concert with these factors to maintain the steady state level of peroxisomes, we evaluated the total number of peroxisomes per cell in different yeast mutants incubated in oleic acid-containing medium (Fig. 1A). Cells lacking Pex30p exhibited more peroxisomes than wild type cells (9.8 ± 1.5 vs. 7.8 ± 1.5; $n > 100$). When Pex30p was expressed in *pex30*Δ cells the number of peroxisomes decreased virtually to wild type levels (Fig. 1A). Interestingly, upon deletion of the last 229 amino acids (PEX30ΔC) ~50% of the cells contained more than 10 peroxisomes showing that full length Pex30p was required to control peroxisome proliferation.

Huber et al. recently demonstrated that in *S. cerevisiae* the two members of the Pex11 family, Pex11p and Pex25p acted differently on peroxisome proliferation. While Pex11p was only required for the proliferation of pre-existing peroxisomes, the presence of Pex25p was indispensable for the regeneration of peroxisomes de novo from the ER (Huber et al., 2011). Thus, we examined the effect of deleting *PEX30* on cells lacking the latter proteins. We consistently found few peroxisomes in *pex11*Δ and *pex25*Δ mutant cells whereas overexpression of Pex11p led to higher number of peroxisomes (Fig. 1A). Strikingly, when *PEX30* was deleted in *pex11*Δ mutant cells (6.6 ± 1.6 vs. 2.1 ± 1.8; $n > 100$), the number of peroxisomes rose near wild type levels demonstrating the potential of peroxisomes to proliferate in the absence of Pex11p. In contrast, deletion of *PEX30* in *pex25*Δ mutant cells did not lead to an increase in peroxisome number (1.2 ± 1.4 vs. 1.3 ± 1.4; $n > 100$) and, in the absence of all three proteins, cells hardly contained peroxisomes (0.6 ± 1.0; $n>100$). Thus, our data demonstrate that the function of Pex30p is required downstream of Pex25p suggesting that Pex30p might rather be involved in the regulation of de novo formation of peroxisomes (see Fig. 1A; lower panel).

We analyzed whether variations in the number of peroxisomes per cell correlated with peroxisomal function in several mutant yeasts. In contrast to wild type cells, *pex3*Δ mutant cells lack peroxisomes and, thus, are unable to perform ß-oxidation and cannot utilize oleate as sole carbon source (Fig. 1B). The ability to consume the fatty acid was reduced in cells lacking Pex30p (Fig. 1B). Cells lacking *PEX11* were hampered in their ability to utilize oleate and peroxisomal function did not fully recover through overexpression of Pex11p. This, together with the statistical analyses, suggests that high levels of Pex11p lead to uncontrolled peroxisome propagation. The phenotype became even stronger when Pex30p was absent. Plasmid-borne expression of both Pex11p and Pex30p only partially recovered the phenotype in *pex11*Δ*pex30*Δ mutant cells (Fig. 1B) suggesting that in wild types, the levels of these proteins must be delicately regulated in the cell to obtain metabolically active organelles. In agreement with the idea that Pex11p and Pex25p act in independent routes during peroxisome proliferation, peroxisomes were partially functional in *pex25*Δ cells, whereas they were non-functional in *pex11*Δ*pex25*Δ or *pex11*Δ*pex25*Δ*pex30*Δ mutant cells. Expression of Pex25p only, led to recovery of peroxisomal function in these cells (Fig. 1B). Furthermore, in favor of the notion that Pex30p and Pex25p act on the same pathway, mutant cells lacking both Pex25p and Pex30p could utilize oleate better than cells lacking only Pex25p.

Pex30p interacts with ER-resident proteins

To accurately identify Pex30p membrane protein interactions in *S. cerevisiae*, we employed SILAC-based quantitative affinity-purification mass spectrometry, a strategy that allows for unbiased and comprehensive analysis of protein complexes and large interaction networks (Hubner et al., 2010; Oeljeklaus et al., 2009; Vermeulen et al., 2010). To this end, differentially labeled cells were grown under peroxisome-proliferating conditions and mixed. Then, following a one-step purification protocol, Pex30p complexes were purified from digitonin-solubilized crude membrane fractions (affinity-purification after mixing, AP-AM; Fig. 2A). This method allows the identification of true protein complexes that form in vivo, that are not due to rearrangement during the purification procedure. Quantitative analysis of the mass spectrometric data obtained from three independent experiments by MaxQuant (Cox and Mann, 2008) was followed by statistical data evaluation and

resulted in the classification of 20 proteins as specific constituents of the Pex30p membrane protein complex (Fig. 2A and Table S1A). Among these were the bait Pex30p and Pex28p, Pex29p, and Pex32p, known factors in peroxisome proliferation (Vizeacoumar et al., 2004; Vizeacoumar et al., 2003) as well as Rtn1p, Rtn2p, Yop1p, Pom33p, Spf1p, Scs2p, Ncp1p, Hfd1p, Dpm1p, Pho88p, Vac8p, Gsf2p, Sop4p, Pga3p, Emc4p and Yet1p, all ER-membrane proteins involved in ER maintenance and function. Among all specific binding partners, our quantitative MS data point to the reticulon homology domain-containing proteins (RHPs) Rtn1p, Rtn2p and Yop1p as the most abundant ER factors in the Pex30p complexes.

Identification of transient interaction partners for the membrane proteins Pex30p and Pex29p

Our initial AP-AM strategy applied to Pex30p complexes accurately identified stable interaction partners and, thus, defined the core components of the Pex30p interactome. However, in the AP-AM approach, identification of transient interaction partners exhibiting high on-/off-rates is biased due to exchange of components during affinity-purification (Mousson et al., 2008; Oeljeklaus et al., 2009; Wang and Huang, 2008). To adequately tackle this issue, we performed affinity-purification separately on the cell extracts containing untagged ("Heavy") or TAP-tagged ("Light") Pex30p before combining the differentially-labeled samples for quantitative MS analyses (AP-PM; cartoon Fig. 2B). Application of the AP-PM strategy holds the potential to identify proteins with essential roles in modulating Pex30p function or localization that only transiently associate with the "core" complexes. As a result of triplicate experiments, 36 proteins were classified as Pex30p interaction partners including numerous proteins previously identified in the AP-AM experiments. Among these were Pex28p, Pex29p, and Pex32p as well as the RHPs (Fig. 2B, and Table S1B). We also identified the GTPase Sey1p, a functional ortholog of atlastins known to cooperate with Rtn1p and Yop1p to maintain the ER morphology (Hu et al., 2009). Especially interesting was that the entire COPI coatomer complex transiently associated with Pex30p complexes. The specificity of this association is reflected by the absence or very low abundance of peptide species derived from wild type yeasts as compared to the co-purified contaminant Kgd1p (Fig. 2C).

Pex30p and Pex29p are believed to play a role in the same process and Pex29p has been proposed to act upstream of Pex30p in the regulation of peroxisome proliferation (Vizeacoumar et al., 2004) suggesting that these proteins might assemble in a protein complex. We addressed this issue in reverse AP-PM experiments using Pex29p as bait. Here, we identified a subset of the proteins present in Pex30p complexes such as Pex30p, Pex32p, Rtn1, Rtn2p, Yop1p, and Sey1p as well as Ret1p, Sec21p, Sec26p, Sec27p, four components of the COP1 coatomer (Fig. 2D and Table S1C). Note that although not classified as specific in the Pex30p complexes, Pex11p was found as specific binding partner in the Pex29p complexes.

We performed reverse affinity-purification experiments using cells expressing TAP-tagged copies of COPI coatomer subunits and tested for the presence of Pex30p in the eluates (Fig. 2E). In all cases, the intensity of the detected Pex30p signal correlated well with the amounts of coatomer complex purified, whereas no signal was detected in the purified importomer complexes using Pex14p as bait. The data, thus, demonstrate that Pex30p associated with the coatomer complex.

Pex30p interacts with Rtn1p and localizes to the cortical ER

To evaluate whether the interaction of Pex30p with Rtn1p relied on the presence of the other peroxisomal proliferation factors, we performed affinity-purification of Rtn1p-TAP from different yeast mutants. Pex30p specifically co-purified with Rtn1p-TAP (Fig. 3A). However, in the absence of Pex29p, higher levels of Pex30p co-precipitated with Rtn1p suggesting a competition between Pex29p and Rtn1p for Pex30p binding. To test this hypothesis, we compared the amounts of Pex30p co-purifying with Rtn1p in different mutant backgrounds. In cells lacking Rtn2p, known to interact with Rtn1p, higher amounts of Pex30p co-purified with Rtn1p and similarly in *pex29Δ* or *pex28Δpex29Δ* *pex31Δpex32Δ* mutant cells. Concurring, in cells expressing elevated levels of Pex29p, smaller amounts of Pex30p co-purified with Rtn1p (Fig. 3A). These data further illustrate that Pex30p and Rtn1p are likely to be involved in several interactions.

The results of our proteomic analysis prompted us to assess the localization of Pex30p in yeast cells co-expressing Sec63p-RFP, a subunit of the ER translocon complex, and Pex30p-GFP in *pex30Δ* cells.

To tune the *PEX30* expression levels, we transformed *pex30*Δ mutant cells with a plasmid expressing Pex30p-GFP controlled by the *GAL*-promoter. Our western blot analysis of Pex30p-GFP levels established that induction through a 15 min galactose pulse followed by incubation of the cells in medium containing oleic acid was adequate to produce a signal for Pex30p-GFP comparable to wild type levels (Fig. S1). As illustrated in Figure 3B, Pex30p-GFP partially co-localized with Sec63p-RFP, in particular, at the nuclear periphery (correlation coefficient $I_{corr} = 0.752 \pm 0.227$, n = 30). In contrast, the fluorescent signals were higher for Pex30p-GFP than for Sec63p-RFP at the cortical ER and Pex30p-GFP structures were observed at the cell periphery that lacked Sec63p-GFP signal.

Next, we examined whether the observed Pex30p-GFP punctae represented peroxisomes and co-expressed the peroxisomal marker mCherry-Px and Pex30p-GFP. Interestingly, most mCherry-Px signal accumulated close to or coincided with Pex30p-GFP punctae as illustrated by the colocalization map (Fig. 3C). These observations suggest either that a portion of Pex30p trafficked to peroxisomes or that peroxisomes gathered to the Pex30p-GFP punctae.

To investigate the ER localization of Pex30p in more detail, we monitored the fluorescent signals in cells co-expressing Rtn1p-mCherry and Pex30p-GFP (Fig. 3D). As previously reported (Lee et al., 2010), Rtn1p-mCherry localized to ER tubules. Likewise, Pex30p-GFP localized throughout the ER, but both proteins accumulated in punctate structures predominantly located at the cell periphery as illustrated by the line profiles ($I_{corr} = 0.646 \pm 0.078$, n = 30; Fig. 3D).

Our microscopic analyses revealed that under conditions that promoted peroxisome proliferation, Pex30p localized primarily to subdomains of the cortical ER at boundaries between ER tubules and peroxisomes (Fig. 3CD) whereas on glucose no drastic accumulation of Pex30p-GFP could be observed (Fig. 3E). In agreement, in early peroxisome proliferation (8 h), the Pex30p binding partners Pex29p and Pex32p also localized to the ER. However, at a later time point (16 h) while Pex29p was still mostly present in the ER, Pex30p accumulated in punctae with peroxisomes in close vicinity and Pex32p trafficked to peroxisomes (Fig. S2).

Reticulon proteins control peroxisome proliferation

The RHPs Rtn1p, Rtn2p and Yop1p are known to participate in the maintenance of the positive membrane curvature and tubulation and primarily partition to cortical ER tubules (De Craene et al., 2006; Voeltz et al., 2006). Because RHPs were found to specifically associate with Pex30p via AP-AM experiments and Rtn1p partially colocalized with Pex30p-GFP, we assessed the effect of deleting Rtn1p on Pex30p localization. In these cells, another RHP, Yop1p-mCherry, localized to tubular ER structures and accumulated in spots containing Pex30p-GFP (Fig. 4A). Our data show that overproduction of Rtn1p altered the distribution of Pex30p-GFP since no dotted structures could be seen.

To evaluate whether all three RHPs acted on peroxisome proliferation, we next analyzed the effect of deleting these three factors on peroxisome abundance (Fig. 4B). The *rtn1Δrtn2Δyop1Δ* mutant cells presented an atypical ER structure lacking the characteristic tubules (Fig. 4C). These cells contained more peroxisomes than wild type cells (11 ± 2.3 vs. 7.8 ± 1.5; $n > 130$) and, strikingly, peroxisomes accumulated in small clusters of two to four peroxisomes similar to the effect observed in cells lacking Pex28p, Pex29p or both proteins (Vizeacoumar et al., 2003). Furthermore, overexpression of Pex30p-mCherry in cells lacking the RHPs did not significantly alter the number of peroxisomes as compared to control cells (Fig. 3BC and 4C left panel) suggesting that the presence of the RHPs is required for Pex30p to control the peroxisomal number (Fig. 4C).

RHPs regulate the efficiency of de novo peroxisome biogenesis from the ER

To test whether RHPs were involved in peroxisome biogenesis, we established an in vivo biogenesis assay based on the transcriptional control of the essential peroxin *PEX3* through the *GAL*-promoter. Cells lacking Pex3p are devoid of peroxisomes and when Pex3p is reintroduced in these cells, peroxisomes are slowly generated de novo from the ER (Hoepfner et al., 2005). We performed time lapse imaging of cells with (control) or without RHPs (*rtn1Δrtn2Δyop1Δ*) expressing *GAL*-driven *PEX3* during galactose induction and evaluated the time required to obtain mature peroxisomes. No peroxisome was present in cells cultured on glucose medium (Fig. 4D, left panel). Strikingly, while control cells needed 3 to 4 hours galactose induction to produce peroxisomes, 1 hour after induction the first cells containing peroxisomes were already visualized in mutants lacking the RHPs. After 4

hour induction the difference was even greater between the two strains (Fig. 4D, right panel). Moreover, similar to cells lacking RHPs only, peroxisomes accumulated in small clusters. These observations were not due to varying levels of Pex3p between both strains (Fig. 4D, bottom panel). In consequence, our data illustrate that de novo peroxisome biogenesis from the ER is enhanced in the absence of RHPs. This strongly suggests that the ER membrane architecture plays an essential role in the regulation of peroxisome maintenance in yeasts.

Pex30p traffics from the perinuclear area to static punctae at the cortical ER

We further tested whether Pex30p trafficked through the ER and measured fluorescence recovery after photobleaching (FRAP) on living yeast cells expressing either Sec63p-GFP or Pex30p-GFP. The Sec63p-GFP signal recovered within few seconds after bleaching regardless of whether the perinuclear ER or the cortical ER had been bleached (Fig. 5A). For Pex30p-GFP, we bleached three different regions, namely, *(i)* the perinuclear area, *(ii)* the cortical ER, and, *(iii)* a punctate structure at the cell periphery. The first two areas showed fast and full recovery whereas the fluorescent signal of the Pex30p-GFP puncta did not recover. Instead, a tubular structure with fluorescence appeared at the bleached position. These data demonstrate that Pex30p-GFP molecules are freely diffusible when present in the perinuclear region and in the cortical ER but fairly static in the punctate structures at the cell periphery. Considering that peroxisomes do not contain Rtn1p (Perry et al., 2009) and that the Pex30p punctae colocalized with Rtn1p (Fig. 3D), these most likely represent specific ER subdomains.

ER-to-peroxisome contact sites control peroxisomal dynamics

Peroxisomes (mCherry-Px) accumulated near the Pex30p-GFP signals (Fig. 3C). This observation suggested that a portion of the peroxisomal population might be in contact with the ER through interaction with Pex30p. To test this possibility, we analyzed living yeast cells grown under peroxisome proliferating conditions following a short galactose pulse to induce Pex30p-GFP expression and monitored the fate of fluorescent signals with time. Pex30p-GFP exhibited clear ER staining that rapidly accumulated into punctate structures. Moreover, peroxisomes (mCherry-Px) appeared to concentrate at Pex30p-GFP accumulation sites suggesting a molecular link between peroxisomes and Pex30p at specific ER domains (Fig. 5B; Movie 1A). Notably, shortly before cell

division, Pex30p-GFP concentrated at the tip of the bud and peroxisomes were consistently present near these structures. Upon cell division, Pex30p-GFP molecules present at the bud tip diffused back into the cortical ER after cytokinesis (Movie 1A).

We noticed that the peroxisomes present near the Pex30p-GFP punctae at the ER clearly lacked rapid movement. In contrast, free peroxisomes showed rapid motion. Thus, to quantify the effect of Pex30p expression on peroxisome motility, we recorded peroxisome movements in *pex30*Δ cells with or without Pex30p-GFP. Peroxisomes in contact with Pex30p-GFP punctae remained associated for the entire time of the experiment (30 min), which clearly points to a stable connection between these two entities. The average center-to-center distance between ER-attached peroxisomes (mCherry-Px) and Pex30p-GFP punctae was smaller than 850 nm. In some cases, the structures even intersected confirming the association (Fig. 5B, Movies 1A and 1B).

In the absence of Pex30p, we frequently observed an increase in peroxisomal movement and sought to examine the trajectories of several peroxisomal populations. In *pex30*Δ cells, most peroxisomes were extremely mobile and exhibited a high percentage of directed trajectories, some of which even breaking the sub-diffusive regime. Accordingly, in *pex30*Δ cells expressing Pex30p-GFP, peroxisomes displayed slower diffusion (low D_2) and less directed movement (low MSS slopes; Fig. 5C-E). Similar observations were made when we compared the trajectories of peroxisomes in cells lacking the RHPs and in cells lacking Pex30p in addition. Considering that half of the peroxisomal population was present on Pex30p-GFP punctae (Fig. 5B), these data point to a direct role of Pex30p as ER-associated peroxisome tethering factor and suggest that RHPs provide an architectural advantage during this process.

Pex30p coordinates de novo biogenesis from the ER and peroxisome growth/ division

Although peroxisomes can be formed de novo from the ER, studies in yeasts showed that under normal conditions, peroxisomes rather use the growth/ division pathway to proliferate (Motley and Hettema, 2007). De novo formation only occurs in mutant cells devoid of peroxisomes in which the missing gene is reintroduced. This is associated with the budding of pre-peroxisomal vesicles from

specialized ER subdomains that represent peroxisome exit sites (Geuze et al., 2003). These exit sites might correspond to peroxisome contact sites during proliferation in wild type cells and both ways might integrate at Pex30p. To test this hypothesis, we needed cells that could proceed to both de novo formation and growth/ division of peroxisomes and established an assay based on the recent finding that in *S. cerevisiae* cells peroxisomes are actively inherited during cell division. Here, the antagonistic action of two proteins, Inp1p and Inp2p, controls inheritance of the peroxisomal population. While Inp1p anchors peroxisomes to the cortex of the mother cell, Inp2p counters it by connecting peroxisomes to Myo2p, a myosin motor protein that transports peroxisomes in the daughter cell along actin cables (Fagarasanu et al., 2007). We used these findings and performed live-cell imaging to analyze peroxisomes in microcolonies growing from single cells lacking either Inp1p or Inp2p in combination with Pex30p and/ or Pex29p absence (Fig. 6 and S3). Figure 6 illustrates that control cells contained peroxisomes all through cell division, while after division *inp2*-mutant daughter cells lacked peroxisomes. However, these daughter cells slowly regained peroxisomes through de novo biogenesis leading to about 50% of cells with peroxisomes in the microcolonies. Interestingly, in the additional absence of Pex30p more cells contained peroxisomes in the microcolonies. This was not due to a rescue of the inheritance defect as shown in the first division steps. Note that these cells also contained more peroxisomes than *inp2*Δ cells showing that absence of Pex30p affected both, de novo formation and growth/ division of peroxisomes. The additional lack of Pex29p enhanced the observed phenotype confirming that these two proteins act in concert (Fig. 6B).

DISCUSSION

The work presented herein provides a thorough analysis of membrane proteins acting as tethering complex between the ER and peroxisomes. Our findings expand the current insights into peroxisome proliferation by demonstrating for the first time that peroxisome maintenance entirely relies on proteins residing in the ER and on the integrity of the cortical ER tubular architecture.

Here, we studied the ER-to-peroxisome connection and identified authentic contact sites of the organelles via quantitative proteomics and imaging analyses. The Pex30p core complex contains two groups of proteins; *i)* proteins regulating peroxisome proliferation, and *ii)* ER resident proteins involved in the maintenance of the ER. Although in the yeast *P. pastoris* Pex30p was reported to localize both to the ER and to peroxisomes (Yan et al., 2008), *Sc*Pex30p was previously described as a peroxisomal membrane protein (Vizeacoumar et al., 2004). Our data demonstrate that similar to *P. pastoris*, *Sc*Pex30p is present at several locations (Fig. 3-5). This finding raises new questions with regard to Pex30p trafficking and function on whether this protein transits through the ER and back and whether these events are associated to its function in peroxisome proliferation. Our study indicates that Pex30p molecules undergo rapid transport through the ER network and segregate to subdomains of the cortical ER tubules (Fig. 3B). Our analyses of peroxisome dynamics show that Pex30p accumulates at specialized ER subdomains in which RHPs are also present (Fig. 3D, 4A) and suggest that these domains represent ER-to-peroxisomes contact sites (EPCONS; Fig. 5B-E).

Organelles can communicate and exchange material through inter-organellar membrane contact sites, whereby two organelles come into close apposition (Toulmay and Prinz, 2011). With regard to peroxisomes, the minimal requirement for their formation and function includes intensive crosstalk with other organelles (Braschi et al., 2010; Hoepfner et al., 2005; Lam et al., 2010). Associations of peroxisomes with the ER thus may enable the transfer of material between both compartments. Furthermore, a recent study in yeast suggested that non-vesicular phospholipid transfer could occur between peroxisomes and the ER (Raychaudhuri and Prinz, 2008).

The proteins RTN4a and DP1 as well as Rtn1p, Rtn2p and Yop1 affect the curvature of the ER membrane in mammalian and yeast cells, respectively (Hu et al., 2009; Shibata et al., 2009; Voeltz

et al., 2006). These integral membrane proteins bend the phospholipid bilayer through generation of a wedge in the outer leaflet explaining their partitioning into and stabilization of highly curved ER tubules (Voeltz et al., 2006; Zurek et al., 2011). While deletion of Rtn1p and Rtn2p affects the ER morphology only under stress conditions, the additional absence of Yop1p results in a disrupted tubular ER under normal growth conditions (Voeltz et al., 2006). Besides, atlastin proteins (Sey1p in yeast) were shown to be involved in the fusion of ER tubules to generate an interconnected network (Rismanchi et al., 2008). These proteins are therefore essential to maintain the net-like structure of the cortical ER. The different architectures of the ER membrane restrict biological processes to specialized areas (Lynes and Simmen, 2011). Moreover, very recent observations illustrate that specialized areas of the cortical ER mark sites for the division of mitochondria (Friedman et al., 2011). Likewise, our data show that RHPs are involved in the regulation of peroxisome proliferation (Fig. 4D).

While peroxisomes are known to originate from the ER, no resident ER protein had previously been identified that regulated this process. The existence of EPCONS is in good agreement with previous observations that ER subdomains exist which extend to peroxisomes (Geuze et al., 2003). We propose that EPCONS serve as molecular platform to regulate the formation of peroxisomes in time and space and that the tubular architecture of the ER achieved through RHPs is essential to regulate this process.

Our biochemical studies also substantiate a role for vesicular trafficking in the transport of peroxisomal membrane proteins. Indeed, we identified all subunits of the COPI coatomer complex as transient interacting partners of Pex30p suggesting a role in the retrograde transport of Pex30p (Fig. 2B-E). Alternatively, the transport of Pex30p en route to peroxisomes occurs through formation of vesicular tubular clusters originating from the cortical ER, an event that might require the function of COPI vesicles. The early peroxin Pex3p accumulated to tubular-vesicular structures in cells with hampered expression of COPI-vesicles tethering factors suggesting the involvement of coatomer in peroxisome biogenesis (Perry et al., 2009). In agreement, ADP-ribosylation factors (Arf), inherent components of coatomer, were reported to bind peroxisomes in vitro (Passreiter et al., 1998). In *S.*

cerevisiae, Arf1p was shown to be essential for the division of peroxisomes from existing peroxisomes and Arf3p stimulated proliferation (Lay et al., 2005). Interestingly, Pex30p is the only peroxin in *S. cerevisiae* whose amino acid sequence ends with an ER-retrieval dilysine motif (-KKXX).

Old morphological data already showed the proximity of peroxisomes and the ER in mammalian cells (Novikoff et al., 1974) but whether both organelles effectively connect and how the organellar interface is established is not known. It is tempting to speculate that EPCONS not only host pre-existing peroxisomes for growth/ divison but also represent ER exit sites during de novo biogenesis allowing the tight coordination of these two processes (Fig. 6). Indeed, our results suggest that peroxisomes in contact with the ER membrane do not proliferate (Fig. 1A, 5B, E).

Our finding that Pex30p regulates the association of peroxisomes to the ER sheds new light on how coordination of cellular functions is achieved and on how sub-cellular organelles interact and influence each other´s function to maintain cellular organization.

MATERIAL AND METHODS

Yeast Strains, Culture Media and Plasmids

The *S. cerevisiae* strains used are listed in Table 1. Cells were grown in SC medium (0.67% yeast nitrogen base) or in SC medium supplemented with 1% yeast extract and the appropriate amino acids as well as 2% D-glucose (Dextrose) or 2% galactose. Unless otherwise stated, cells were shifted to galactose medium for 15 min to induce *GAL*-driven *PEX30* expression followed by 15 min incubation in glucose medium. To induce peroxisome proliferation, cells were grown in YNO (0.67% yeast nitrogen base, 0.1% oleic acid, 0.05% Tween 80, pH 6.0 with KOH). For inheritance assays, cells were diluted to OD600 nm = 0.1 and 0.5 µl were plated onto agarose pads. For biogenesis assays, cells were cultured in glucose containing medium for three days prior to galactose induction. Standard techniques were used for transformation, sporulation and tetrad analysis of yeasts. Oleic acid utilization assays were performed as described (Brocard et al., 1997). Standard procedures were used for DNA engineering and bacterial transformation. The oligonucleotide sequences used in this study are listed in Table 2 and the cloning details are provided in Supplemental material.

Antibodies

Rabbit-anti-Pex30p antibodies (1:30,000) were produced using an affinity-purified fraction of the N-terminal 164 amino acids of Pex30p expressed in *E. coli* BL21. Mouse-anti-Porin antibodies (1:10,000) were purchased from Molecular Probes. Rabbit-anti-COPI coatomer antibodies (1:1,000) were a kind gift from Anne Spang (Basel, Switzerland). Rabbit-anti-Pex3p antibodies (1:5,000) were described before (Hohfeld et al., 1991). Rabbit-anti-Kar2p antibodies (1:20,000) were a kind gift from Ben Distel (University of Amsterdam, The Netherlands). HRP-conjugated sheep-anti-mouse and donkey-anti-rabbit antibodies (1:10,000) were purchased from GE Healthcare. Rabbit-anti-Protein A antibodies (1:10,000) were purchased from Sigma Aldrich.

Membrane Protein Complex Isolation using SILAC and coimmunoprecipitations

Cells were grown to OD600 nm = 1 in glucose medium (0.3% glucose) and 5 x YNO was then added to induce peroxisome proliferation for 16 h. Control yeast cells auxotrophic for lysine and arginine

were grown in SC medium containing 0.3% glucose complemented with $^{13}C_6$-coded "heavy" lysine and arginine (50 mg/l each), whereas the cells from TAP-tagged strains were grown in medium containing 50 mg/l $^{12}C_6$-coded "light" lysine and arginine. In AP-AM experiments, differentially labeled cells were mixed at 1:1 ratio in net weight immediately after harvest. Affinity purification of native protein complexes using IgG-sepharose was carried out as described previously (Agne et al., 2003) with some modifications. Briefly, the cells were lysed with glass beads in buffer containing 20 mM HEPES pH 7.5, 100 mM KOAc, 5 mM $Mg(OAc)_2$, 2 mM PMSF, 10 mM NaF and 10% glycerol, 0.3% digitonin, 2 µg/ml aprotinin, 0.35 µg/ml bestatin, 2.5 µg/ml leupeptin, 1 µg/ml pepstatin. The lysates were centrifuged at 100,000 g and the resulting pellets (adjusted to 3.3 mg protein/ml) were solubilized using 1% digitonin. Affinity-purification was performed from the solubilized extracts using human IgG conjugated to a CNBr-activated-Sepharose matrix and crosslinked with DMP. For mass spectrometric analysis, proteins in TEV eluates were precipitated using ice-cold acetone and resuspended in 0.1 M NaOH, 1% SDS. 2 x Laemmli sample buffer containing 8 M urea was added and proteins were separated on a 4-12% Bis-Tris NuPAGE gel (Invitrogen) according to the manufacturer's instructions.

In AP-PM experiments, isolation of membrane protein complexes was carried out separately for each strain. The TEV-eluates originating from control and TAP-tagged strains were equally mixed prior to acetone precipitation and proteins were separated by SDS-PAGE as described in Supplemental material. For co-immunoprecipitations using Rtn1p as bait, cells were lysed with glass beads in lysis buffer supplemented with 1 mM EDTA. Crude extracts (5 mg protein/ml) were directly solubilized with 1% digitonin for 30 min at 4°C. Solubilized extracts were incubated with human IgG-Sepharose matrix and further processed as described above.

Mass Spectrometry

Following separation of affinity-purified proteins by SDS-PAGE and staining with colloidal Coomassie Blue G-250, gel lanes were cut into 20 slices and processed for mass spectrometric analysis of tryptic peptide mixtures as described previously (Kaller et al., 2011). Nano HPLC/ESI-MS/MS analyses were performed using the UltiMate™ 3000 HPLC system (Dionex LC Packings,

Idstein, Germany) directly coupled to an LTQ-Orbitrap XL instrument (Thermo Fisher Scientific, Bremen, Germany). Full scan MS spectra (*m/z* 300 – 1,500; resolution of 30,000 at *m/z* 400) were acquired. After a brief survey scan, the four most intense multiply charged ions were selected for fragmentation by low energy collision-induced dissociation in the linear ion trap simultaneously with the completion of the MS scan in the orbitrap. More detailed information is provided in Supplemental material.

Mass Spectrometric Data Analysis and Statistics

For protein identification and SILAC-based relative protein quantification, mass spectrometric raw data were processed using MaxQuant (version 1.0.13.13) (Cox and Mann, 2008). MS/MS datasets were searched against a decoy version of the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org) using Mascot (version 2.2, Matrix Science) (Perkins et al., 1999). Proteins were identified based on at least one unique peptide with a minimum length of six amino acids and a false discovery rate of < 1% (see Supplemental material). To identify proteins significantly enriched with Pex29p or Pex30p in AP-AM and AP-PM experiments (n = 3 each), a one-sided t-test for each protein was performed. In addition, significance thresholds for proteins expressed in two or three experiments were calculated separately using power analysis. The standard deviation for this test was the 90% quantile of the observed protein standard deviations. Proteins with p-values < 0.05 and significant abundance ratios as determined by power analysis in the respective affinity-purification dataset were considered true components of Pex29p and Pex30p complexes.

Microscopy and Live-Cell Imaging

After galactose induction, cells were cultured in YNO for 8 h and subsequently transferred onto agarose pads precast on objective slides and imaged immediately. For live-cell imaging, the pad medium was identical to the growth medium supplemented with 1.7% low-melting agarose. Microscopy was performed on an Olympus Cell^R workstation (Objective: UPLSAPO 60x1.42; Camera: Hamamatsu Orca ER; Filter sets: BFP exc: 330-385 nm, em: LP420 nm; GFP exc: 457-487 nm; em: 503-538 nm; mCherry exc: 579-596 nm, em: 618-664 nm; Pixel spacing 107 x 107 x 300 nm or 107 x 107 x 1000 nm for live-cell imaging).

FRAP Experiments

For FRAP experiments, cells were treated as described above except for induction of *GAL*-expression for 2 h prior to microscopic analysis. Cells were immediately transferred onto agarose pads and imaged on an LSM 510 META unit (Objective α-NeoFluar 100 x 1.45; Filter set: MBS488, LP505; Laser: 488 nm; Pixel spacing: 30 x 30 nm; Pixel dwell time: 2.7 µs). Bleaching was performed after three prescans on multiple small areas of 0.5 μm^2 and recovery was monitored until saturation.

Trajectory Analysis

Cells lacking Pex30p and cells expressing Pex30p-GFP were imaged live as described above, but in 1 min intervals for 0.5 h. After deconvolution, the tracks were extracted and analyzed using the SPT software package as described before (Sbalzarini and Koumoutsakos, 2005). Briefly, the trajectory analysis consists of two parts, *i*) particle recognition and trajectory creation, and *ii*) trajectory analysis. The parameters used for particle recognition were the same for all images and tracks longer than 20 (of 30) frames were used for analysis. The trajectories of all mCherry-Px dots (peroxisomes) from more than 10 different cells were analyzed for each strain. The analysis is based on the calculation of the moments of displacements μ_ν (Sbalzarini and Koumoutsakos, 2005) and plotting log μ_ν versus the total time interval. Plots were created for the first 10 moments of displacement μ_ν (ν = 1,2,...,10) and the respective scaling coefficient γ_ν was extracted through a linear least squares fit. To calculate the mean square displacement (MSD), the second order of displacement (ν = 2) was used to calculate the two-dimensional diffusion coefficient D_2 (y-intercepts). For strong self-similar movements D_2 represents the regular diffusion coefficient. The moment scaling spectrum (MSS) was produced by plotting the first 10 scaling coefficients in function of the order of the respective moment. In the analyzed scenario, most trajectories were strongly self-similar as confirmed by the linearity of the MSS curve. Here, the slope gives appraise for the type of motion: slopes < 0.5 correspond to sub-diffusive, slopes > 0.5 to super-diffusive and slopes around 1 to ballistic movements. Values for the generalized two-dimensional diffusion coefficients D_2 and for the MSS-slopes (S_{MSS}) were exported from the SPT software, and a scatter plot was arranged with a two-dimensional box plot. Each box ranging from the first to the third quartile contains 50% of the data points. The whiskers encompass

95% of the data, excluding the topmost and bottommost 2.5%. To estimate the statistical difference between the datasets a Wilcoxon rank sum test was performed on http://elegans.swmed.edu/~leon/stats/utest.html.

Image Processing, Analysis and Counting of Peroxisomes

All images presented but Fig. 4D were deconvolved as 3D data sets with an experimentally derived PSF using the CMLE algorithm in Huygens Professional (SVI, The Netherlands). Blue or white cell surroundings were obtained from transmission images through blurring and artificial colorizing. For statistical analysis of the total number of peroxisomes per cell, fluorescent spots (mCherry-Px) were counted manually on 3D data sets of more than 100 single cells and at least three independent cultures for each strain. Colocalization analyses were performed using the ImageJ Plugin "Colocalization Colormap" based on the normalized mean deviation product (nMDP) described previously (Jaskolski et al., 2005). A colorized heat map provides spatial information about colocalizing pixels and the correlation coefficient, I_{corr} represents a global measurement for the whole image. I_{corr} values were obtained for 30 cells of three independent cultures. For FRAP analyses, image intensities were quantified in ImageJ (NIH). Background was subtracted and the bleached signal was normalized to non-bleached areas. Signal intensity at time point 0 was set to 100%. Recovery was fitted using the function $y=a[1-\exp(-bx)]+c$.

SUPPLEMENTAL MATERIAL

Figure S1 shows the protein levels of Pex30p and Pex30p-GFP after induction with galactose followed by incubation on media containing either glucose or oleic acid. Figure S2 shows colocalization of Pex29p-GFP, Pex30p-GFP or Pex32p-GFP with the ER marker Sec63p-RFP 8 h after induction of peroxisome proliferation on medium containing oleic acid and colocalization of Pex29p-GFP, Pex30p-GFP or Pex32p-GFP with the peroxisomal matrix marker mCherry-Px 16 h after induction of peroxisome proliferation. Figure S3 shows inheritance assays using *inp1*Δ cells. Supplemental material can also be found with this manuscript.

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ABBREVIATIONS LIST

AP-AM, affinity-purification after mixing; AP-PM, affinity-purification prior to mixing; EPCONS, ER-to-peroxisome contact sites; MS, mass spectrometry; PEX, peroxin; RFP, red fluorescent protein; RHPs, reticulon homology-domain proteins; SILAC, stable isotope labeling with amino acid in cell culture; TAP, tandem affinity-purification tag; TEV, tobacco etch virus protease.

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FIGURE LEGENDS

Figure 1: Pex30p and Pex11p act at different steps of peroxisome proliferation. (A) Quantitative distribution of peroxisomes in yeast cells with various genetic backgrounds (as indicated) expressing mCherry-Px (red channel) as illustrated in a 3D model. Yeast cells were transformed with plasmids expressing *PEX30* driven by the *GAL* promoter or *GPD*-controlled *PEX11* or *PEX25*. The mutant cells *PEX30ΔC* expressed a truncated version of Pex30p lacking amino acids 375-523 from the *PEX30* genomic locus. After galactose induction, cells were further incubated in medium containing oleic acid to induce peroxisome proliferation and observed 8 h later. For each yeast strain, fluorescent dots (mCherry-Px) were counted in three dimensions through a whole Z-stack in at least 100 cells from three independent cultures. The histograms illustrate the frequency distributions of cells (in percent) with a distinct number of peroxisomes. The average number of fluorescent mCherry-Px dots per cell is indicated as mean ± SD and tested for statistical significance (bottom panel). The dashed red lines represent the average number of peroxisomes in wild type cells. (B) Yeast cells with various genetic backgrounds, as indicated untransformed or transformed with plasmids expressing Pex11p, Pex25p, Pex30p or combinations were grown to logarithmic phase in glucose medium and 10-fold serial dilutions were spotted onto solid medium containing either glucose or oleic acid. The function of peroxisomes was monitored through visualization of oleate utilization which led to the occurrence of transparency in the solid medium.

Figure 2: SILAC-based analysis of Pex30p-containing membrane protein complexes via affinity-purification and quantitative mass spectrometry. (A) Yeast cells auxotrophic for arginine and lysine were grown in medium containing oleic acid (left panel). (1) Differentially labeled cells expressing either native (control, "Heavy" amino acids) or TAP-tagged Pex30p (bait, "Light" amino acids) were mixed in equal ratio. (2) Affinity-purification after mixing (AP-AM) experiments were performed from purified membrane fractions and (3) allowed for accurate characterization of the Pex30p-containing complexes by quantitative mass spectrometry. The bait and specific binding partners exhibited high SILAC ratios, while proteins with ratios of approximately one were considered

co-purified contaminants. The peroxisomal membrane proteins Pex28p, Pex29p, and Pex32p as well as the reticulon homology proteins Rtn1p, Rtn2p and Yop1p are core components of Pex30p complexes (right panel). Proteins were plotted according to their p-values ($-\log_{10}$) against the mean $\log_{10}$ SILAC ratios determined in AP-AM experiments (n = 3). Specific binding partners illustrated in black and colored dots exhibit p-values of ≤ 0.5 and mean $\log_{10}$ ratios "light-to-heavy" of ≥ 0.66 when quantified in 3/3 replicates and ≥ 1.66 in 2/3 replicates. (B) Experimental approach of SILAC-based analyses of Pex30p-containing membrane protein complexes via affinity-purification prior to mixing (AP-PM). Yeast cells were prepared (left panel, 1) as described in (A). AP-PM experiments from purified membranes were performed to allow for identification of stable and transient interaction partners by quantitative mass spectrometry (2). The bait, core components and specific transient binding partners exhibited high SILAC ratios, while proteins with ratios of approximately one were considered co-purified contaminants (3). Interactomes of the peroxisomal membrane protein Pex30p (right panel). Proteins were plotted according to their p-values ($-\log_{10}$) versus the mean $\log_{10}$ SILAC ratios determined in AP-PM experiments (n = 3). Specific binding partners of Pex30p exhibit p-values of ≤ 0.5 and mean $\log_{10}$ ratios "light-to-heavy" of ≥ 1.10 when quantified in 3/3 replicates and ≥ 2.79 in 2/3 replicates as well as ≥ 0.75 and ≥ 1.89, respectively. Specific components of Pex30p complexes are labeled in black, red for peroxins involved in peroxisome proliferation, green for ER proteins with a function in ER maintenance or in blue for subunits of the COPI coatomer complex. (C) Specific association between COPI components and Pex30p. Shown are zoom-in spectra of SILAC-encoded peptides derived from the bait Pex30p, subunits of the COPI coatomer complex and co-purified mitochondrial protein Kgd1p. (D) AP-PM experiments for Pex29p-containing membrane protein complexes were performed as described in (B). (E) Cells expressing TAP-tagged subunits of the coatomer complex or Pex14p^{-TAP}, a subunit of the peroxisomal importomer, from their respective genomic locus were used for affinity purification with IgG-coated sepharose beads. Whole cell lysates (Input) and eluates including supernatants after TEV digestion (TEV) and non-eluted material (SDS) were analyzed by immunoblotting with anti-Protein A, anti-Pex30p, anti-COPI or anti-Porin antibodies. Signals for the TAP-tagged COPI subunits show slower electrophoretic mobility in the input lanes due to the Protein A repeats (arrowheads) but not in the TEV eluates.

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Figure 3: Pex30p localizes to the ER and accumulates at subdomains of the cortical ER in patches that coincide with Rtn1p but not with Sec63p. (A) Yeast cells with various genetic backgrounds (as indicated) expressing TAP-tagged Rtn1p (Rtn1p^{-TAP}) from their genomic locus were used for affinity-purification. Equal amounts of proteins from digitonin solubilized membrane fractions (Inputs) and supernatants from fractions treated with the TEV protease (Eluates) were analyzed by immunoblotting using anti-Pex30p antibodies. The levels of Rtn1^{-TAP} detected in the input fractions by the rabbit IgG serve as loading control. In the last two lanes of the right panel wild type cells contained multicopy plasmids expressing *PEX29* either controlled by the *ADH1*- or the endogenous *PEX29*-promoter. (B) Mutant *pex30*Δ yeast cells expressing plasmid-borne Sec63p-RFP (red channel) and Pex30p-GFP (green channel) controlled by the *GAL*-promoter were treated as indicated in Fig. 1. Pex30p-GFP partially colocalized with Sec63p-RFP and accumulated in punctae (arrow) devoid of Sec63p-RFP as illustrated by the fluorescence intensity distribution (dashed lines and profiles). The blue color depicts the cell wall obtained from transmission images. (C) Cells lacking the endogenous *PEX30* gene expressing the peroxisomal marker protein mCherry-Px (red channel) and Pex30p-GFP (green channel) were cultured as described in (A). Colocalization was analyzed for the indicated cells as depicted in the color-coded maps for spatial discrimination. Correlation between peroxisomes and Pex30p is indicated (arrowheads). (D) Cells expressed Rtn1p-mCherry (red channel) from the *RTN1* genomic locus and plasmid-borne Pex30p-GFP (green channel). Rtn1p-mCherry colocalized with Pex30p-GFP in the ER. The perinuclear ER contained less Rtn1p-mCherry signal (open arrowhead) which rather distributed to the cortical ER. Especially, Rtn1p-mCherry accumulations coincided with Pex30p-GFP punctae as illustrated by the line profiles (arrow). (E) In cells grown with glucose, Pex30p-GFP did not form punctae, whereas on both growth conditions the Rtn1p-mCherry stainings were indistinguishable (D, E).

Figure 4: Maintenance of the cortical ER tubular structure through RHPs contributes to the regulation of peroxisome number. (A) Mutant cells *rtn1*Δ (left panel) and *rtn1*Δ with plasmid-

encoded Rtn1p (right panel) expressing Yop1p-mCherry (red channel) from the *YOP1* genomic locus were transformed with plasmids coding for Pex30p-GFP (green channel). Cells were treated as indicated in Fig. 1. (B) Cells lacking Rtn1p, Rtn2p and Yop1p were treated as indicated in Fig. 1 and images were acquired for mCherry-Px (red channel). Note the high number of peroxisomes in these cells (11 ± 2.3; $n > 100$) and their tendency to cluster (arrowheads). (C) Localization of Pex30p-mCherry with respect to Sec63p-GFP (ER) and BFP-Px (peroxisomes) in cells lacking Pex30p or the RHPs as indicated. (D) Peroxisome biogenesis was monitored through expression of *GAL*-driven *PEX3* in control cells or in cells lacking the RHPs chromosomally expressing mCherry-Px as indicated. The graph shows the percentage of cells containing peroxisomes in function of the incubation time in galactose medium. Protein extracts were analyzed by immunoblotting for the indicated time points (D, Dextrose; G, Galactose). Equal amounts of proteins were loaded in each lane. After protein transfer onto nitrocellulose, the membrane was cut in two parts; the upper part was probed with anti-Kar2p (Kar2p, 74.4 kD) and the lower part with anti-Pex3p (Pex3p, 50.6 kD) antibodies.

Figure 5: Pex30p traffics through the ER and peroxisomes adhere to Pex30p patches at ER subdomains. (A) FRAP experiments were performed in *pex30*Δ yeast cells expressing either Sec63p-GFP or Pex30p-GFP (green channel). Small regions of the perinuclear or cortical ER fluorescence were bleached as indicated and fluorescence recovery was monitored (closed arrowhead, upper panel). For Pex30p-GFP, three regions were chosen for bleaching as indicated and fluorescence recovery was monitored within the perinuclear and the cortical ER (closed arrowheads) as well as in a fluorescent dot at the cell periphery (open arrowhead). The images show representative experiments. Quantifications of fluorescence intensities from at least five independent experiments are illustrated for each studied region. Error bars represent the standard error. (B) The fluorescence emitted by mCherry-Px (red channel) and Pex30p-GFP (green channel) was monitored live starting 3 h after plating the cells on agarose pads containing oleic acid. A portion of the mCherry-Px signal gathered to the Pex30p-GFP accumulations forming at later time points (closed arrowheads) and at the bud tip

(open arrowhead). (C) Mutant *pex30*Δ cells expressing either mCherry-Px (red channel) only or Pex30p-GFP (green channel) in addition were imaged in 60 sec intervals. Representative trajectories of mCherry-Px dots were visualized in a *pex30*Δ cell (#1) and in a *pex30*Δ cell expressing Pex30p-GFP. Trajectory #2 corresponds to a mobile mCherry-Px dot whereas #3 adhered to the depicted Pex30p-GFP puncta (#4). Cell walls are shown based on transmission images (blue color). (D) Analysis of the mCherry-Px trajectories (#1-3) shown in (C) for two different parameters. The mean square displacement (MSD, upper plot) allows the calculation of the diffusion coefficients (D_2). The type of motion can be discriminated via the slopes of the corresponding moment scaling spectra (MSS, lower plot). (E) The scatter plot summarizes all trajectories studied by showing the diffusion coefficients (D_2) extracted from the MSD diagram versus the slope from the MSS (S_{MSS}). Peroxisomal trajectories are indicated for *pex30*Δ mutant cells (blue circles; n = 251), *pex30*Δ expressing Pex30p-GFP (red crosses; n = 108), *rtn1*Δ*rtn2*Δ*yop1*Δ cells (green crosses; n = 327) and for *pex30*Δ*rtn1*Δ*rtn2*Δ*yop1*Δ (gray triangles; n = 197). For each strain studied, trajectories derived from measurements of all visible mCherry-Px dots (peroxisomes) in more than 10 cells. For each peroxisomal population a two-dimensional box plot was overlaid and the statistical significance was assessed ($p<0.05$).

Figure 6: De novo biogenesis of peroxisomes is improved in cells lacking Pex30p. (A) In wild type cells, the number of peroxisomes doubles shortly before cell division and both mother and daughter inherit half of the peroxisome pool. In the absence of Inp2p, all peroxisomes are retained in the mother cell upon cell division. Peroxisomes are slowly regenerated de novo in daughter cells. (B) Cells expressing mCherry-Px were plated onto agarose pads containing glucose medium and the formation of microcolonies originating from single cells were observed live for a total of 12 h in 15 min intervals. The experiments were performed on three independent cultures. Each time, 8 single cells were imaged simultaneously, one example of which is shown for each strain as indicated. Note that cells lacking Pex30p or Pex29p and Pex30p contain many peroxisomes. The arrowheads indicate mother cells.

Table 1: Yeast strains used in this study.

Table 2: Oligonucleotide sequences used in this study.

LEGEND TO SUPPLEMENTAL FIGURES AND TABLE

Supplemental Figure 1: Analysis of Pex30p and Pex30p-GFP expression. Wild type cells or *pex30*Δ cells containing a plasmid coding for Pex30p-GFP controlled by the *GAL*-promoter were analyzed for the presence of Pex30p. Cells were grown to logarithmic phase on glucose medium, incubated for 15 min in galactose medium and immediately transferred to glucose medium for 15 min. Cells were then incubated for 3, 8 or 16 hours in glucose or oleic acid-containing medium. Whole cell lysates were prepared and equal amounts of total proteins were analyzed by immunoblotting using antibodies specific to Pex30p or Kar2p, an ER membrane protein, which served as control for protein loading.

Supplemental Figure 2: Localization of Pex29p-GFP, Pex30p-GFP and Pex32p-GFP 8h and 16h after induction of peroxisome proliferation. *pex29*Δ, *pex30*Δ or *pex32*Δ mutant cells expressed Pex29p-GFP, Pex30p-GFP or Pex32p-GFP from plasmids controlled by the *GAL*-promoter and either Sec63p-RFP (left panels) or mCherry-Px (right panels). After 4 hours galactose induction, expression was stopped by incubation of the cells in glucose medium (15 min). Then the cells were further incubated in medium containing oleic acid and observed either 8 hours (left panels) or 16 hours (right panels) later. Colocalization was analyzed for the indicated cells as illustrated in the color-coded maps for spatial discrimination.

Supplemental Figure 3: De novo formation of peroxisomes is more efficient in the absence of Pex30p. (A) Upon cell division cells lacking Inp1p segregate all peroxisomes in the daugther cell. Peroxisomes are slowly generated de novo in mother cells. (B) Cells expressing mCherry-Px were plated onto agarose pads containing glucose medium and the formation of microcolonies originating from single cells were observed live for a total of 12 hours in 15 minute intervals. The experiments were performed on three independent cultures. Each time 8 single cells were imaged simultaneously, one example of which is shown for each strain as indicated. The arrowheads indicate the mother cells.

Supplemental Table 1: (A) Proteins identified and quantified in triplicate AP-AM experiments using Pex30p^{-TAP} as bait. For peptide and protein identification and SILAC-based relative quantification, mass spectrometric data were processed by MaxQuant (version 1.0.13.13) and Mascot (version 2.2). Proteins classified as components of Pex30p protein complexes were required to exhibit a sequence coverage of ≥ 5%, a posterior error probability (PEP) of < 0.01, a p-value of < 0.05 and a mean $\log_{10}$ ratio light-to-heavy (L/H) of ≥ 0.66 when quantified in 3/3 replicates and ≥ 1.66 in 2/3 replicates as determined by power analysis. (B) Proteins identified and quantified in triplicate AP-PM experiments using Pex30p-TAP as bait. Peptide and protein identification and SILAC-based relative quantification were performed using MaxQuant (version 1.0.13.13) and Mascot (version 2.2). Proteins classified as components of Pex30p protein complexes were required to meet the following criteria: sequence coverage, ≥ 5%; posterior error probability (PEP), < 0.01; p-value, < 0.05; mean log10 ratio light-to-heavy (L/H) of ≥ 1.10 when quantified in 3/3 replicates and ≥ 2.79 in 2/3 replicates as determined by power analysis. (C) Proteins identified and quantified in triplicate AP-PM experiments using Pex29p-TAP as bait. Identification and SILAC-based relative quantification of peptides/proteins was performed by processing mass spectrometric data with MaxQuant (version 1.0.13.13) and Mascot (version 2.2). Criteria applied to proteins that were considered as specific components of Pex29p complexes were as follows: sequence coverage of ≥ 5%, posterior error probability (PEP) value of < 0.01, p-value of < 0.05, mean log10 ratio light-to-heavy (L/H) of ≥ 0.75 when quantified in 3/3 replicates and ≥ 1.89 in 2/3 replicates (determined by power analysis).

For details on protein identification, relative quantification and statistical data evaluation, see Materials and Methods in the manuscript and Supplemental material. SEM, standard error of the mean; NA, not available.

Movie 1A: Dynamics of Pex30p-GFP in cells grown on medium containing oleic acid

Mutant cells lacking the *PEX30* gene were transformed with plasmids coding for Pex30p-GFP (green channel) and mCherry-Px (red channel) and imaged live under peroxisome proliferating conditions. Expression of Pex30p-GFP was induced by incubating the cells in galactose medium (15 min) and stopped by incubating the cells in glucose medium (15 min). Next, cells were incubated in liquid medium containing oleic acid for 3 hours and plated out on oleic acid-containing agarose pads and imaged immediately for a total of 12 hours. The blue color represents the cell walls obtained from transmission images. Selected time points are shown in Fig. 5B. Note the accumulation of Pex30p-GFP fluorescence at the bud tip and the presence of mCherry-Px nearby Pex30p-GFP ER punctae.

Movie 1B: Tracking analysis of peroxisomes in *pex30*Δ cells with and without Pex30p

*pex30*Δ mutant cells were transformed with plasmids coding for mCherry-Px (red channel) only (A) or mCherry-Px and Pex30p-GFP (green channel) (B). Cells were induced on galactose medium (15 min) and incubated for 8 h in oleic acid medium. Cell walls were drawn based on the transmission images (blue color). Cells were subsequently imaged live for 30 min with a one minute time interval to ensure suitable resolution of the peroxisomal and Pex30p punctae tracks. Tracking of the selected features is shown in Fig. 5C-E.

SUPPLEMENTAL MATERIAL

Plasmids

Plasmids were amplified in the *Escherichia coli* strain DH5α (Hanahan et al., 1983). *ADH1p*-mCherry-Px (pCB741) was created by cloning the amplified *ADH1*-promoter (SacI/ XbaI; primers: H911/H912) and mCherry-Px (XbaI/ HindIII; primers CB293/CB112) into YEplac195. *MLS*prom-BFP-Px (pCB 579) was created by cloning the amplified BFP-Px (primer: CB180/181) via a pGEM-T vector into pJR233 conferring ura auxotrophy (BamHI/ HindIII). pBFP-Px (pCB 858) BFP-Px was shuffled into pCB633 (BamHI/ HindIII) for leucine prototrophy. *GALSp-PEX30-GFP* was created by PCR using genomic DNA as template (CB517, primers CB320/CB244), cloned into the pGEM-T vector (Promega) to give rise to pCB822 and subcloned into a YEplac181 (SphI/PstI) resulting in pCB823. *GALS*prom-PEX30-mCherry (pCB855) was established by replacing DsRed against mCherry (BstXI/ KpnI) from a PCR product (primer: CB406/407) in pCB854 previously obtained via cloning the amplified *GALS*prom-PEX30-DsRed from genomic DNA (CB509, primer: CB388/389) into a YEplac112 (SphI/ KpnI). *GALS*p-*PEX32-GFP* (pCB849) was obtained by replacing *PEX30-GFP* (pCB823; XbaI/ PstI) through *PEX32-GFP* amplified from genomic DNA (CB214, primer CB381/CB382). Similarly, for *GALS*p-*PEX29-GFP* (pCB851), *PEX32-GFP* was replaced by *PEX29-GFP* (BglII/ PstI) amplified from genomic DNA (CB208, primers: CB383/CB382). *PEX29* and its cognate promoter were amplified by PCR using genomic DNA as template (CB80A; primers: CB203/H869) and cloned into YEplac195 (SalI; pCB747). The *ADH1p-PEX29* construct (pCB746) was created by cloning the PCR generated *PEX29* (primers: CB301/H891) into the YEplac195-*ADH1*p (SalI/PstI) construct mentioned above. The plasmid containing *GPDp-PEX*11 has been described previously (Huber et al., 2011). pCB861 containing GPDprom-*PEX25* was obtained from recombination of tpENTR4-*PEX25* (Koch et al., 2010) with the destination vector pAG416-*GPD* (Euroscarf) using the GATEWAY technology (Invitrogene). The plasmids coding for Sec63p-RFP and Sec63p-GFP controlled by the *SEC63* promoter were a kind gift from Jeffrey Gerst (Rehovot, Isreal). pCB842 was created from pFA6-natNT2 (Euroscarf) by inserting *ADH1*prom-mCherry-Px (SacI) obtained via PCR (pCB741, primers: CB348/ CB349). To create pCB856, *ADH1*prom-mCherry-Px

was cloned into pCB840 (SacI, Huber et al., 2011). For the integration into the *S. cerevisiae* genome, PCR fragments were generated using the templates pFA6-hphNT1 (Euroscarf), pCB842 (*ADH1*prom-mCherry-Px::natNT2), or pCB856 (*ADH1*prom-mCherry-Px::hphNT1) and the appropriate primer pairs as indicated in Table 2 and transformed using standard procedures. All genomic integrations were tested by PCR and southern blot when required.

LC/MS Analysis

The UltiMate™ 3000 system (Dionex LC Packings, Idstein, Germany) used for peptide separation by nanoflow reversed-phase (RP) capillary HPLC was equipped with two C18 µ-precolumns (0.3 mm inner diameter x 5 mm, particle size 5 µm; PepMap, Dionex LC Packings) and a C18 RP nano LC column (75 µm inner diameter x 150 mm; particle size 5 µm; PepMap, Dionex LC Packings). Peptides were eluted using a 30-min linear gradient ranging from 4% - 40% acetonitrile in 0.1% formic acid. The LTQ-Orbitrap XL, equipped with a nanoelectrospray ion source (Thermo Fisher Scientific) and distal coated SilicaTips, was operated using the following parameters: spray voltage, 1.6 kV; capillary voltage, 45 V; capillary temperature, 200°C; tube lens voltage, 100 V; automatic gain control, 5 x 10^5 ions in the orbitrap and 10,000 ions in the LTQ; maximum fill time, 750 msec (orbitrap) and 150 msec (LTQ); normalized collision energy, 35% with an activation $q = 0.25$ and an activation time of 30 msec; ion selection threshold, 500; dynamic exlusion time for fragmentation of previously selected precursor ions, 60 sec.

Protein Identification and Quantification

MaxQuant data analyses (version 1.0.13.13) and data base searches by Mascot [version 2.2, Matrix Science (Perkins et al., 1999)] were performed using the following parameters: mass tolerances for parent and fragment ions, 7 ppm and 0.5 Da, respectively; tryptic specificity allowing two missed cleavages and three labeled amino acids; variable modifications, oxidation of methionine; no fixed modifications; $^{13}C_6$-arginine and -lysine were set as heavy labels; peptide and protein false discovery rate, 0.01. The decoy version of the *Saccharomyces* Genome Database used for peptide and protein identification included the original and a shuffled variant of each protein sequence. For relative protein quantification by MaxQuant, the following parameters were selected: only unique peptides were

quantified; minimum ratio count, one; "Re-quantify" was enabled; "Filter labeled amino acids" and "Keep low-scoring versions of identified peptides" were disabled.

SUPPLEMENTAL REFERENCES

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Table 1: Yeast strains used in this study

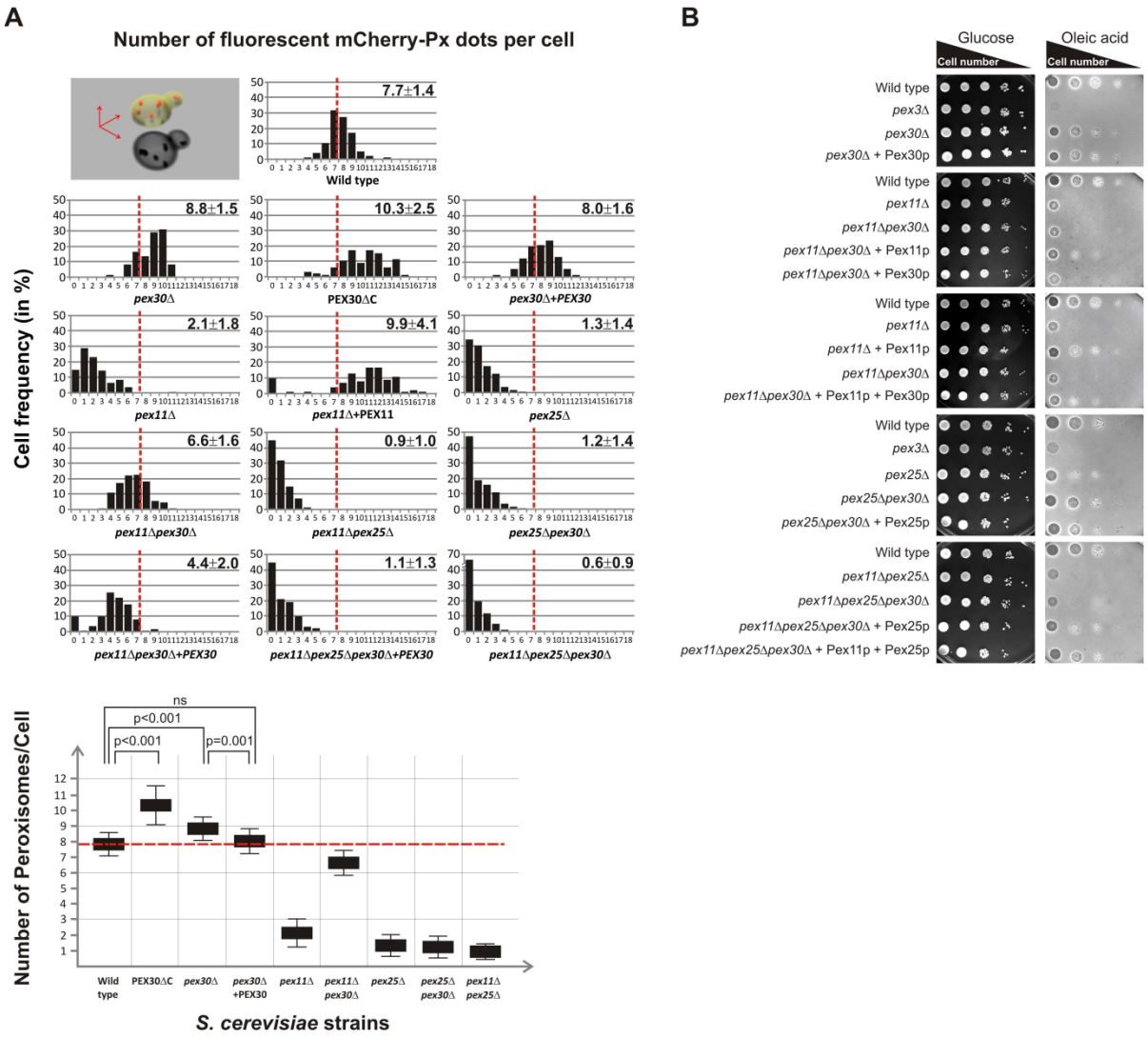
Name	Genotype	Source
CB80	Mat A, *ura3-52, leu2-1, trp1-63, his3-200*	*Brocard et al., 1997*
CB199	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, lys1::NatMX3, arg4::NatMX3*	*This study*
CB200	Mat α, *ura3-52, leu2-1, trp1-63, his3-200, lys1::NatMX3, arg4::NatMX3*	*This study*
CB251	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex29::KanMX4*	*This study*
CB254	Mat α, *ura3-52, leu2-1, trp1-63, his3-200, pex30::KanMX4*	*This study*
CB257	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex32::KanMX4*	*This study*
CB267	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex29::KanMX4, pex30::KanMX4*	*This study*
CB369	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex11::KanMX4*	*Huber et al., 2011*
CB371	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex25::KanMX4*	*Huber et al., 2011*
CB374	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, lys2Δ0, pex11::KanMX4, pex25::KanMX4*	*Huber et al., 2011*
CB386	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, rtn1::KanMX4, rtn2::KanMX4*	*This study*
CB392	Mat α, *ura3-52, leu2-1, trp1-63, his3-200, lys1::NatMX3, arg4::NatMX3,* ***PEX30::TAP****::His3MX4*	*This study*
CB404	Mat A, *ura3-52, leu2-1, trp1-63, his3-200,* ***RTN1::TAP****::KlTRP1*	*This study*
CB406	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex28::KanMX4,* ***RTN1::TAP****::KlTRP1*	*This study*
CB407	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex29::KanMX4,* ***RTN1::TAP****::KlTRP1*	*This study*
CB408	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex30::KanMX4,* ***RTN1::TAP****::KlTRP1*	*This study*
CB409	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex31::KanMX4,* ***RTN1::TAP****::KlTRP1*	*This study*
CB461	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex32::KanMX4,* ***RTN1::TAP****::KlTRP1*	*This study*
CB472	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex28::KanMX4, pex29::KanMX4, pex31::KanMX4, pex32::KanMX4,* ***RTN1::TAP****::KlTRP1*	*This study*
CB476	Mat A, *ura3-52, leu2-1, trp1-63, his3-200,* ***PEX30ΔC::TEV-ProteinA****::KanMx4*	*This study*
CB509	Mat α, *ura3-52, leu2-1, trp1-63, his3-200,* ***GALS*****prom*****::natNT2****,* ***Pex30-dsRed****::klTRP1, pex3::KanMX4*	*This study*
CB510	Mat α, *ura3-52, leu2-1, trp1-63, his3-200, rtn2::KanMX4,* ***RTN1::TAP****::KlTRP1*	*This study*
CB511	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, yop1::NatMX4, rtn1::KanMX4, rtn2::KanMX4*	*This study*
CB513	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, lys1::NatMX3, arg4::NatMX3,* ***PEX29::TAP****::KanMX4*	*This study*
CB528	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex11::KanMX4, pex30::KanMX4*	*This study*
CB548	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex3::KanMX4*	*This study*
CB549	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex30::KanMX4, pex25::NatNT2*	*This study*
CB550	Mat A, *ura3-52, leu2-2, trp1-63, his3-200, pex11::KanMX4, pex30::KanMX4, pex25::NatNT2*	*This study*
CB557	Mat A, *ura3-52, leu2-1, trp1-63, his3-200,* ***inp1::ADH1*****prom-*****mCherry-Px****::natNT2*	*This study*
CB559	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex30::KanMX4,* ***inp1::ADH1*****prom-*****mCherry-Px****::natNT2*	*This study*
CB560	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, pex29::KanMX4, pex30::KanMX4,* ***inp1::ADH1*****prom-*****mCherry-Px****::natNT2*	*This study*
CB562	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, rtn1::KanMX4, rtn2::KanMX4,* ***pex11::ADH1*****prom-*****mCherry-Px****::natNT2*	*This study*
CB564	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, rtn1::KanMX4, rtn2::KanMX4,* ***pex30::ADH1*****prom-*****mCherry-Px****::natNT2*	*This study*
CB575	Mat A, *ura3-52, leu2-1, trp1-63, his3-200,* ***leu2Δ0::ADH1*****prom-*****mCherry-Px****::natNT2*	*This study*
CB578	Mat A, *ura3-52, leu2-1, trp1-63, his3-200, rtn1::KanMX4, rtn2::KanMX4, yop1::hphNT1*	*This study*

CB579	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, *rtn1::KanMX4*, *rtn2::KanMX4*, *yop1::hphNT1*, ***leu2Δ0::ADH1*prom-*mCherry-Px***::*natNT2*	*This study*
CB584	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, ***PEX3prom::GALSprom***::*natNT2*, ***leu2Δ0::ADH1*prom-*mCherry-Px***::*hphNT1*	*This study*
CB586	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, *rtn1::KanMX4*, *rtn2::KanMX4*, ***PEX3prom::GALSprom***::*natNT2*, ***yop1::ADH1*prom-*mCherry-Px***::*hphNT1*	*This study*
CB587	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, ***inp2::ADH1*prom-*mCherry-Px***::*natNT2*	*This study*
CB589	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, *pex30::KanMX4*, ***inp2::ADH1*prom-*mCherry-Px***::*natNT2*	*This study*
CB590	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, *pex29::KanMX4*, *pex30::KanMX4*, ***inp2::ADH1*prom-*mCherry-Px***::*natNT2*	*This study*
CB592	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, *rtn1::KanMX4*, *rtn2::KanMX4*, ***pex11::ADH1*prom-*mCherry-Px***::*natNT2*, *yop1::hphNT1*	*This study*
CB593	Mat A, *ura3-52*, *leu2-1*, *trp1-63*, *his3-200*, *rtn1::KanMX4*, *rtn2::KanMX4*, ***pex30::ADH1*prom-*mCherry-Px***::*natNT2*, *yop1::hphNT1*	*This study*
CWY2754	BY4742, ***RTN1::mCherry***::*HIS*	*Lee et al 2010*
CWY2762	BY4742, *rtn1::KanMX4*, ***YOP1::mCherry***::*HIS3*	*Lee et al 2010*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***PEX29::GFP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***PEX32::GFP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***RET1::TAP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***SEC26::TAP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***SEC27::TAP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***SEC28::TAP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***SEC21::TAP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***RET2::TAP***::*HisMX6*	*Open Biosystems*
BY4741	Mat A, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*, ***PEX14::TAP***::*HisMX6*	*Open Biosystems*

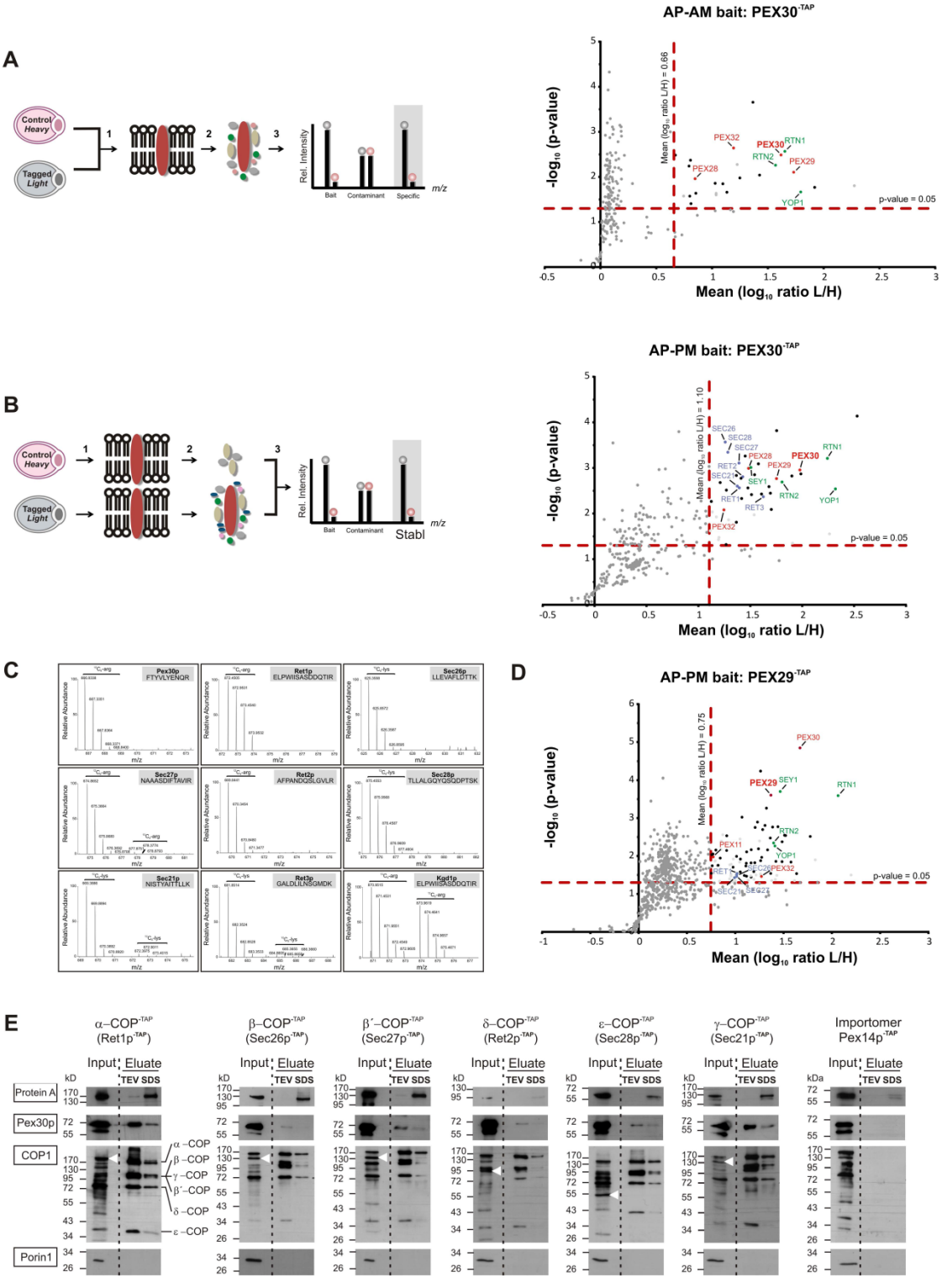
Table 2: Oligonucleotides used in this study

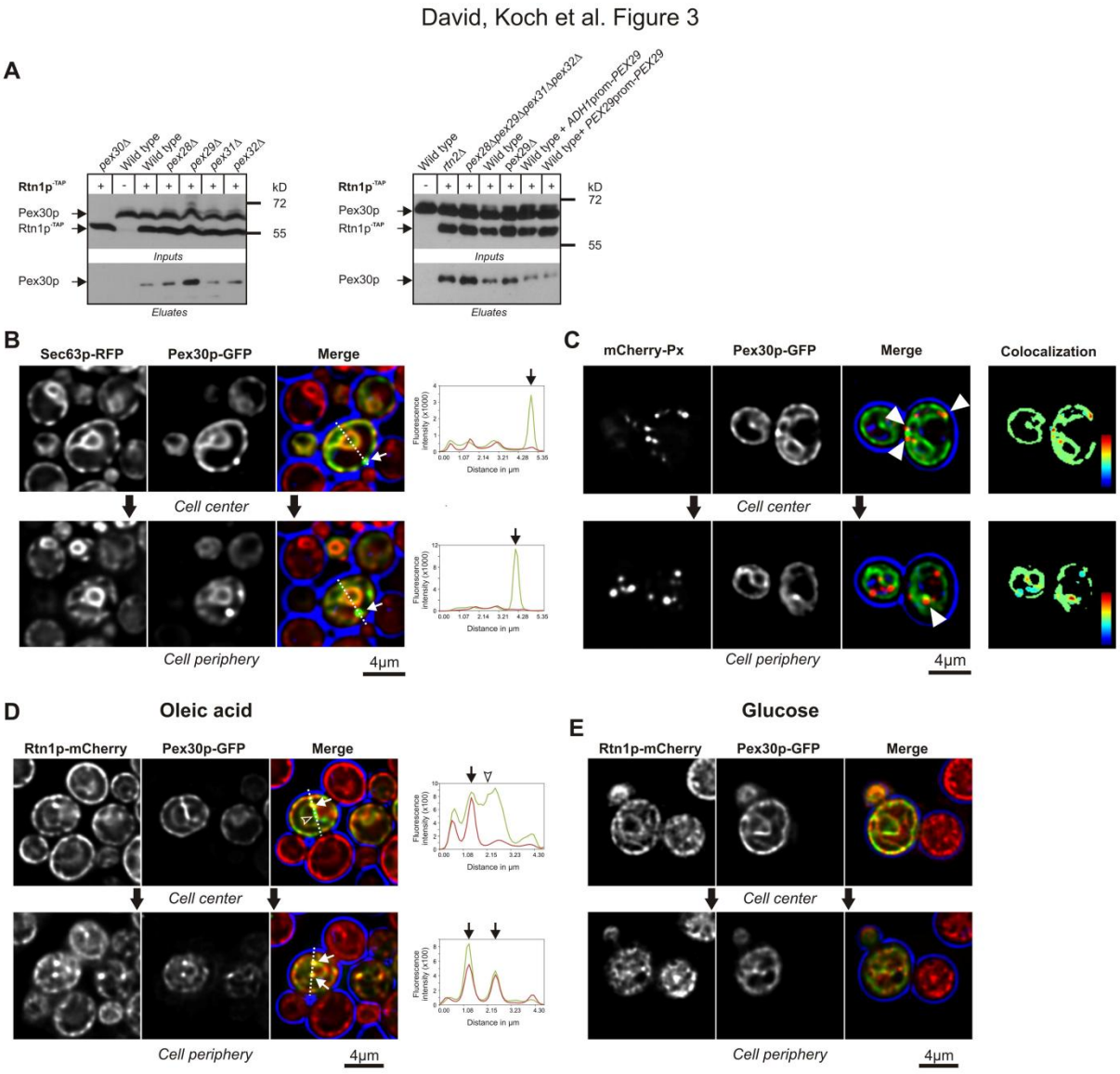
Name	Sequence
H869	5′CGAGTCGACTTATATAGTTGAATTGAGAGTGTCTG3′
H891	5′TCTGCAGTTATATAGTTGAATTGAGAGTGTC3′
H911	5′ATGCGAGCTCCCCCTCCGCGCTCTTTTCCG3′
H912	5′ATGCTCTAGAATCCGGGTGTATATGAGATAGTTGATTGTATGC3′
CB112	5′GCAAGCTTTTATAATTTGGACTCGATGTTTTTCTTGTACAGCTCGTCCATGCC3′
CB153	5′GCAATTGGTAGTGAAAACAAATAAACAAAGACATAACCGCACTCCAATCCGTACGCTGCAGGTCGAC3′
CB154	5′GAGAACAAAAACGAGAGTTTGATTTGAGGATATAGGTGAGTTGCCTCATCGATGAATTCGAGCTCG3′
CB180	5′ATTGGATCCTCTAGAATGGCTAGCAAAGGAGAAGAAC3′
CB181	5′AATAAGCTTGATATCTTATAATTTGGACAGGTGGTGGCGGTTGTACAGTTCATCCATG3′
CB203	5′GGCGTCGACTCCTGCAGGCTTAACTATACCAC3′
CB206	5′TACTAGTCATCGTAAAAGCAGAAGCACGAAACAAGGAGGCAAACCACTAAAAGGATGCGTACGCTGCAGGTCG3′
CB207	5′ATGAGTACCTTTCCTCGATGTCTCTGCAGAAGCGAACGTGATCTTTGATTTGGGGCCATCGATGAATTCTCTGTCG3′
CB244	5′GCGCATGCCTGCAGTTATTTGTACAATTCATCCATACC3′
CB285	5′GACCATGGTGAGTGATATCGTTGAATATCTTGCGTTCC3′
CB286	5′CGCAAGATATTCAACGATATCACTCACCATGGTCTAAAG3′
CB293	5′GCTCTAGAATGGTGAGCAAGGGCGAGGAGGATAAC3′
CB301	5′CGGTCGACATGGACTCGGTGACAAATTTTTTCTGG3′
CB320	5′CGAGCTCGGAAGACTCTCCTCCG3′
CB321	5′CTATTTGTATAGTTCATCCATGCC3′
CB346	5′CCTCACGCTTATTGCAACAAGTTTGTTTTTACTTACTTGTGAAACGTTTGTTG CTGAAGCTTCGTACGCTGCAGGTCGACGG3′
CB347	5′GTAATTAGTTATTTCAAAGTACATATTAAAATATATTATCATGAATCAGGATCTGATATCATCGATGAATTCGAGCTC3′
CB352	5′CCTCAAAAAGATCCATGTATAATCTTCATTATTACAGCCCTCTTGACCCTGAAGCTTCGTACGCTGCAGGTCGACGG3′
CB353	5′GTATGTAGATTGCGTATATAGTTTCGTCTACCCTATGAACATATTCCGGATCTGATATCATCGATGAATTCGAGCTC3′
CB379	5′CCCTGTAAGTCTTCACCTATAGAAACTGGTCGTAAAACACGTACGCTGCAGGTCGAC3′
CB380	5′CATATATATGTACATATCTATATGTATACATATTTTTATATAATCGATGAATTCGAGCTCG3′
CB381	5′AGTAGTCTAGACGAAGATCTATCGATGGACACAAATTCTAAAACC3′
CB382	5′AGTAGCTGCAGCTATTTGTATAGTTCATCCATGCC3′
CB383	5′AGTAGAGATCTATCGATGGACTCGGTGACAAATTTTTTC3′
CB388	5′TACACGCATGCGAGCTCGGAAGACTCTCCTCCG3′
CB389	5′CATCCGGTACCCTACAGGAACAGGTGGTGGCG3′
CB390	5′ATGGTTTTATCAAGGGGAGAAACAAAGAAAAATAGCGTGAGATTACGTACGCTGCAGGTCGAC3′
CB392	5′ATGAGTGGTAACACAACTAACGTGCATGAGACTAGAGCCAAGTTTCGTACGCTGCAGGTCGAC3′
CB393	5′TCATACGGCCTTCTTGCTATCGCGACCAATGGTTGGATTTGATTGATCGATGAATTCGAGCTC3′
CB394	5′ATGGTCTGTGATACACTGGTATATCATCCCTCCGTGACGAGATTCCGTACGCTGCAGGTCGAC3′
CB395	5′CTATGTAGCTTTCCACATGTCTTGCATACCAAGGATAGATGTGACATCGATGAATTCGAGCTC3′
CB391	5′TCAAAGGTCGCCAAGACCAGATATGGATCTCCTCTGAAATCTTCTATCGATGAATTCGAGCTC3′
CB406	5′TACACCCACTGCAGTGGGTGAGCAAGGGCGAGGAG3′
CB407	5′CATCCGGTACCCTACTTGTACAGCTCGTCCATG3′

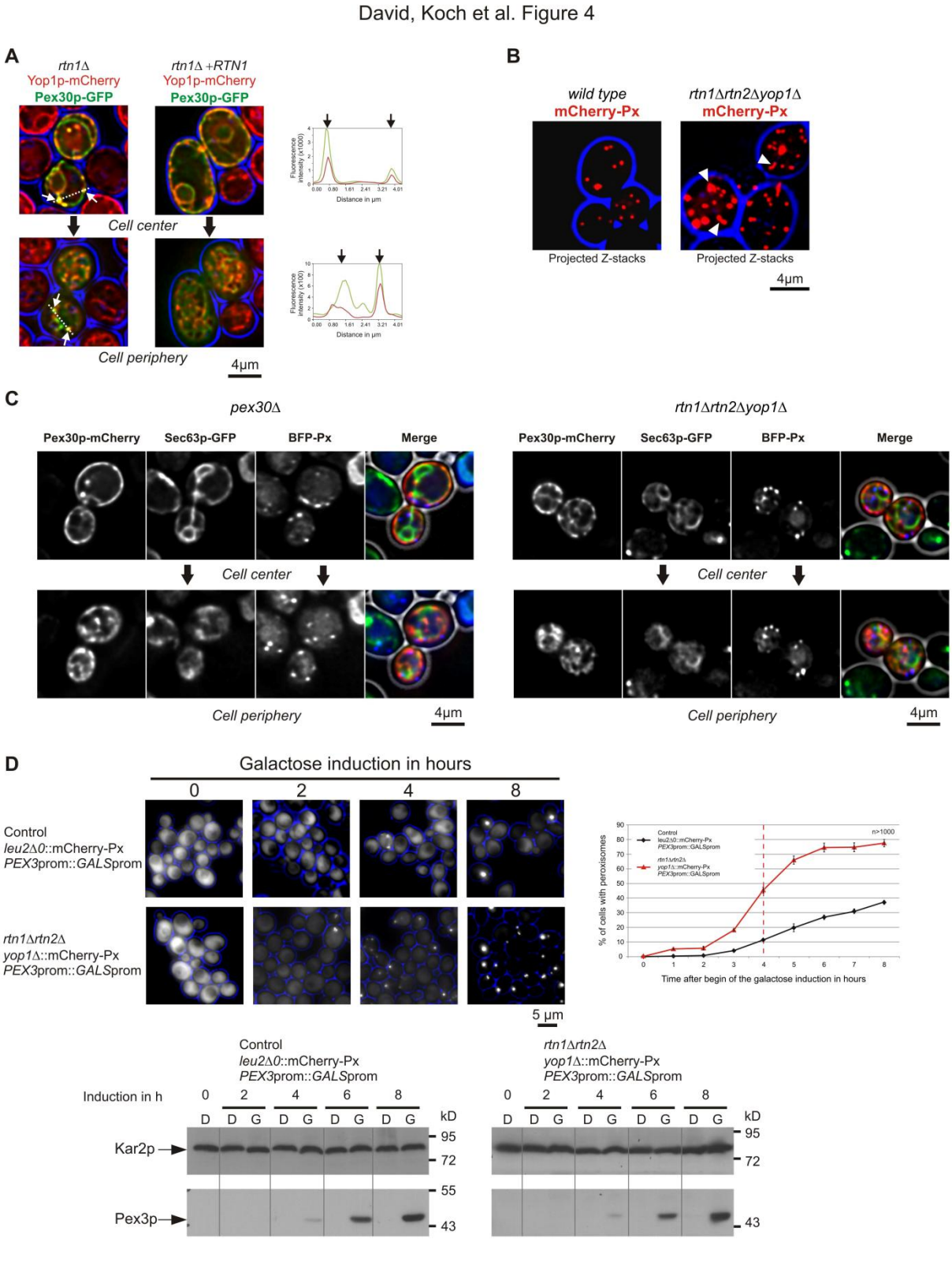
David, Koch et al. Figure 1

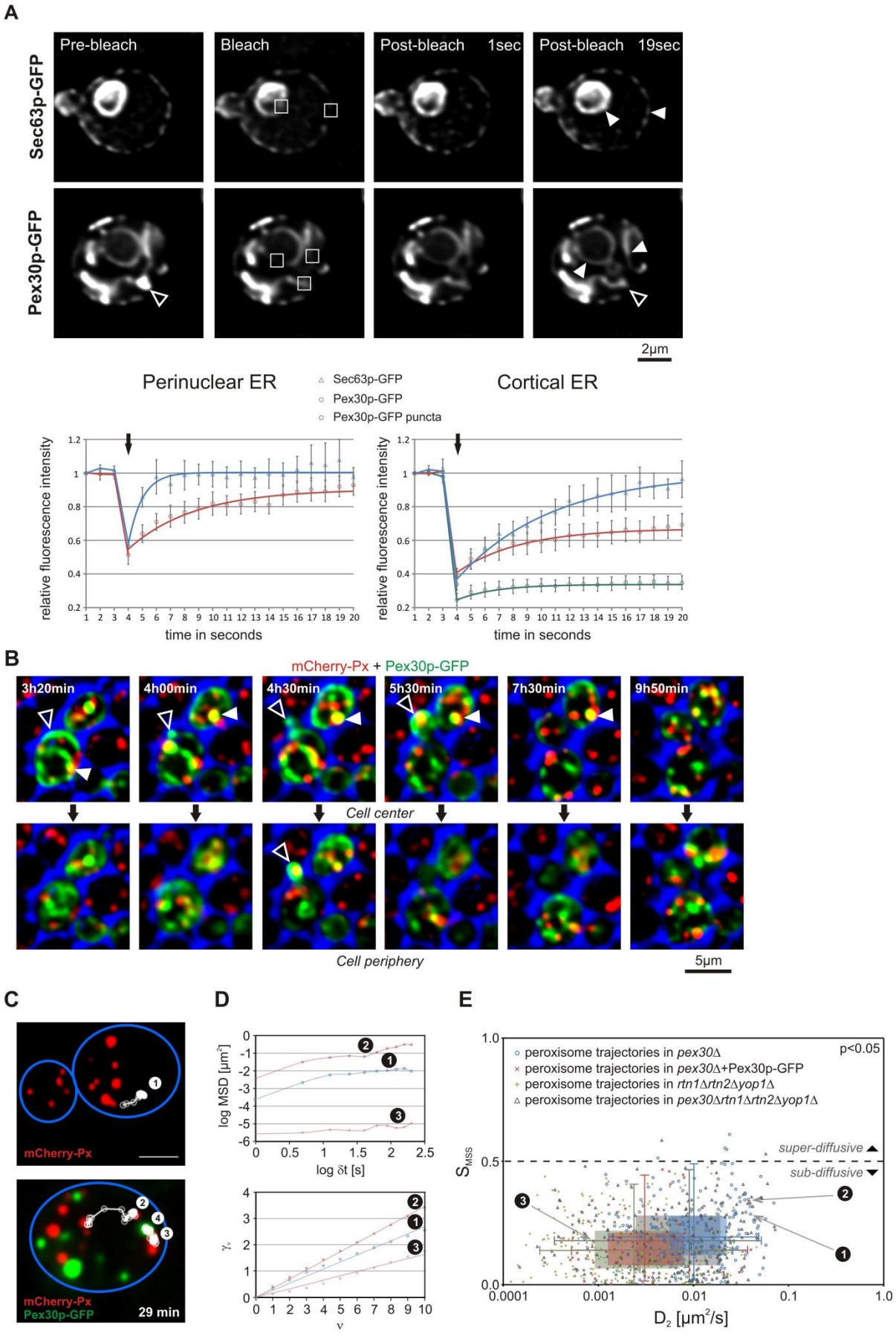


David, Koch et al. Figure 2

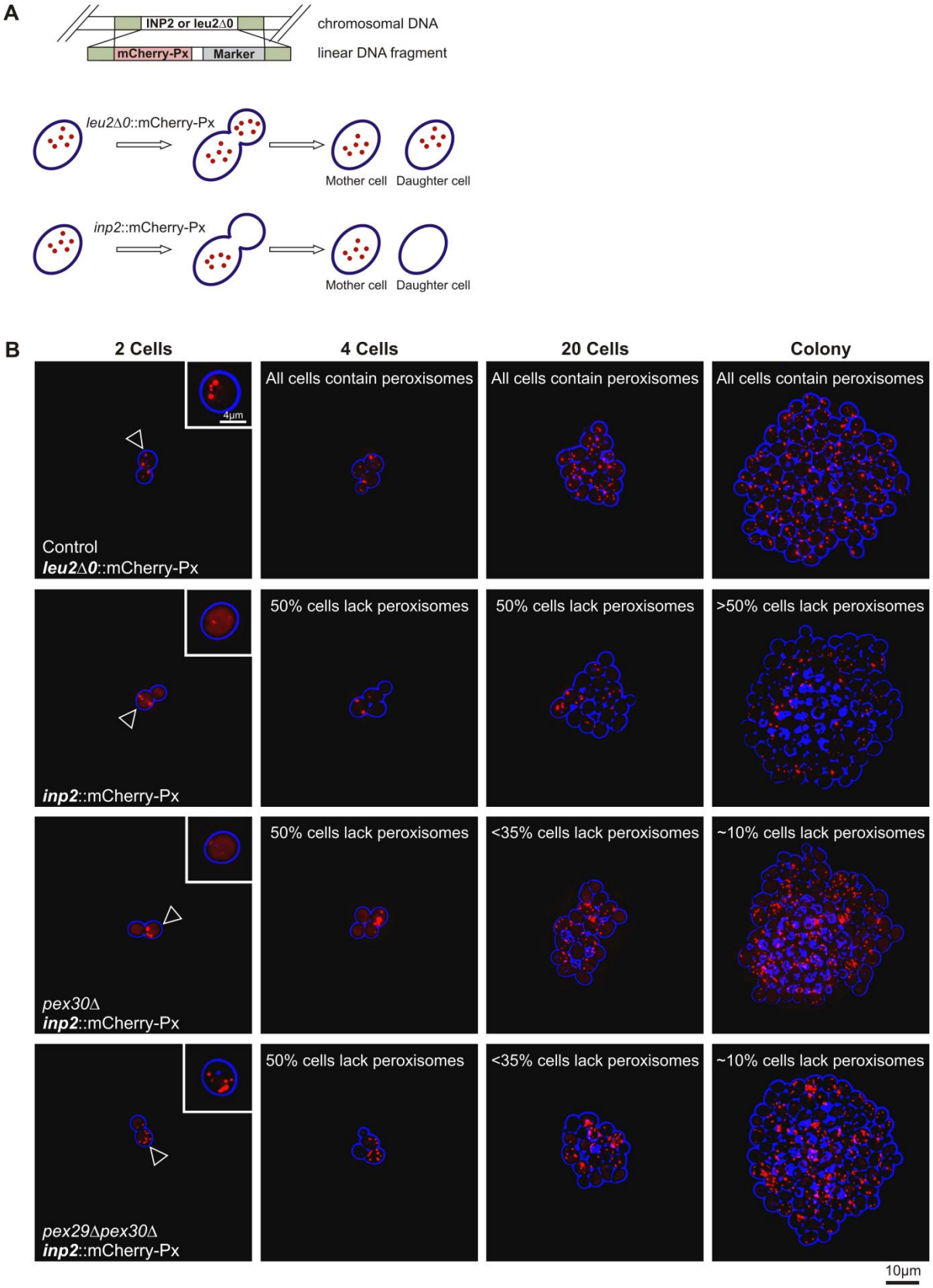



David, Koch et al. Figure 3
A
pex30Δ
Wild type
Wild type
pex28Δ
pex29Δ
pex31Δ
pex32Δ
Rtn1p-TAP
Pex30p
Rtn1p-TAP
Inputs
Pex30p
Eluates
kD
72
55
Wild type
rtn2Δ
pex28Δpex29Δpex31Δpex32Δ
Wild type
pex29Δ
Wild type + ADH1prom-PEX29
Wild type+ PEX29prom-PEX29
B
Sec63p-RFP
Pex30p-GFP
Merge
Cell center
Cell periphery
4µm
Fluorescence intensity (x1000)
Distance in µm
C
mCherry-Px
Pex30p-GFP
Merge
Colocalization
D
Oleic acid
Rtn1p-mCherry
E
Glucose

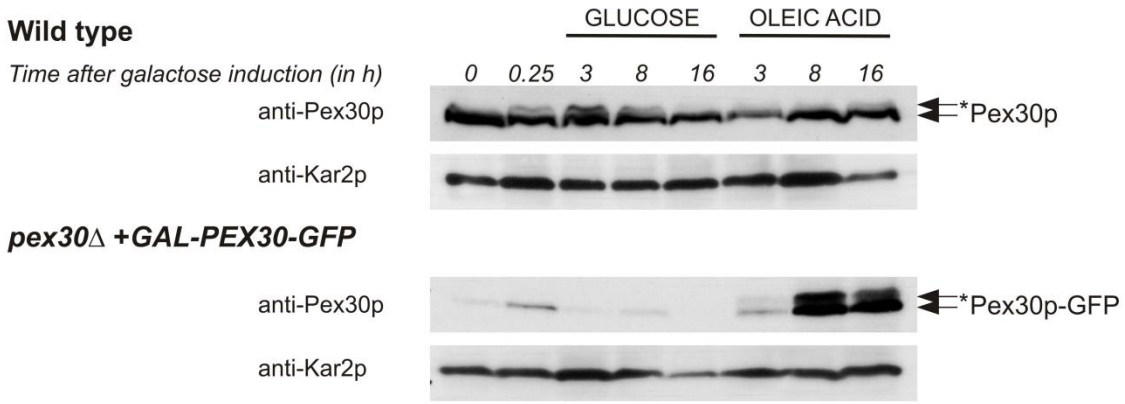

David, Koch et al. Figure 4
A
rtn1Δ
Yop1p-mCherry
Pex30p-GFP
rtn1Δ +RTN1
Yop1p-mCherry
Pex30p-GFP
Cell center
Cell periphery
4µm
Fluorescence intensity (x1000)
Distance in µm
B
wild type
mCherry-Px
rtn1Δrtn2Δyop1Δ
mCherry-Px
Projected Z-stacks
Projected Z-stacks
4µm
C
pex30Δ
rtn1Δrtn2Δyop1Δ
Pex30p-mCherry
Sec63p-GFP
BFP-Px
Merge
Cell center
Cell periphery
4µm
D
Galactose induction in hours
0
2
4
8
Control
leu2Δ0::mCherry-Px
PEX3prom::GALSprom
rtn1Δrtn2Δ
yop1Δ::mCherry-Px
PEX3prom::GALSprom
5 µm
% of cells with peroxisomes
Time after begin of the galactose induction in hours
n>1000
Induction in h
D
G
kD
Kar2p
Pex3p
95
72
55
43


David, Koch et al. Figure 5
A
Pre-bleach
Bleach
Post-bleach 1sec
Post-bleach 19sec
Sec63p-GFP
Pex30p-GFP
2µm
Perinuclear ER
Cortical ER
Sec63p-GFP
Pex30p-GFP
Pex30p-GFP puncta
relative fluorescence intensity
time in seconds
B
mCherry-Px + Pex30p-GFP
3h20min
4h00min
4h30min
5h30min
7h30min
9h50min
Cell center
Cell periphery
5µm
C
mCherry-Px
mCherry-Px
Pex30p-GFP
29 min
D
log MSD [µm²]
log δt [s]
γ
ν
E
peroxisome trajectories in pex30Δ
peroxisome trajectories in pex30Δ+Pex30p-GFP
peroxisome trajectories in rtn1Δrtn2Δyop1Δ
peroxisome trajectories in pex30Δrtn1Δrtn2Δyop1Δ
p<0.05
super-diffusive
sub-diffusive
S_MSS
D₂ [µm²/s]

David, Koch et al. Figure 6

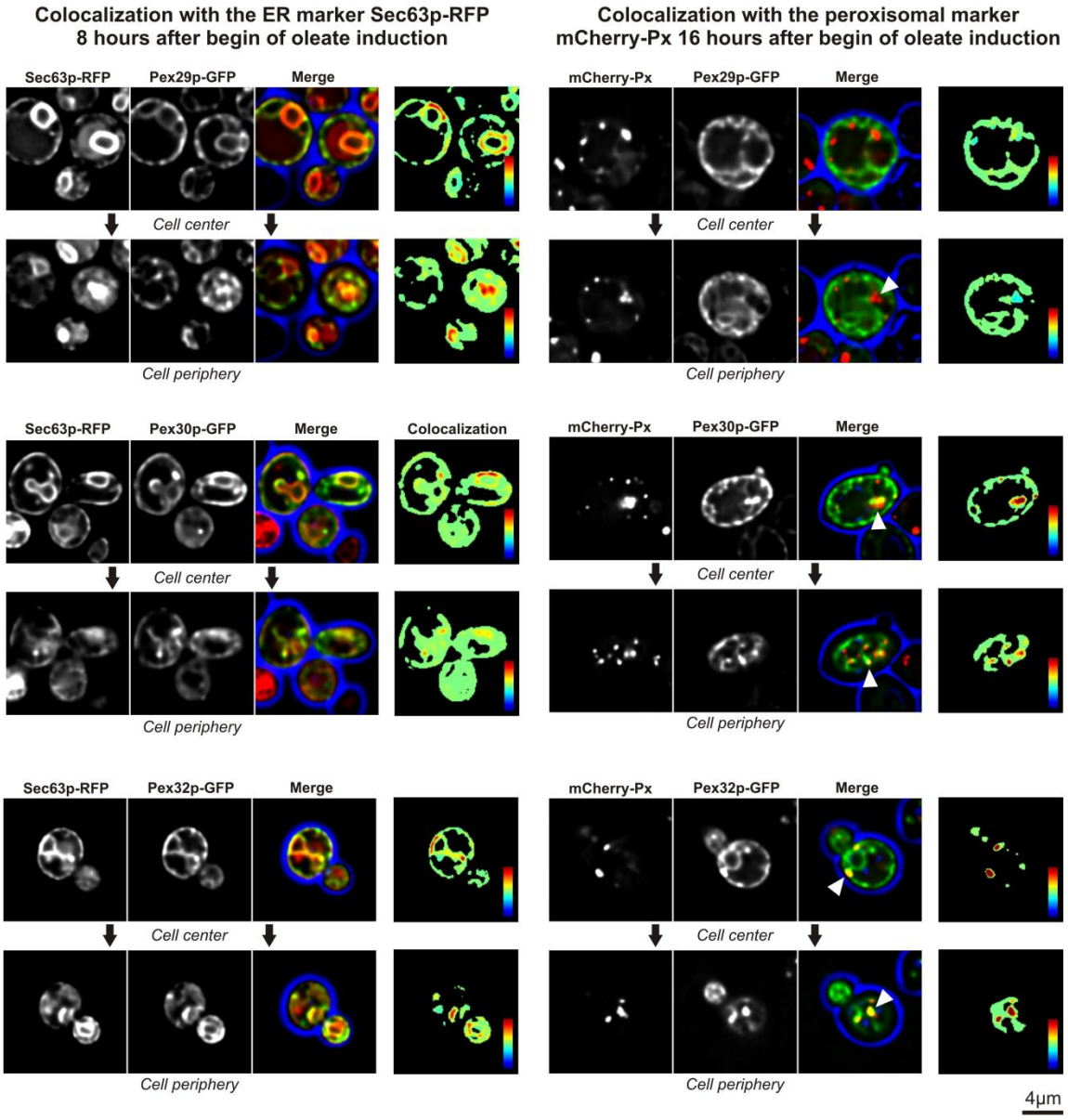


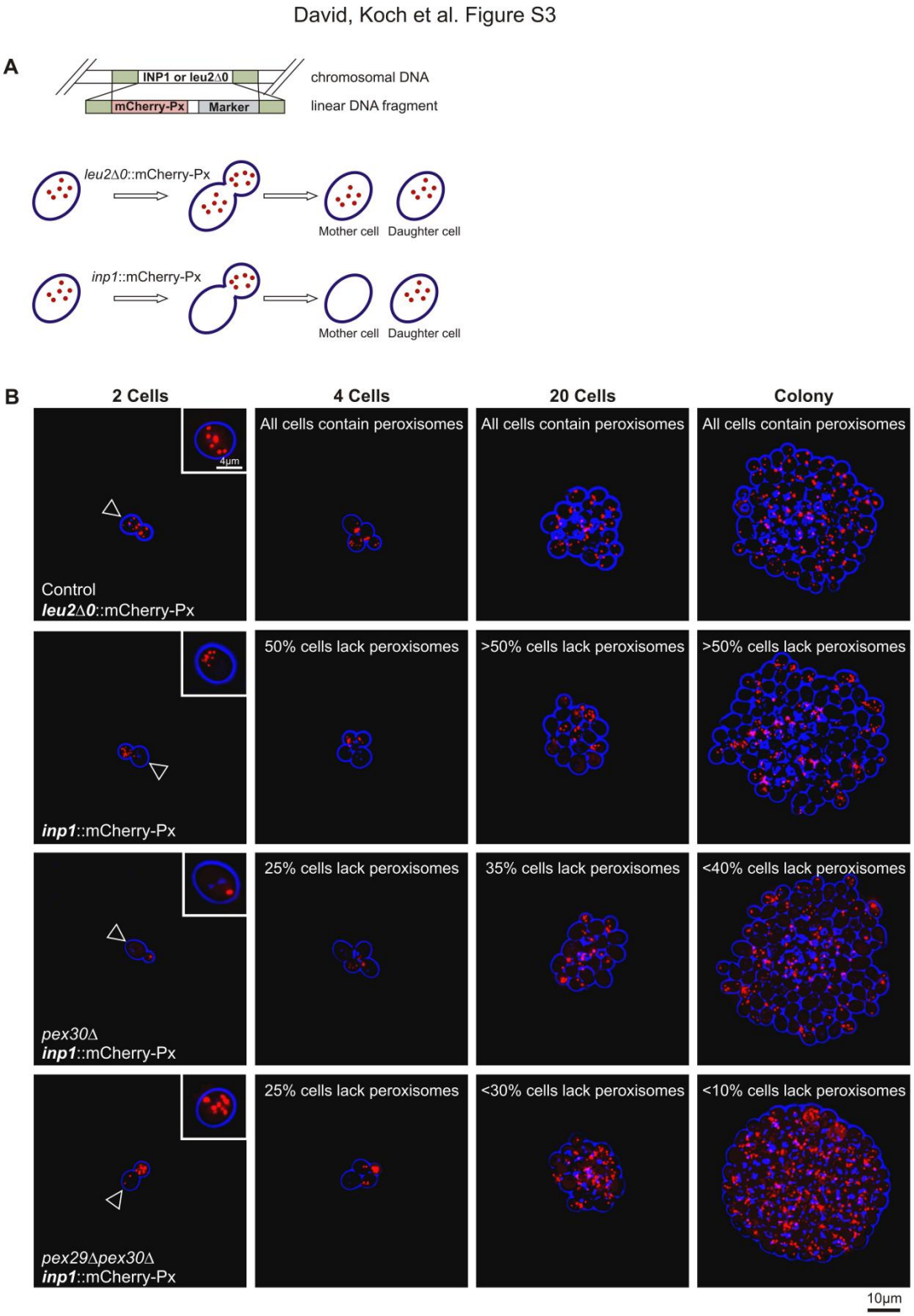
David, Koch et al. Figure S1



* Asterisks represent specifc Pex30p bands with lower electrophoretic mobility which disappear upon treatment with lambda-phophatase indicating that they correspond to phosphorylated Pex30p.

David, Koch et al. Figure S2




David, Koch et al. Figure S3
A
INP1 or leu2Δ0
mCherry-Px
Marker
chromosomal DNA
linear DNA fragment
leu2Δ0::mCherry-Px
Mother cell
Daughter cell
inp1::mCherry-Px
Mother cell
Daughter cell
B
2 Cells
4 Cells
20 Cells
Colony
4µm
All cells contain peroxisomes
All cells contain peroxisomes
All cells contain peroxisomes
Control
leu2Δ0::mCherry-Px
50% cells lack peroxisomes
>50% cells lack peroxisomes
>50% cells lack peroxisomes
inp1::mCherry-Px
25% cells lack peroxisomes
35% cells lack peroxisomes
<40% cells lack peroxisomes
pex30Δ
inp1::mCherry-Px
25% cells lack peroxisomes
<30% cells lack peroxisomes
<10% cells lack peroxisomes
pex29Δpex30Δ
inp1::mCherry-Px
10µm

6.2 A Subtle Interplay Between Three PEX11 Proteins Shapes *De Novo* Formation and Fission of Peroxisomes

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Author contributions:

I contributed to the experimental setup, established RT-PCR, and performed part of the clonings. I established the microscopy including automated counting techniques for peroxisomes in yeast cells and image processing including deconvolution.

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A Subtle Interplay Between Three Pex11 Proteins Shapes *De Novo* Formation and Fission of Peroxisomes

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The organization of eukaryotic cells into membrane-bound compartments must be faithfully sustained for survival of the cell. A subtle equilibrium exists between the degradation and the proliferation of organelles. Commonly, proliferation is initiated by a membrane remodeling process. Here, we dissect the function of proteins driving organelle proliferation in the particular case of peroxisomes. These organelles are formed either through a growth and division process from existing peroxisomes or *de novo* from the endoplasmic reticulum (ER). Among the proteins involved in the biogenesis of peroxisomes, peroxins, members of the Pex11 protein family participate in peroxisomal membrane alterations. In the yeast *Saccharomyces cerevisiae*, the Pex11 family consists of three proteins, Pex11p, Pex25p and Pex27p. Here we demonstrate that yeast mutants lacking peroxisomes require the presence of Pex25p to regenerate this organelle *de novo*. We also provide evidence showing that Pex27p inhibits peroxisomal function and illustrate that Pex25p initiates elongation of the peroxisomal membrane. Our data establish that although structurally conserved each of the three Pex11 protein family members plays a distinct role. While *Sc*Pex11p promotes the proliferation of peroxisomes already present in the cell, *Sc*Pex25p initiates remodeling at the peroxisomal membrane and *Sc*Pex27p acts to counter this activity. In addition, we reveal that *Sc*Pex25p acts in concert with Pex3p in the initiation of *de novo* peroxisome biogenesis from the ER.

Key words: fatty acid consumption, inheritance assay, membrane elongation, membrane proteins, organelle biogenesis, peroxisomes, PEX11, PEX25, PEX27, proliferation

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A consequence of the modular organization of the eukaryotic cytoplasm into membrane-bound organelles is an increase in the efficiency of metabolic processes. Such arrangement provides tailored microenvironments for chemical reactions in the cell. The modular organization is associated with a subtle equilibrium between proliferation and degradation of all subcellular compartments. For proliferation, organellar membranes are remodeled in a restricted area to accommodate altered protein and lipid composition, leading to polarization of the organelle. Polarizing events are usually initiated by the insertion of morphogenic proteins which alter the membrane curvature and sustain protrusion of this membrane (1,2). A consequence thereof is local membrane instability, which is ultimately resolved by fission. Accordingly at the onset of peroxisome proliferation, extensions form at the peroxisomal membrane (3,4). The number of peroxisomes per cell increases through growth and division of pre-existing peroxisomes (5) or, when required, peroxisome biogenesis is initiated *de novo* at the endoplasmic reticulum (ER) (6–11). These processes are controlled and executed by peroxins (PEX proteins) which act to maintain the peroxisomal compartment thereby sustaining cellular homeostasis.

Conceptually, peroxisome proliferation can be divided into five steps. Initially, proliferation needs to be spatiotemporally defined at the peroxisomal membrane (step 1), leading to polarized growth of the membrane, its protrusion (step 2) and elongation (step 3). Step 4 comprises the import of matrix proteins into the elongated area and recruitment of the fission machinery coinciding with constriction of the organellar membrane. Finally, scission and separation into individual peroxisomes (step 5) is carried out by fission factors shared with mitochondria (3,12,13).

Among the peroxins implicated in peroxisome proliferation, Pex11 proteins directly influence the elongation of the peroxisomal membrane (3,13–15). We explored the role of the Pex11 proteins employing a panoply of *Saccharomyces cerevisiae* mutants with peroxisome biogenesis defects. Previous work focusing on members of the Pex11 family in yeast, Pex11p, Pex25p and Pex27p suggested that each plays a different role in peroxisome function (16–19). However, comprehensive insight regarding their interplay and specific function in forming new peroxisomes is still missing. Here we present data demonstrating that Pex11p acts to maintain the peroxisomes in a metabolically active state and to proliferate already existing peroxisomes. Based on *in vivo* studies we established that Pex25p serves as an initiating factor in the process of membrane proliferation. In addition, we showed that after the complete loss of peroxisomes, Pex25p is the main factor of this family responsible for the regeneration of the organelle. Our data also support a model in which Pex27p competes with Pex25p and negatively affects peroxisomal function.

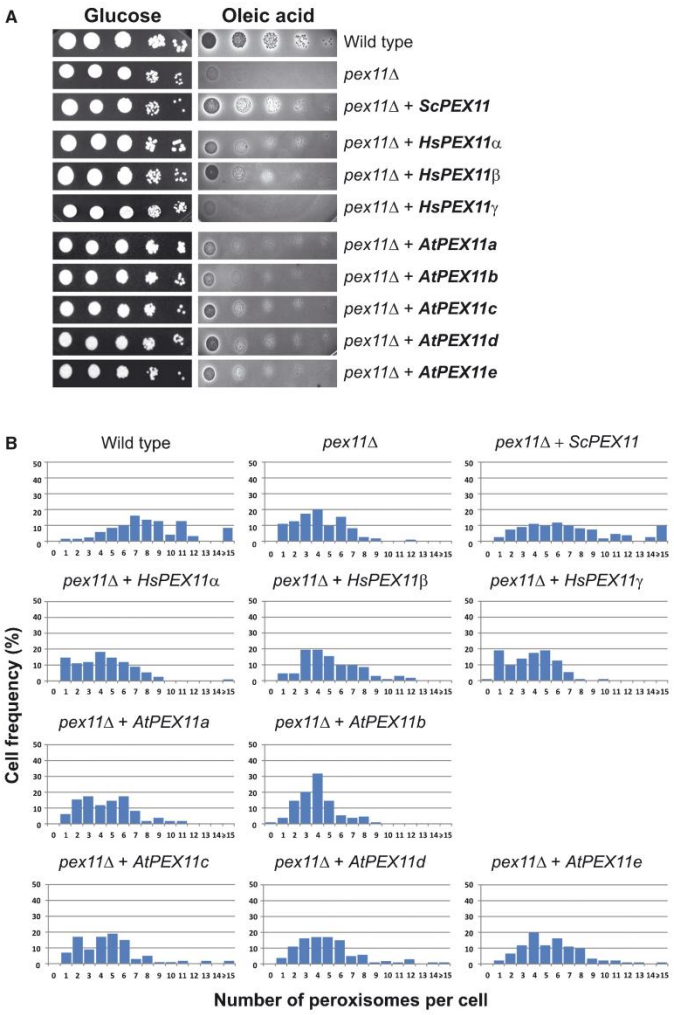

Figure 1: Heterologous Pex11 proteins can compensate for the peroxisomal defect of *pex11*Δ yeast cells. A) Yeast cells lacking the *PEX11* gene were transformed with plasmids expressing Pex11 proteins from different organisms, grown to logarithmic phase in medium containing 0.3% glucose and 10-fold serial dilutions were spotted onto glucose or oleic acid-containing agar plates. Oleate utilization was monitored by the formation of a clear zone (halo assay). B) Peroxisomes were visualized through mCherry-px (red channel) in cells, described in (A), incubated for 16 h in oleic acid-containing medium. For each strain, the fluorescent dots were counted in 100 non-budding cells. The histograms illustrate the frequency of cells with a distinct number of peroxisomes.

Results

Heterologous Pex11 proteins can substitute for PEX11 in S. cerevisiae

Yeast cells lacking *PEX11* contain few enlarged peroxisomes and are unable to utilize fatty acids as a carbon source (16,17,20). Conversely, overexpression of *PEX11* leads to the occurrence of many small peroxisomes. Hence, there seems to be a correlation between the number, the size and the function of peroxisomes. Generally, cells lacking Pex11 proteins present reduced peroxisomal function (21–23). To explore the evolutionary conservation of Pex11 protein function, we examined the effect of expressing human (PEX11α, PEX11β, PEX11γ; 24–26) or plant (PEX11a to e; 22) Pex11-proteins in *pex11*Δ yeast cells. We assessed the ability of the cells to utilize oleic acid and determined the number of peroxisomes per cell (Figure 1). The human PEX11α and PEX11β and the plant PEX11c, PEX11d and PEX11e complemented the oleate utilization defect of the yeast mutant. Expression of the plant PEX11a or PEX11b proteins in *pex11*Δ cells partially complemented the oleate utilization defect, whereas no complementation could be observed with the human PEX11γ (Figure 1A).

We visualized peroxisomes through expression of the marker protein mCherry-px and counted red fluorescent dots in individual cells after induction of peroxisome proliferation. While most wild-type cells contained 4–9 peroxisomes, a significant fraction (>20% of the cells) contained 10 peroxisomes or more. In contrast, *pex11*Δ cells rarely contained more than seven peroxisomes (Figures 1B and S1A). Expression of the heterologous proteins *Hs*Pex11α, *Hs*Pex11β or *At*Pex11a-d in *pex11*Δ cells did not substantially alter the number of peroxisomes per cell and in most cases 20% of the cells contained one or two peroxisomes. However, upon expression of *Hs*Pex11β or *At*Pex11e more than 15% of the cells contained more than seven peroxisomes and only few cells contained one or two peroxisomes (<10%).

EGFP-tagged Pex11 proteins localize to peroxisomes

Considering the functional differences between the various heterologous Pex11 proteins we sought to analyze whether these also localize to peroxisomes in yeast cells. Interestingly, regardless of their ability to complement the oleic acid utilization phenotype, all enhanced green fluorescent protein (EGFP)-tagged Pex11 proteins localized to peroxisomes in wild-type cells (Figure 2A). Similarly, N-terminally EGFP-tagged Pex11p, Pex25p and Pex27p colocalized with mCherry-px (Figure 2B). These observations illustrate that even highly expressed, all Pex11 proteins localize to peroxisomes (see Figure 3B–D).

Pex27p negatively affects peroxisomal function

We examined whether the three members of the yeast Pex11 family could mutually compensate for each other's function. We employed a variety of yeast mutants deleted for *PEX11, PEX25, PEX27* or combinations thereof, tested their ability to utilize oleate and counted peroxisomes in the cells. In addition, to study the effects of individual Pex11 family members, we expressed one of the three Pex11 proteins in these mutant cells (Figure 3A). Generally, cells lacking Pex11p did not utilize oleate, but the concomitant absence of Pex27p restored the ability of *pex11*Δ cells to utilize oleate (*pex11*Δ*pex27*Δ and *pex11*Δ*pex27*Δ + *PEX25*). These results point for the first time to a negative effect of *Sc*Pex27p on peroxisomal function. In accordance with such a negative effect, overexpression of *Sc*Pex27p in *pex25*Δ*pex27*Δ cells resulted in the inability to utilize oleate (Figure 3A) and in *pex11*Δ*pex25*Δ*pex27*Δ cells significantly increased the fraction of cells without peroxisomes (50%; Figure 4). The negative action of *Sc*Pex27p could be based on competition with *Sc*Pex25p. In agreement, overexpression of *Sc*Pex27p in *pex11*Δ*pex27*Δ cells resulted in the reduced ability to utilize oleate (Figure 3A).

Pex25p catalyzes membrane elongation

Furthermore, we asked whether Pex11p, Pex25p and Pex27p exerted different functions when expressed in *pex11*Δ*pex25*Δ*pex27*Δ cells (Figure 4). As reported previously (18,19), the expression of Pex11p in these cells

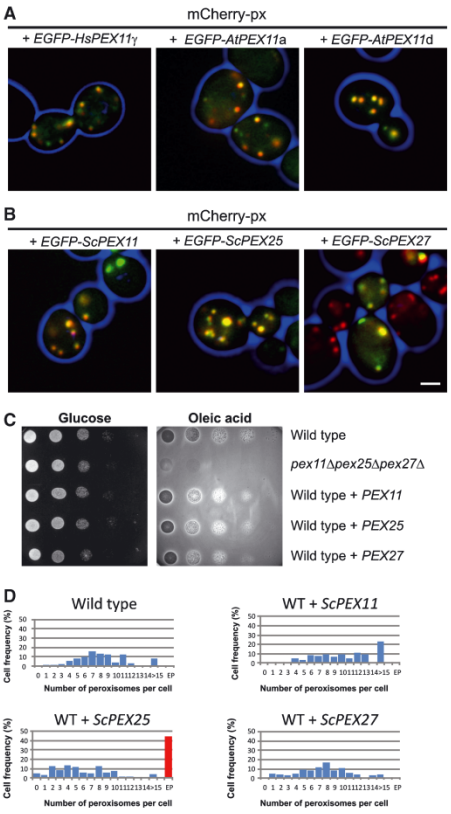

Figure 2: Localization of EGFP-tagged Pex11 proteins in yeast cells. A) Wild-type yeast cells expressing the fluorescent peroxisomal marker protein mCherry-px (red channel) and transformed with plasmids expressing the EGFP-tagged version of *Hs*Pex11γ, *At*Pex11a or *At*Pex11d (green channel) under the control of the *GAL1* promoter were incubated overnight in medium containing glucose, and then in galactose for 4 h. B) Wild-type yeast cells expressing mCherry-px (red channel) and EGFP-tagged *Sc*Pex11p, *Sc*Pex25p or *Sc*Pex27p (green channel) controlled by the GPD promoter were incubated with oleic acid-containing medium for 16 h. Images represent single *Z*-layers. Bar: 2 mm. C) Wild-type yeast cells expressing mCherry-px and in addition *GPD*-controlled ScPex11p, ScPex25p or ScPex27p were spotted onto agar plates, and oleate utilization was monitored as described in Figure 1B. D) Wild-type yeast cells expressing mCherry-px (red channel) and *Sc*Pex11p, *Sc*Pex25p or *Sc*Pex27p were incubated in oleic acid-containing medium for 16 h. For each strain the fluorescent dots were counted in 100 non-budding cells. The histograms illustrate the frequency of cells with a distinct number of peroxisomes. The red bars indicate the frequency of cells containing elongated peroxisomes (EP). Note that the fraction of cells with elongated peroxisomal structures (red bar) is not included in the histograms presenting the peroxisome counts (blue bars).

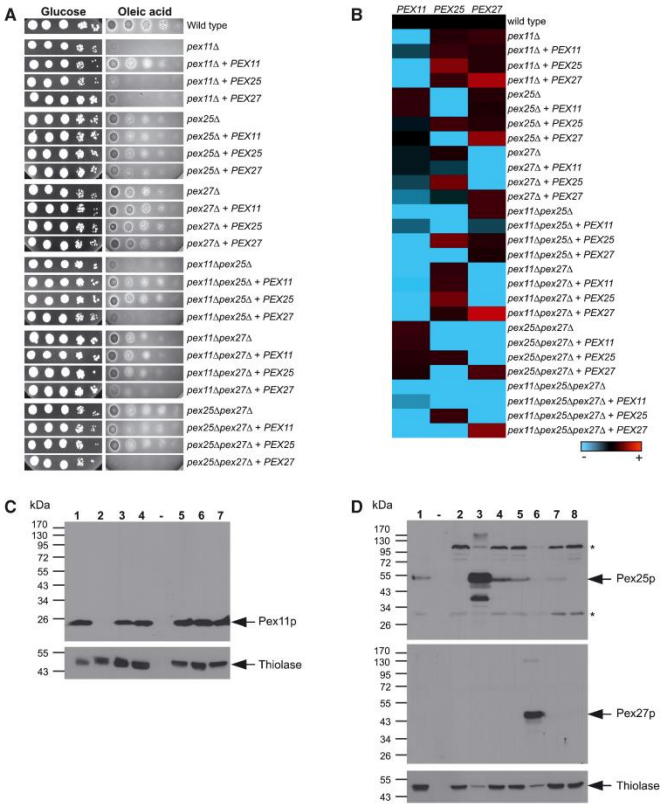

Figure 3: Overexpression of *Sc*Pex11p, *Sc*Pex25p or *Sc*Pex27p in yeast cells affects the function of peroxisomes. A) Yeast mutant cells expressing Pex11p, Pex25p or Pex27p as indicated were grown to logarithmic phase in medium containing glucose. Then, 10-fold serial dilutions were spotted onto agar plates and oleate utilization was monitored by means of halo formation in the agar. B) Quantitative real-time PCRs were performed with mRNAs obtained from yeast cells lacking one, two or all three *PEX11* genes and from mutants expressing *PEX11*, *PEX25* or *PEX27* from plasmids as indicated. Cells were incubated in oleic acid-containing medium for 16 h. The levels were compared to mRNA levels in wild-type cells. Black indicates wild-type mRNA levels; a decrease in mRNA level is indicated by varying intensities of blue color; intensities of red color correspond to an increase in mRNA levels. The colored bar represents mRNA levels between 1/10 and 10-fold of wild-type levels. C) Western blot analysis of protein levels in wild-type cells (lane 1), *pex11*Δ (lane 2), *pex25*Δ (lane 3), *pex27*Δ cells (lane 4), and in *pex11*Δ*pex25*Δ cells (lane 5), *pex25*Δ*pex27*Δ cells (lane 6) and *pex11*Δ*pex25*Δ*pex27*Δ cells (lane 7) expressing *GPD*-promoter-controlled *Sc*Pex11p. Anti-Pex11p antibodies were used to visualize the Pex11 protein and thiolase was analyzed as loading control. D) Western blot analysis of protein levels in wild-type cells (lane 1), *pex11*Δ*pex25*Δ*pex27*Δ cells (lane 2), *pex11*Δ*pex25*Δ cells expressing *Sc*Pex25p from the *GPD* promoter (lane 3) or the *PEX25* promoter (lane 4), *pex11*Δ*pex25*Δ*pex27*Δ cells expressing *Sc*Pex25p from the *PEX25* promoter (lane 5), *pex11*Δ*pex27*Δ cells expressing *Sc*Pex27p either from the *GPD* promoter (lane 6) or the *PEX27* promoter (lane 7), and in *pex11*Δ*pex25*Δ*pex27*Δ cells expressing *Sc*Pex27p from the *PEX27* promoter (lane 8). Anti-Pex25p or anti-Pex27p antibodies were used to visualize the respective proteins and thiolase was used as the loading control. Less amount of protein was loaded in lanes 3 and 6 to avoid interference of strong signals with neighboring lanes. Asterisks indicate non-specific protein bands recognized by the antibody.

only partially restored oleate utilization and resulted in a slight increase in the fraction of cells (33%) lacking peroxisomes. In contrast to the negative effect of Pex27p, when Pex25p was overexpressed, the cells could utilize oleate and produced more peroxisomes per cell leaving only a small portion (11%) of cells without peroxisomes. Strikingly, the expression of Pex25p from a plasmid was always associated with the appearance of elongated organelles (Figure 4B,C) reminiscent of juxtaposed elongated peroxisomes (JEPs) previously described in human

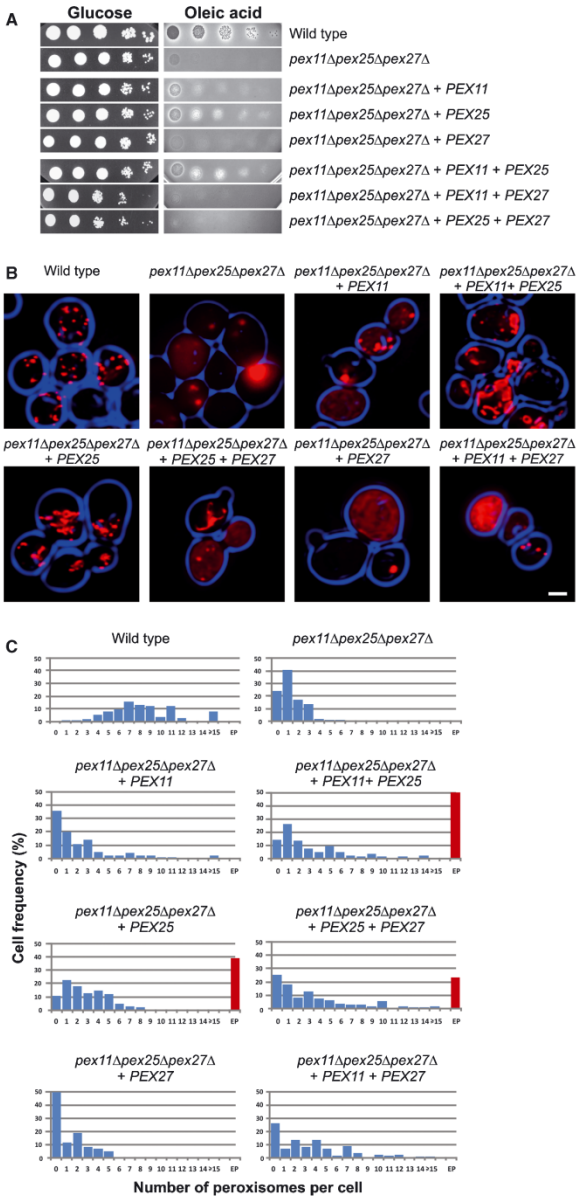

Figure 4: Each member of the Pex11p family differently affects function and number of peroxisomes. A) Yeast mutants lacking *PEX11*, *PEX25* and *PEX27* (*pex11Δpex25Δpex27Δ*) and transformed with plasmids expressing Pex11p, Pex25p or Pex27p, Pex11p and Pex25p, Pex11p and Pex27p or Pex25p and Pex27p were tested for peroxisomal function through the halo assay. B) Cells described in (A) were incubated in medium containing oleic acid as the sole carbon source for 16 h. Peroxisomes were visualized by fluorescence microscopy (mCherry-px; red channel). Images represent deconvolved projected *Z*-stacks. Bar: 4 μm. C) Quantitative distribution of peroxisomes in cells described in (A) incubated for 16 h in oleic acid-containing medium. For each strain, fluorescent dots (mCherry-px) were counted in 100 non-budding cells. The histograms illustrate the frequency of cells with a distinct number of peroxisomes. The red bars indicate the frequency of cells containing elongated peroxisomes (EP). Note that the fraction of cells with elongated peroxisomal structures is not included in the histograms presenting the peroxisome counts (blue bars).

cells upon ectopic expression of several members of the Pex11 protein family (3). The occurrence of these structures was enhanced upon concomitant expression of *PEX11* and *PEX25* from plasmids (Figure 4C). In contrast, when Pex27p was simultaneously expressed with either Pex11p or Pex25p, the negative effect of Pex27p on peroxisome function prevailed. Overexpression of *Sc*Pex25p from a plasmid compensated for the oleate utilization defect of *pex11Δpex25Δ* mutants (Figure 3A). These combined data suggest that *Sc*Pex25p plays a key role in peroxisome biogenesis.

Pex11p, Pex25p and Pex27p are expressed independently

The various effects of Pex11p, Pex25p and Pex27p on the number of peroxisomes and the formation of elongated peroxisomes could be the result of functional interdependence of the three proteins. Alternatively, this could reflect a mutual influence on gene expression. To distinguish between these possibilities, we analyzed gene expression and protein levels in yeast cells transformed with different plasmids (Figure 3B–D). Deletion or ectopic expression of any of the Pex11 protein-encoding genes had no significant influence on the mRNA levels of the others ruling out a mutual effect on transcription. The mRNA and protein of genomically expressed *PEX11* reached higher levels compared to plasmid-born expression controlled by the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter. This observation reflects the abundant production of Pex11p upon oleate induction. In contrast, *GPD*-driven expression of *PEX25* and *PEX27* was drastically increased as compared to their genomic expression levels (Figure 3B–D) and the proteins localized to peroxisomes (Figure 2B). These results indicated that expression levels of the three proteins are independent of each other, but do not exclude a mutual control through post-translational modifications.

ScPex11p and ScPex25p alter the number of peroxisomes in wild-type cells

To investigate their diverse functions we expressed the Pex11 proteins from plasmids in wild-type cells. The ability of these cells to utilize oleic acid was not drastically altered compared to wild-type cells (Figure 2C). However, additional expression of Pex11p resulted in a higher number of peroxisomes per cell, and overexpression of Pex25p correlated with the appearance of elongated peroxisomal structures (Figure 2D). In contrast, cells overexpressing Pex27p displayed almost wild-type levels of peroxisomes. A plausible explanation for the lack of effect due to Pex27p overexpression is that its function might only be required under exceptional circumstances.

Pex25p is a key player in de novo *formation of peroxisomes*

Yeast cells lacking the protein Pex3p are devoid of peroxisomes but reintroduction of a functional Pex3 protein leads to full peroxisomal recovery (27,28). While mutant cells lacking all three Pex11-related proteins (pex11Δpex25Δpex27Δ) contained up to three peroxisomes (see Figure 4), the additional lack of Pex3p led, as expected, to the complete absence of peroxisomes (Figure 5A). To analyze the effects of Pex11 family members on peroxisome biogenesis, we established an experimental set up allowing the reintroduction of Pex3p upon change of carbon source. We replaced the genuine *PEX3* promoter with a galactose-inducible *GAL* promoter, whose expression is turned off in the presence of glucose. The cells (*pex11Δpex25Δpex27Δ pex3Δ::GAL-PEX3*) remained void of peroxisomes even after activation of Pex3p synthesis by growth on galactose (Figure 5A). This result demonstrated that at least one of the three Pex11 family members is required for the regeneration of peroxisomes after their complete loss. Using simultaneous expression of *PEX3* via growth of cells on galactose and either *PEX11*, *PEX25* or *PEX27* from plasmids, we asked which one of the three proteins is required for *de novo* formation of peroxisomes (Figure 5A). Wild-type levels of peroxisomes were only restored in mutant cells when *PEX25* was expressed together with *PEX3*. Moreover, elongated peroxisomes were visible in the course of peroxisome generation. The expression of *PEX27* in conjunction with *PEX3* allowed the formation of few peroxisomes in a limited number of cells (<10%). Indicating that Pex11p has no function in *de novo* formation of peroxisomes, the combined expression of *PEX3* and *PEX11* did not lead to the formation of peroxisomes. Taken together, these data suggest that *Sc*Pex25p is an essential factor for *de novo* biogenesis of peroxisomes and that *Sc*Pex27p has the capacity to partially substitute for the role of *Sc*Pex25p.

If Pex25p is indeed required for *de novo* biogenesis, this protein should be essential to regain peroxisomes in inheritance mutants. Therefore, we employed *inp2Δ* mutant cells, in which peroxisomes are retained in the mother cells during cell division (29). However, peroxisomes can slowly form in *inp2Δ* cells. While daughter cells are temporarily devoid of peroxisomes, after a full generation they seem to assemble these organelles *de novo* (10). Consequently, in a colony arising from a single budding *inp2Δ* cell, approximately half of the cells are expected to contain peroxisomes. If *de novo* biogenesis is impaired then most cells will lack peroxisomes. To visualize peroxisomes the coding sequence for the marker protein GFP-px was integrated into the genome replacing *INP2* in cells additionally deleted for different combinations of *PEX11*, *PEX25* and *PEX27*. The cells were thinly seeded onto agarose containing growth medium and allowed to form microcolonies prior to microscopic analysis (Figure 5B). As expected, in *inp2Δ* colonies half of the cells contained green fluorescent dots indicating the presence of peroxisomes. The *inp2Δ* mutants additionally lacking all three Pex11 proteins presented a cytosolic green staining without punctae, indicating the absence of peroxisomes. Few peroxisomes were visualized in most *pex11Δpex25Δpex27Δ* mutant cells (see Figure 4). The additional loss of *INP2* would require *de novo* formation of

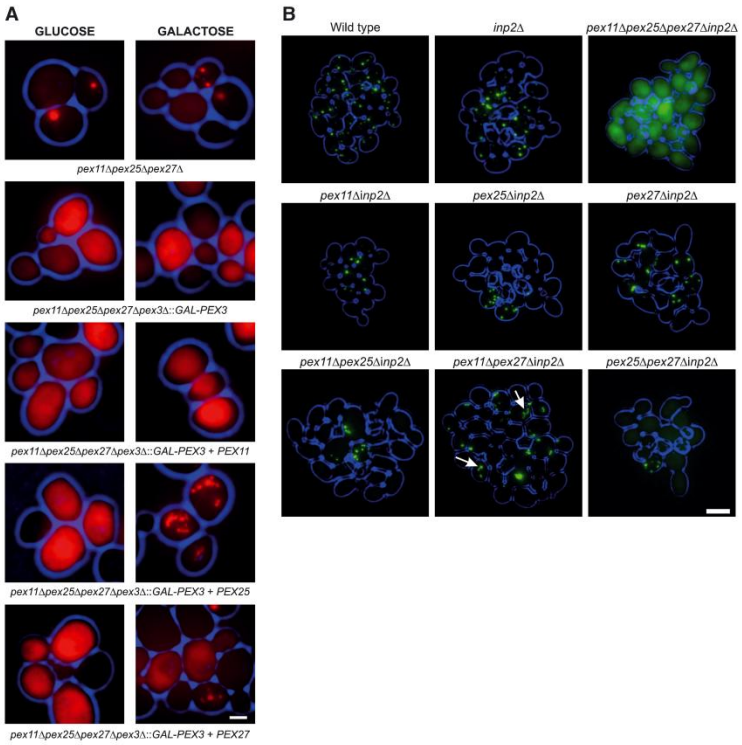

Figure 5: *De novo* formation of peroxisomes requires Pex25p. A) Mutant cells lacking *PEX11*, *PEX25* and *PEX27*, expressing *PEX3* under the control of the *GAL* promoter (*pex11Δpex25Δpex27Δpex3Δ::GAL-PEX3*) and transformed with plasmids expressing Pex11p, Pex25p or Pex27p were grown in the presence of 2% glucose with or without a short period of exposure to 2% galactose 8 h prior to microscopic inspection. Peroxisomes were visualized by fluorescence microscopy (mCherry-px; red channel). Bar: 2 μm. B) Microscopic analysis of yeast cells lacking *INP2* and one, two or three members of the *PEX11* family and expressing GFP-px under the control of the *GAL* promoter (green channel). The corresponding DNA sequence was integrated into the *INP2* locus. After growth on glucose for 16 h, cells were shifted to medium containing 1% raffinose and 2% galactose for 6 h and thinly seeded on microscope slides covered with agarose containing the same medium. After 10 h, colonies originating from single cells were inspected for the distribution of green fluorescence emitted from GFP-px. Arrows point to elongated peroxisomes. Images in (A) and (B) represent projected *Z*-stacks. Bar: 5 μm.

peroxisomes in the daughter cells which obviously did not occur in the absence of the three Pex11 family members. In colonies originating from cells lacking *INP2* and *PEX11* (*pex11Δinp2Δ*), half of the cells contained peroxisomes, and these cells contained a smaller number of peroxisomes. These results suggest that Pex11p is not involved in *de novo* biogenesis but rather functions in determining the number of peroxisomes present in each peroxisome-containing cell. In the absence of Pex27p (*inp2Δpex27Δ*), colonies were indistinguishable from those originating from *inp2Δ* mutant cells (Figure 5B). Similarly, when only Pex25p was expressed (*pex11Δpex27Δinp2Δ*), half of the cells contained peroxisomes. Moreover, elongated peroxisomes were visible in a number of these cells (Figure 5B). In colonies originating from *pex25Δinp2Δ* cells, only 10% of cells contained peroxisomes, and the majority of cells showed a diffuse cytosolic fluorescence. These inheritance assays demonstrated that Pex25p plays an essential role in the *de novo* formation of peroxisomes. Its function can at least be partially substituted by Pex27p, because in colonies originating from *pex11Δpex25Δinp2Δ* single cells, about 10% of cells enclosed peroxisomes. Further supporting this notion, less than 5% of *pex25Δpex27Δinp2Δ* mutants contained peroxisomes, and several colonies originating from these mutants were observed with cells completely devoid of peroxisomes. Notably, numerous peroxisomes could be observed in the very few peroxisome-containing cells

found in *pex25*Δ*pex27*Δ*inp2*Δ mutant colonies. Again this finding supports the central role for Pex11p in the regulation of the number of peroxisomes per cell.

Discussion

To determine the overall function of Pex11 proteins, we explored the potential of heterologous Pex11 proteins to complement the *pex11*Δ phenotype of *S. cerevisiae* cells (Figure 1). The *Sc*Pex11 protein is more closely related to the heterologous Pex11 proteins than to the two other family members present in *S. cerevisiae*, Pex25p and Pex27p (30). Expression of the heterologous Pex11 proteins did not significantly change the number of peroxisomes in *pex11*Δ cells. While they all localized to peroxisomes, only five of the eight proteins tested allowed the cells to consume oleic acid (Figures 1A and 2A). Noteworthy, among the five proteins complementing the oleate utilization phenotype, all but *Hs*PEX11β contain at their C-terminus a –KXKXX motif known as ER-retrieval signal (31). This motif may already indicate a connection of these peroxisomal proteins with the ER.

The three members of the Pex11 family in *S. cerevisiae* were originally identified as factors controlling peroxisome number and function (16,18,19,32). However, their individual contribution remained unknown. To discern their particular roles we expressed these proteins in single, double and triple mutants alone or in combinations, analyzed the ability of transformed cells to utilize oleate and evaluated the number of peroxisomes per cell (Figures 3A and 4). Our results provide evidence that each member of the Pex11 family holds a different function in the control of peroxisome number and metabolic activity. This suggests that in *S. cerevisiae*, the delicate balance between Pex11p, Pex25p and Pex27p ensures a variable number of peroxisomes and guarantees that each cell is furnished with adequate peroxisomal metabolism.

Several characteristics suggest a role for *Sc*Pex11p and close heterologous relatives in membrane remodeling. Its conserved localization (Figure 2) (3,23,30), its abundance (16) and the lack of a transmembrane domain but presence of an amphipathic helix (15) might allow for exclusion or specific association of proteins or metabolites at the peroxisomal membrane. Amphipathic helices are thought to sense membrane curvature or to participate in membrane remodeling (33). Thus, Pex11p might act as a sensor to determine the ability of the membrane to proliferate. Alternatively, a continuous gradient of Pex11p might allow for membrane protrusion. In both cases, accumulation of Pex11p at specific membrane sites with a precise form or lipid composition might influence the peroxisomal metabolism, a behavior that seems to have been conserved throughout evolution.

In the mere absence of Pex11p, cells are unable to utilize fatty acids. Interestingly, the additional lack of Pex27p allowed the cells to regain peroxisomal function (Figure 3A), suggesting a negative or competitive role for Pex27p. Consistent with this notion, the reintroduction of Pex27p into *pex11*Δ*pex27*Δ cells and *pex25*Δ*pex27*Δ cells reduced their ability to utilize oleate (Figure 3A). While overexpression of Pex25p in *pex11*Δ*pex25*Δ*pex27*Δ cells led to the occurrence of elongated peroxisomal structures, the concomitant expression of Pex25p and Pex27p in these cells reduced the frequency of elongated peroxisomes (Figure 4B,C). The presence of functional peroxisomes in *pex11*Δ*pex27*Δ cells and the occurrence of fewer cells with elongated peroxisomes in *pex11*Δ*pex25*Δ*pex27*Δ mutants expressing Pex25p and Pex27p from plasmids are observations in agreement with a model in which Pex27p competes with Pex25p during the process of proliferation. The finding that Pex27p can partially substitute for Pex25p in *de novo* formation of peroxisomes strongly supports a competition between these two proteins (Figure 5B), suggesting a similar role and a similar localization for both (18). That this process is slow in the absence of Pex25p could be because of the fact that endogenous Pex27p is only present in small amount in wild-type yeast cells (18). However, in contrast to Pex11p and Pex25p, overexpression of Pex27p does not lead to functional peroxisomes in cells lacking all three proteins (Figure 4A). In wild-type cells, overexpression of Pex27p showed only moderate influence on peroxisomal number. An explanation could be that Pex27p is only active when the balance between Pex11p and Pex25p is perturbed which could endanger the propagation of peroxisomes.

The negative effect exerted by Pex27p on peroxisomal function most likely takes place at the peroxisomal membrane. In wild-type cells, the presence of Pex25p or Pex27p at the peroxisomal membrane could locally alter the lipid-to-protein ratio, thereby enhancing the association of Pex11p with the membrane at this site. This in turn would result in Pex11p accumulation, membrane remodeling and proliferation at this exact site. The property of Pex11p to oligomerize (20) might support a co-operative association with the peroxisomal membrane, which, in turn, could explain its function in proliferating peroxisomes already present in the cell.

In the absence of Pex11p and Pex27p, Pex25p is sufficient to provide the cells with functional peroxisomes. The occurrence of elongated peroxisomes (Figure 5B), strongly increased upon ectopic expression of Pex25p (Figure 4B), suggests that this protein triggers membrane elongation, a step essential to prime peroxisome proliferation. Pex27p might compete with Pex25p in the process of membrane association or at the level of protein interaction, e.g. with Pex11p. However, as there is no evidence for heteromeric interactions between Pex11 family members, the interplay between these proteins might rather rely on the interaction of each individual protein with lipids of the same (peroxisomal) membrane. We propose that the interaction between each member of the Pex11 protein family and the peroxisomal membrane has been

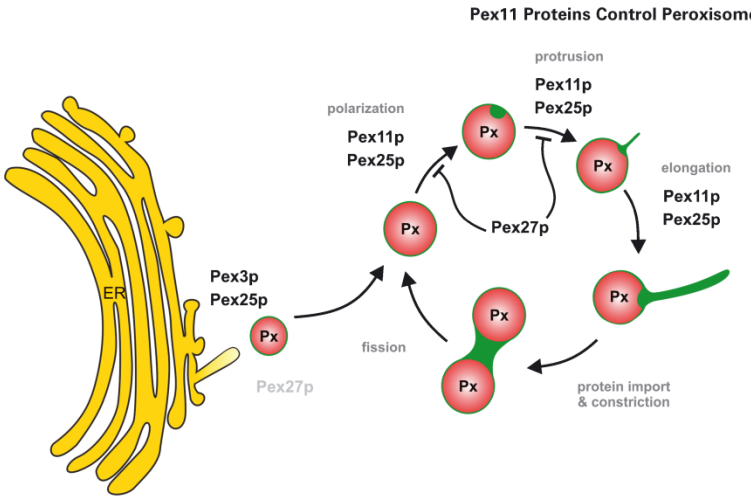

Figure 6: A model for the function of Pex11p, Pex25p and Pex27p in peroxisome biogenesis. Consistent with the previous models on Pex11 protein function (3,14,15), *Sc*Pex11p serves as a sensor to determine the ability of the peroxisomal membrane to proliferate. Supported by its ability to co-operatively associate, *Sc*Pex11p accumulates at specific membrane sites, which leads to membrane elongation and protrusion. *Sc*Pex25p might catalyze this priming event for peroxisomal membrane elongation preparing the membrane for association and accumulation of *Sc*Pex11p. And *Sc*Pex25p is also able to provide yeast cells with functional peroxisomes on its own. These particular functions of *Sc*Pex25p in proliferation of existing peroxisomes are inhibited or competed by *Sc*Pex27p. In addition, *Sc*Pex25p plays together with Pex3p an important role in initiating the *de novo* formation of peroxisomes. Here, *Sc*Pex27p most likely acts as a structural component (indicated in gray) which partially substitutes for the function of *Sc*Pex25p.

conserved throughout evolution. This hypothesis fits the observation that heterologous Pex11 proteins localize to peroxisomes and compensate for the loss of peroxisomal function to various degrees (Figure 2) (3,23,30).

Growth and division of existing peroxisomes and *de novo* formation from the ER constitute the peroxisome biogenesis (10,34). The protein Pex3p was previously described as an early peroxisome biogenesis factor and it was shown to be the initiating factor for peroxisome biogenesis from the ER (9,35). While proliferation from existing peroxisomes could take place in the absence of the Pex11-family members, after loss of peroxisomes, Pex25p was required to generate wild-type levels of peroxisomes. Hence, we demonstrate that Pex25p acts in intimate co-operation with Pex3p and that both are equally required for *de novo* formation. Similar results were obtained in the yeast *Hansenula polymorpha* (36).

We present a model (Figure 6), in which each one of the yeast Pex11 proteins holds an individual function in the formation of peroxisomes. In conclusion, (i) we demonstrate that Pex25p participates in membrane elongation of existing peroxisomes and in the initiation of *de novo* biogenesis from the ER, (ii) we provide evidence that Pex27p exerts an inhibitory or competitive function and (iii) we show that Pex11p only promotes the proliferation of peroxisomes already present in the cell.

Materials and Methods

Plasmids

The pENTR4-*PEX11* plasmids (3) were recombined (Gateway, Invitrogen) with plasmid pRS413-*GPD*prom-*ccdB* (pCB623) to generate plasmids expressing the *PEX11* genes under the control of the *GPD* promoter (for plasmids used in this study see Table S1). In addition, plasmids #1 (*ScPEX11*), #25 (*ScPEX25*) and #74 (*ScPEX27*) were recombined with plasmid pRS415-*GPD*prom-*ccdB* (pCB826). To generate plasmids expressing N-terminally EGFP-tagged versions of the Pex11 proteins, plasmids #1 (*Sc*PEX11), #25 (*Sc*PEX25), #74 (*Sc*PEX27), #217 (*Hs*PEX11$_\gamma$), #102 (*At*PEX11A) and #105 (*At*PEX11D) were recombined with plasmids pRS413-GALprom-EGFP-ccdB (pCB630) or pRS413-GPDprom-EGFP-ccdB (pCB631). The promoter and coding sequences of PEX25 and PEX27 were amplified by PCR using genomic yeast DNA as template and the primer pairs Pex25y-1/Pex25y-2 and Pex27y-1/Pex27y-2, respectively. The DNA fragments obtained were cloned into pGEM-T (Stratagene) and then into pRS313 (*Xba*I/*Not*I) to produce plasmids 1087 and 1088, respectively. mCherry-px was amplified by PCR using pCB314 as a template and primer pair CB111/CB112 and cloned into pCB441 (*Bam*HI/*Hin*dIII) to produce pCB367. The primer pair H911/H912 and plasmid pCB761 were used to amplify the *ADH1* promoter. The PCR fragment was cloned into YEplac195 (37; *Sac*I/*Xba*I) to obtain pCB619. Then, the mCherry-px coding sequence was amplified using primer pair CB293/CB112 (template pCB314) and subcloned into pCB619 resulting in plasmid pCB741. A PCR fragment coding for *GAL*-Sprom-*yeGFP*-px was produced using the primer pair CB344/CB345 and template pCB516 and introduced into plasmid pCB447 (*Sac*I) to obtain plasmid pCB840.

Strains, media and growth conditions

Escherichia coli strains DH5α or DB3.1 (DEST vectors) were used for cloning. The *PEX11* gene was deleted via homologous recombination

in CB80 (38) using a PCR fragment amplified from plasmid pFA6-KanMX4 (Euroscarf) with primer pair Pex11-y-24 and Pex11-y-25 giving rise to strains CB369 and CB370 (for yeast strains used in this study see Table S3). Strains CB371, CB372, CB374, CB375, CB376, CB417 and CB419 were obtained by crossing. Plasmid pCB367 was linearized (EcoRV) and integrated into the URA3 locus of the yeast genomes of CB80, CB369 and CB419 to obtain the strains CB515, CB516, CB545, respectively. In strain CB419, the *PEX3* promoter was replaced by the *GAL-S* promoter using plasmid pCB514 and primers CB206/CB207 to obtain strain CB547. The *LEU2* locus in CB80 was deleted using a PCR fragment (primers CB352/CB353) derived from plasmid pCB840 resulting in strain CB537. To obtain strains CB541, CB542, CB543, CB544, CB532, CB533 and CB535, CB536 *INP2* was deleted using a PCR fragment amplified from plasmid pCB840 with primer pair CB346/CB347 in strains CB80, CB369, CB371, CB372, CB374, CB376, CB417 and CB419, respectively. Yeast strains were grown to optical density at 600nm (OD_{600}) = 1 in glucose medium (0.3% glucose, 0.67% yeast nitrogen base without amino acids (YNB), 0.1% yeast extract, supplemented with amino acids and bases as required, pH 6 with KOH). Then, 5xYNO medium (3.35% yeast nitrogen base without amino acids, supplemented with amino acids and bases as required, pH 6 with KOH, 0.25% Tween-80, 0.5% oleic acid) was added and cells were grown for 16 h. For halo assays, 10-fold serial dilutions of logarithmically growing cultures (OD_{600} = 1) were spotted onto yeast-extract peptone dextrose (YPD) plates (2% glucose, 1% yeast extract, 2% peptone, 3% agar) or oleic acid plates (0.67% YNB, 0.1% yeast extract, 0.125% oleic acid, 0.5% Tween-80, 0.5% KH_2PO_4, pH 6 with K_2HPO_4, supplemented with amino acids and bases as required, 3% agar). Cells expressing *PEX3* under the *GAL-S* promoter were incubated in medium containing 2% glucose with or without a short incubation period in medium containing 2% galactose 8 h prior to microscopic inspection. Cells expressing GFP-px from the *GAL-S* promoter after integration into the *INP2* locus were grown in a synthetic complete medium (SC) with 2% glucose, transferred to SC with 2% galactose/1% raffinose for overnight growth, thinly seeded onto agarose pads containing SC with 2% galactose/1% raffinose and incubated at 30°C for 10 h. Cells expressing heterologous EGFP-tagged Pex11 proteins from the GAL1 promoter on plasmids were grown overnight in glucose medium (0.3% glucose, 0.67% yeast nitrogen base without amino acids, 0.1% yeast extract, supplemented with amino acids and bases as required, pH 6 with KOH), transferred to glucose-free medium containing 1% galactose as carbon source and grown for 4 h prior to fluorescence imaging.

Quantitative real-time PCR

Total RNAs were isolated from yeast using standard procedures. cDNAs were synthesized using $Oligo(dT)_{18}$ primer and RevertAid™ Premium Reverse Transcriptase (Fermentas). PCRs were performed in triplicates with RedTaq2.0xMasterMix (1.5 mM $MgCl_2$; VWR), SYBR Green (Invitrogen), fluorescein isothiocyanate (FITC) (BioRad), deoxyribonucleotide triphosphates (dNTPs) (Roche) and primer pairs Pex11-y-50/51, Pex25-y-12/13 or Pex27-y-15/16 in 96-well plates using a BioRad ICycler. $\Delta\Delta C_T$ values were calculated and the TreeView software was used to illustrate the results.

Antibodies

Anti-Pex11p and anti-Pex25p antibodies were generated in rabbits using the following peptides Pex11: KAKSQSQGDEHEDHKKVLG and Pex25: GASYQDAQDDNTHPHSSDA (Davids Biotechnologie GmbH). Horseradish peroxidase-conjugated antibodies sheep-anti-mouse and donkey-anti-rabbit (GE Healthcare) were purchased. Rabbit anti-thiolase antibodies and rabbit anti-Pex27p antibodies were kindly supplied by Wolf Kunau (Bochum, Germany) and Richard Rachubinski (Edmonton, Canada), respectively.

Microscopy and statistical analysis

All images were acquired with a wide-field microscope (Olympus CellR Imaging Station) equipped with the following filter sets: BP510-550 excitation; LP590 emission for mCherry; BP457-487 excitation; BP503-538 emission for GFP. Images were processed using ImageJ (NIH). Stacks were projected along the *z*-axis (maximum intensity), and brightness and contrast were adjusted for each channel. Transmission images were acquired, colored in blue and brightness and contrast were adjusted to display the borders of each cell. Images were deconvolved with the cmle algorithm using an experimentally derived PSF and the software Huygens Professional when indicated. The figures were composed in CorelDrawX4. For statistical analysis, images were acquired as described above (Olympus, CellR) and red dots (mCherry-px) were manually counted through the whole image stack for at least 100 randomly chosen cells. Only in cells with red fluorescence, either cytosolic or punctate, and without elongated structures, peroxisomes were counted. The histograms were generated in Microsoft Office Excel.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Overexpression of Pex11 proteins in yeast mutant cells affects peroxisome number. A) Yeast cells lacking *PEX11* were transformed with plasmids expressing Pex11 proteins from different organisms and grown on oleic acid-containing media for 16 h. Peroxisomes were visualized through mCherry-px (red channel). Images represent projected *Z*-stacks. Bar: 2 μm. B) Yeast mutant cells as indicated were transformed with plasmids expressing Pex11p, Pex25p or Pex27p and grown on oleic acid-containing media for 16 h. Peroxisomes were visualized through mCherry-px (red channel). Images represent projected *Z*-stacks. Bar: 2 μm.

Table S1: Plasmids used in this study

Table S2: Oligonucleotides used in this study

Table S3: Yeast strains used in this study

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6.3 PEX11 family members are membrane elongation factors that coordinate peroxisome proliferation and maintenance

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Author contributions:

I contributed to the experimental setup, performed all experiments concerning human cells, including microscopy and co-immunoprecipitation, analyzed and interpreted the data and arranged the figures. I performed part of the clonings and established automated counting techniques for peroxisomes in human cells. Moreover, I participated in writing the manuscript.

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PEX11 family members are membrane elongation factors that coordinate peroxisome proliferation and maintenance

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Summary

Dynamic changes of membrane structure are intrinsic to organelle morphogenesis and homeostasis. Ectopic expression of proteins of the PEX11 family from yeast, plant or human lead to the formation of juxtaposed elongated peroxisomes (JEPs),which is evocative of an evolutionary conserved function of these proteins in membrane tubulation. Microscopic examinations reveal that JEPs are composed of independent elongated peroxisomes with heterogeneous distribution of matrix proteins. We established the homo- and heterodimerization properties of the human PEX11 proteins and their interaction with the fission factor *h*Fis1, which is known to recruit the GTPase DRP1 to the peroxisomal membrane. We show that excess of *h*Fis1 but not of DRP1 is sufficient to fragment JEPs into normal round-shaped organelles, and illustrate the requirement of microtubules for JEP formation. Our results demonstrate that PEX11-induced JEPs represent intermediates in the process of peroxisome membrane proliferation and that *h*Fis1 is the limiting factor for progression. Hence, we propose a model for a conserved role of PEX11 proteins in peroxisome maintenance through peroxisome polarization, membrane elongation and segregation.

Key words: Peroxisome proliferation, Organelle fission, PEX11, *h*Fis1, DRP1/DLP1, Microtubules

Introduction

Peroxisomes are highly versatile organelles whose size, shape, number and protein content adapt to the cell type or metabolic requirements (Subramani, 1993; Wanders and Waterham, 2006). This dynamic behavior is essential for cell survival. Peroxisomes are able to proliferate by growth of pre-existing organelles followed by division, but they might also form de novo from the endoplasmic reticulum (ER) (Geuze et al., 2003; Motley and Hettema, 2007; Toro et al., 2009). In human cells, both pathways seem to contribute to the cellular peroxisome pool (Huybrechts et al., 2009; Kim et al., 2006). Failures in peroxisome formation lead to biogenesis disorders (PBDs) such as the Zellweger syndrome, which belongs to a group of lethal metabolic diseases (Steinberg et al., 2006). Similarly, peroxisomes in plants fulfill vital functions in photorespiration and in the metabolism of essential growth hormones such as auxin and jasmonic acid. The absence of functional peroxisomes has been associated with mutations in *PEX* genes whose products, the peroxins, are required for protein import and organelle maintenance (Distel et al., 1996; Wanders and Waterham, 2005). Peroxins are involved in peroxisomal biogenesis and proliferation, membrane and matrix protein import, and recycling of import receptors to the cytosol. Orthologs of these proteins are found in all eukaryotic organisms.

Proteins of the PEX11 family were shown to directly participate in peroxisome proliferation in yeasts, plants and mammals (Abe and Fujiki, 1998; Erdmann and Blobel, 1995; Lingard and Trelease, 2006; Marshall et al., 1995; Orth et al., 2007; Schrader et al., 1998). The yeast *Saccharomyces cerevisiae* harbors three members of the PEX11 protein family, the founding member PEX11, plus PEX25 and PEX27 (Erdmann and Blobel, 1995; Rottensteiner et al., 2003; Smith et al., 2002; Tam et al., 2003). Five orthologs of PEX11 were found in *Arabidopsis thaliana* (Lingard and Trelease, 2006; Orth et al., 2007) and three in mammals (Abe et al., 1998; Tanaka et al., 2003). The five plant PEX11 proteins (PEX11a–PEX11e) fall into two clades. Members of the first clade (PEX11c–PEX11e) are nearly identical in their amino acid composition. Members of the second clade (PEX11a, PEX11b) are more divergent, with approximately 50% similarity. Interestingly, proteins of the first clade carry a C-terminal amino acid sequence –KXKXX, known as the ER retrieval motif, which is thought to facilitate binding of coatomer (Andersson et al., 1999; Cosson et al., 1998).

In mammalian cells, three *PEX11*-related genes have been identified, *PEX11*α, *PEX11*β and *PEX11*γ. The expression of the *PEX11* genes has been extensively studied and animal models have been generated lacking either PEX11α or PEX11β (Li et al., 2002; Li and Gould, 2002). Mice lacking PEX11β display many of the pathologic characteristics of a Zellweger syndrome mouse, which includes neonatal lethality. Mice lacking PEX11α developed normally, showed no obvious defect in constitutive peroxisome division, and displayed a normal response to peroxisome proliferating agents (Li et al., 2002). However, overproduction of PEX11α is sufficient to induce peroxisome proliferation in mouse and human cultured cells (Li and Gould, 2002).

Conservation in the targeting of PEX11 proteins between kingdoms was suggested because heterologously expressed PEX11 from *Trypanosoma brucei* localizes to peroxisomes in mammalian cells (Lorenz et al., 1998). So far, all identified PEX11 proteins share common features: they are small, very basic, and harbor putative transmembrane regions, as calculated using the algorithm HMMTOP 2.0 (Tusnady and Simon, 2001). PEX11 is the most abundant peroxin at the peroxisomal membrane (Erdmann and Blobel, 1996). PEX11 proteins from *S. cerevisae*, *T. brucei* and human cells (*Sc*PEX11, *Tb*PEX11 and *Hs*PEX11γ, respectively) were reported to remain insensitive to protease digestion, which suggests membrane embedding (Lorenz et al., 1998; Marshall et al., 1996; Tanaka et al., 2003). Although a consensus membrane peroxisomal targeting signal (mPTS) exists in PEX11 proteins and facilitates interaction with the mPTS receptor, PEX19 (Fransen et al., 2005; Lorenz et al., 1998; Rottensteiner et al., 2004; Sacksteder et al., 2000), most aspects of peroxisome membrane protein insertion and membrane proliferation remain unknown (Brocard and Hartig, 2006; Girzalsky et al., 2009).

Peroxisome proliferation seems to be a multistep process including elongation, constriction and fission (Koch et al., 2004). PEX11 proteins are thought to participate in the first two steps, whereas DRP1 (dynamin-related protein) and Fis1 (mitochondrial fission protein 1) seem to facilitate fission (Koch et al., 2005; Motley et al., 2008; Zhang and Hu, 2009). Molecular interactions have been established between *Hs*PEX11β and *h*Fis1, a tail-anchored protein recruiting the DRP1 GTPase to the peroxisome membrane (Kobayashi et al., 2007). Fis1 and DRP1, originally found as components of the mitochondrial fission machinery, also localized to the peroxisomal membrane (Koch et al., 2005; Kuravi et al., 2006; Lingard et al., 2008; Motley et al., 2008; Wells et al., 2007). Only a subset of PEX11 proteins was shown to interact with Fis1 in plant and mammals (Kobayashi et al., 2007; Lingard et al., 2008), raising the question of the specific role of each PEX11 protein.

To address this question, we performed a functional study on proteins of the PEX11 family. We present cross-species studies with all PEX11 proteins from yeast, plant and human that illustrate the evolutionary conservation of the molecular mechanism that governs peroxisome proliferation. Revealing new aspects on peroxisome membrane proliferation and inheritance, we demonstrate that PEX11 proteins from yeast, plant and human stimulate the formation of juxtaposed elongated peroxisomes (JEPs) in human cells. We established specific homo- and heterodimerization properties of human proteins and interaction with fission factors. Live-imaging and biochemical analyses exposed specific roles of distinct PEX11 proteins in membrane tubulation and suggest the involvement of microtubules in peroxisome maintenance.

Results

Heterologous PEX11 proteins localize to peroxisomes in human cells and plants

In general, eukaryotic organisms contain a number of distinct PEX11 proteins. To assess PEX11 expression levels in human embryonic kidney cells (HEK293T), we performed quantitative RT-PCRs with mRNAs. The data revealed that all three *PEX11* genes are expressed, with the ratio 2:5:1 for *PEX11*α, *PEX11*β and *PEX11*γ, respectively. To investigate the effects on the shape, size and number of peroxisomes, we expressed EGFP appended with PEX11 sequences originating from the yeast *S. cerevisae* (*Sc*), the plant *A. thaliana* (*At*) and human (*Hs*) in HEK293T cells. Each recombinant EGFP–PEX11 fusion protein produced showed the correct size on a western blot (supplementary material Fig. S1).

We first examined cells expressing either the marker protein EGFP or EGFP–PEX11 fusion proteins (Fig. 1A) using confocal laser scanning microscopy (CLSM). The expression of the PEX11 fusion proteins resulted in the appearance of diverse fluorescent structures, either punctate or elongated, that could also be decorated with antibodies specific to genuine peroxisomal membrane (*Hs*PEX14; Fig. 1A) proteins or matrix (catalase; supplementary material Fig. S2) proteins. This demonstrates that PEX11 proteins of yeast, plant and human origin could all traffic to peroxisomes in human cells (Fig. 1A). Next, to visualize peroxisomes in living cells we concomitantly expressed the peroxisomal matrix marker mCherry–SKL and EGFP–PEX11 fusion proteins and obtained similar results (supplementary material Fig. S3). To scrutinize whether the PEX11 family members present a similar localization pattern in plant tissue, we transiently coexpressed YFP-tagged PEX11 proteins together with the red fluorescent mCherry–SKL in leaf epidermal cells. Except for *Hs*PEX11β, all PEX11 fluorescent fusion proteins were expressed and detected in association with peroxisomes (Fig. 2). Together, these results suggest that targeting of PEX11 proteins to peroxisomes is evolutionarily conserved.

Ectopic expression of PEX11 proteins leads to peroxisome proliferation in human and plant cells

Because our experiments show that all PEX11 fusion proteins localized to peroxisomes, we evaluated their individual effects on the peroxisome number and size and performed statistical analyses. Here, we coexpressed EGFP–PEX11 fusion proteins together with mCherry–SKL in human cells and quantified peroxisomes 24 hours after transfection. This time point was chosen to observe early effects on peroxisomes (Fig. 1B).

The expression of all EGFP–PEX11 fusion proteins had an effect on peroxisome (Px) number and size, although with various intensities. Although expression of *Sc*PEX11 (281±34 Px), *Hs*PEX11β (405±41 Px) or *At*PEX11d (264±32 Px) led to an increase in the number of peroxisomes counted per cell as compared to control (187±17 Px), the cells expressing other proteins such as *Sc*PEX25 (60±6 Px), *At*PEX11e (50±9 Px) and *Hs*PEX11γ (63±3 Px) presented fewer but larger peroxisomes per cell (Fig. 1B). The expression of *At*PEX11a (186±22 Px) and *Hs*PEX11α (188±17 Px) was associated with the incidence of smaller peroxisomes, whereas expression of *At*PEX11b (140±14 Px) and *At*PEX11c (137±17 Px) led to slightly larger ones. By contrast, peroxisomes in cells expressing *Sc*PEX27 (180±26 Px) were indistinguishable from control cells. On a similar line, ectopic expression of individual PEX11 fusion proteins in plant tissue altered the appearance of peroxisomes to different degrees (Fig. 2; supplementary material Table S1). Our data confirm that overexpression of all PEX11 proteins affects peroxisome appearance in plant and human cells.

Ectopic expression of PEX11 proteins leads to the formation of JEPs in human cells

Expression of PEX11 proteins induced the formation of unusually large peroxisomal structures. Within these, sub-structures were observed that could represent juxtaposed elongated peroxisomes (JEPs). The appearance of such structures could be due to intracellular membrane proliferation and aggregation unrelated to peroxisomes, as a consequence of the overproduction of membrane proteins (Wright et al., 1988). Therefore, we analyzed whether such

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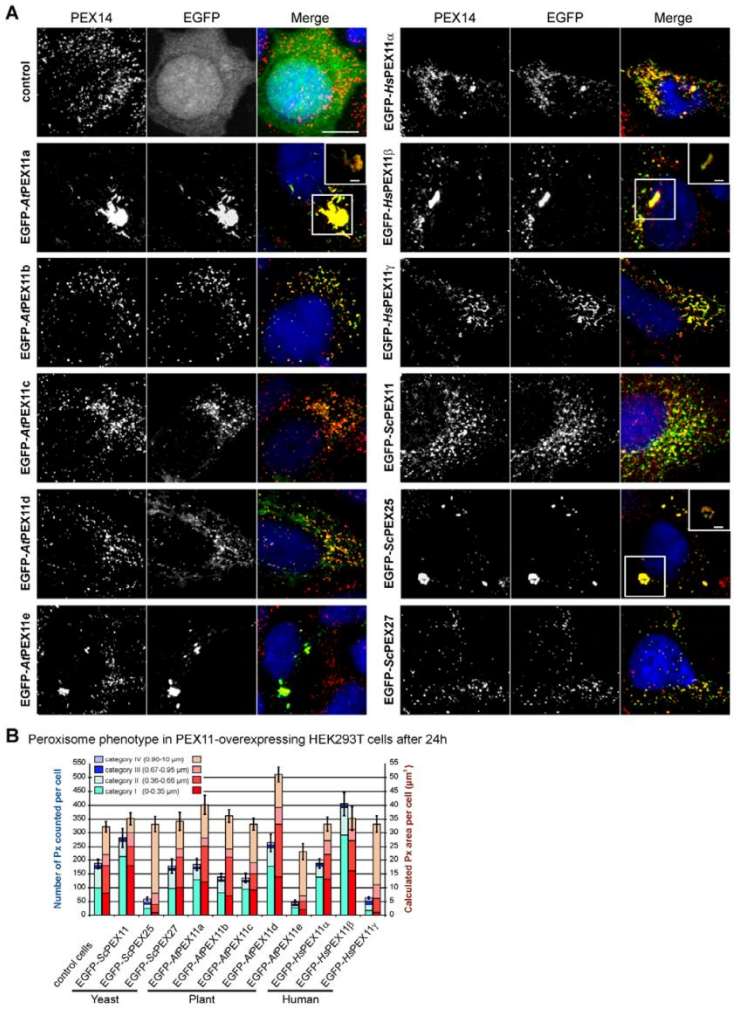

Fig. 1. PEX11 proteins originating from yeast, plant or human all localize to peroxisomes in human cells and affect the number and size of peroxisomes. (A) HEK293T cells were transfected with plasmids coding for EGFP (control) or EGFP–PEX11 fusion proteins as indicated. At 36 hours after transfection, cells were fixed and immunofluorescent stainings were performed with anti-PEX14 antibodies (Alexa Fluor 594, red channel). The fluorescence emitted by GFP and Alexa Fluor 594 was visualized using CLSM. The images represent projected z-stacks. All tested PEX11 fusion proteins localized to human peroxisomes and affected the morphology and number of peroxisomes at various degrees. Nuclear structures were stained with Hoechst 33342 prior to fixation (blue channel). Images in the inserts represent independently acquired pictures of clustered peroxisomes. Scale bars: 10 μm, inserts: 2 μm. (B) Statistical analysis of peroxisome number (blue bars) and area (red bars) in cells overexpressing EGFP–PEX11 fusion proteins 24 hours after transfection. Peroxisomes were visualized by concomitant expression of mCherry–SKL and EGFP (control) or EGFP–PEX11 fusion proteins. Peroxisomes (Px) were counted in cells (n=50) and classified into four categories according to their diameter in order to detect subtle variations of their size. Peroxisomes usually observed in control cells belong to category I (0–0.35 μm) or category II (0.36–0.66 μm). Slightly enlarged peroxisomes are represented in category III (0.67–0.95 μm), whereas elongated and juxtaposed peroxisomes are belong to category IV (0.96–10 μm). Enhanced peroxisomal fission can be statistically observed by a decrease in the peroxisomes, reflected by a shift from category II to category I. The area covered by peroxisomes was calculated for each category ($A=\pi r^2$).

membrane alterations also occurred in cells devoid of peroxisomes. We expressed each one of the EGFP–PEX11 proteins in skin fibroblasts obtained from Zellweger patients lacking a functional PEX19 (Matsuzono et al., 1999). In these cells devoid of detectable peroxisomes, no fluorescent membranous structures were detected. Some of the ectopically expressed proteins appeared essentially in the cytosol, whereas others were not visible by confocal microscopy (our unpublished data), demonstrating that the clusters observed in wild-type cells upon ectopic expression of PEX11 proteins were indeed associated with the presence of peroxisomes and represent JEPs. These structures could also be the consequence of the N-terminal tagging of PEX11 with EGFP. To rule out this possibility, we expressed untagged PEX11 proteins in HEK293T and observed the same effects on peroxisome morphology (supplementary material Fig. S4). Our results clearly show that JEP formation is solely due to overexpression of PEX11 proteins.

At the time point chosen for the statistical analysis (24 hours), JEPs were visualized only in cells expressing certain EGFP–PEX11 fusion proteins. Therefore, we performed time-course experiments and analyzed JEP formation in cells expressing the different PEX11 fusion proteins. After 4 days, expression of all PEX11 fusion proteins led to the occurrence of JEPs, but the kinetics and progression greatly varied depending on which PEX11 was overexpressed (supplementary material Table S1). The expression of *Sc*PEX25, *Hs*PEX11β, *Hs*PEX11γ, *At*PEX11a and *At*PEX11e led to the formation of JEPs 18–24 hours after transfection.

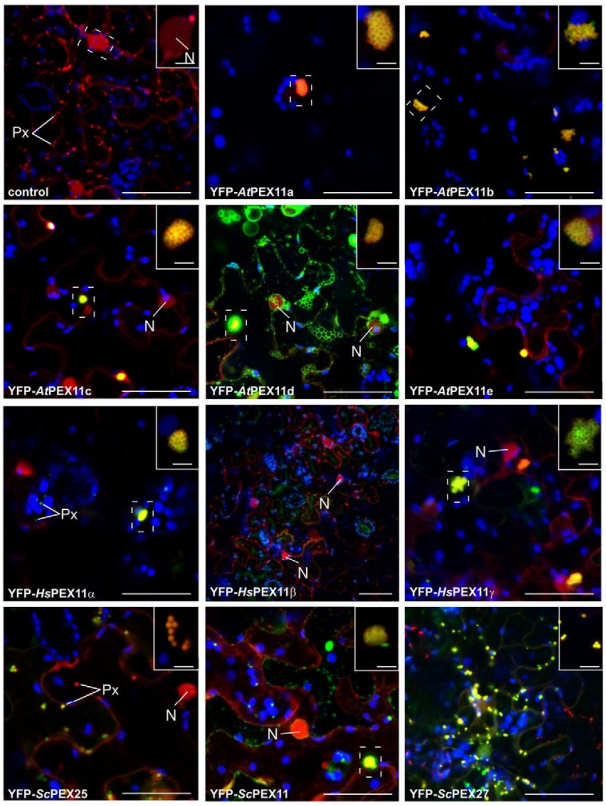

Fig. 2. PEX11 proteins originating from yeast, plant or human all localize to peroxisomes in plant cells. Confocal images of epidermal tissue from *N. benthamiana* leaves transformed with the peroxisomal marker construct mCherry–SKL (control, red channel) and the various plant, human and yeast YFP–PEX11 constructs (green channel). The blue channel indicates chloroplastic autofluorescence. The images represent projected z-stacks and were taken 48 hours after agrobacterial infection. In the inserts, independently acquired single scans of peroxisomal structures appearing in infiltrated cells are shown. These high magnification images indicate that all PEX11 fusion proteins except YFP–*Hs*PEX11β were detected in membranes surrounding peroxisomes labeled with mCherry–SKL. All detected PEX11 constructs altered the size and number of peroxisomes at various degrees. Note that although cell wall autofluorescence was observed, no YFP-specific signal was detected in tissue transformed with the YFP–*Hs*PEX11β-expressing construct. Px peroxisome; N nucleus; Scale bars: 40 μm; inserts: 5 μm.

*Hs*PEX11α led to the occurrence of small JEPs 24 hours after transfection, whereas *Sc*PEX27 or *At*PEX11d induced large JEPs only after 2–3 days (supplementary material Table S1; Fig. 3A). Note that in JEPs fluorescence of peroxisomal matrix proteins did not entirely overlap with the appearance of PEX11 fusion proteins. Instead, the PEX11 fusion proteins localized to structures surrounding the matrix, as indicated with EGFP–*Sc*PEX27 (Fig. 3B,C).

It has been reported that the excess of peroxisomes is selectively degraded by pexophagy (Klionsky, 1997) involving the microtubule-associated protein I light chain 3 (LC3), an essential factor for autophagy in mammalian cells (Hara-Kuge and Fujiki, 2008). To test whether JEPs represent intermediates in the process of pexophagy, we coexpressed the mRFP–LC3 and PEX11 fusion proteins. As expected, mRFP–LC3 associated with few peroxisomes; however, JEPs did not colocalize with mRFP–LC3 (supplementary material Fig. S5), indicating that they do not constitute pexophagy intermediates.

In time-course experiments, we observed that overexpression of PEX16, a peroxisomal membrane peroxin required for peroxisome membrane biogenesis, did not lead to any change in peroxisome morphology. Thus, the detected alterations are correlated with PEX11 protein function (Fig. 1A; Fig. 3D). We analyzed the changes in peroxisomal membrane appearance using human PEX11 proteins and observed that, prior to JEP formation (30 hours), the peroxisomes formed protrusions as visualized by GFP fluorescence (Fig. 3D). Note that mCherry–SKL did not fully colocalize with EGFP–PEX11-labeled protrusions. Similar observations were made using immunofluorescence after staining the cells with anti-catalase antibodies (supplementary material Fig. S2). Hence, peroxisomal matrix proteins seem to be excluded from the PEX11-induced membrane protrusions.

Peroxisome clustering is a membrane dynamic event leading to JEP formation

Live-imaging of cells expressing the PEX11 proteins revealed a dynamic trafficking and clustering of peroxisomes towards the center of the cells (supplementary material Movie 1). Peroxisome remodeling and elongation occurred up to the formation of JEPs, which were inherited during cell division. To determine whether the JEPs are autonomous entities or represent continuous membrane structures we performed fluorescence recovery after photobleaching (FRAP) assays (Fig. 4A,B). Whereas in control cells the expressed ER membrane protein EGFP–Sec61β showed rapid and full

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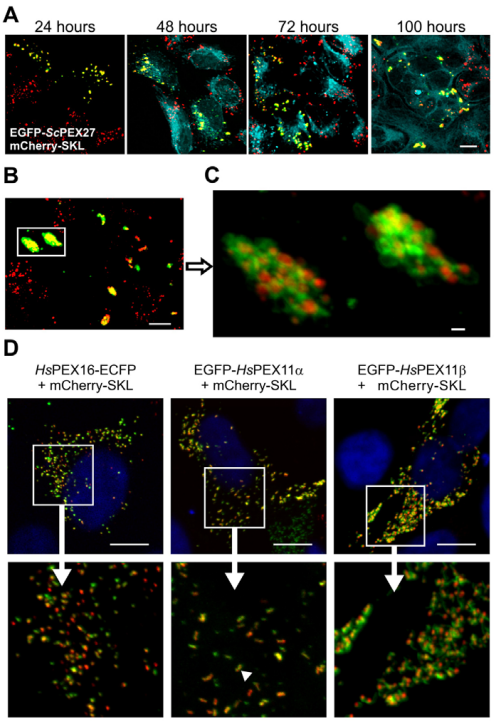

Fig. 3. Ectopic expression of PEX11 proteins influences the peroxisomal morphology in human cells. (**A**) Cells were co-transfected with mCherry–SKL (red channel) and EGFP–*Sc*PEX27 (green channel) encoding plasmids. Cells were fixed after 24, 48, 72 and 100 hours and the fluorescence was visualized by CLSM. EGFP–*Sc*PEX27 localizes almost exclusively to peroxisomes in human cells. Well-separated peroxisomes (24 hours) slowly formed clusters. (**B**) At later timepoints, the peroxisomal structures were enlarged and EGFP–*Sc*PEX27 did not fully colocalize with the peroxisomal marker mCherry–SKL. (**C**) Instead, red and green speckled structures could be visualized, which shows that the fluorescence signal emitted by EGFP–*Sc*PEX27 (green) and the matrix marker mCherry–SKL (red) only partially overlap. Mitochondria were stained using deep-red mitotracker (cyan channel). Scale bars: 10 µm, crop: 1 µm. (**D**) Cells coexpressing mCherry–SKL and *Hs*PEX16–ECFP, EGFP–*Hs*PEX11α or EGFP–*Hs*PEX11β were observed 30 hours after transfection. The fluorescence emitted by the fluorophores was monitored via CLSM. *Hs*PEX16–ECFP colocalized with mCherry–SKL (red channel) without affecting the size, shape and number of peroxisomes. EGFP–*Hs*PEX11α and EGFP–*Hs*PEX11β both appeared to concentrate at specific sites on the peroxisomal membrane and form protrusions. After 30 hours in cells expressing EGFP–*Hs*PEX11α, peroxisomes were elongated. EGFP–*Hs*PEX11β-containing peroxisomes presented multiple protrusions, as depicted in the region of interest. Note that the peroxisomal matrix marker could not be detected in the membranous protrusions induced by *Hs*PEX11β. Nuclear structures were stained with Hoechst 33342 prior fixation (blue channel). Scale bars: 10 µm, inserts: 5 µm.

fluorescence recovery ($t_{1/2}$~7 seconds), the EGFP–*Hs*PEX11β signal did not show significant fluorescence recovery. This clearly shows that the PEX11-containing structures do not share a common membrane. Thus, it might well be that the clusters that emerge upon overexpression of PEX11 proteins represent peroxisomes that remain juxtaposed due to interorganellar protein interactions. Analysis of PEX11γ-induced structures via electron microscopy illustrates the presence of tubular smooth membranes that are absent in wild-type HEK293T cells (Fig. 4C). The abundant presence of PEX11 proteins enhances peroxisome elongation and concomitantly delays peroxisome fission, generating the opportunity to follow several steps of peroxisome proliferation. JEPs seem to represent intermediates formed during peroxisome proliferation. Interaction of PEX11 with components of the cytoskeleton might lead to such clustering and influence peroxisome inheritance during cell division, as proposed for yeast (Krikken et al., 2009).

JEPs are the result of exhausted fission machinery

To test whether JEPs are the consequence of incomplete peroxisome fission, we analyzed the in vivo effect of fission factors on JEP formation. Coexpression of myc-*h*Fis1 and EGFP–PEX11 fusion proteins gave rise to well-separated peroxisomes (Fig. 5A). Thus, the fission factor *h*Fis1 counterbalanced PEX11-induced JEP formation. Supporting the notion that membrane elongation is part of the proliferation process coexpression of ECFP–DRP1 and EGFP–PEX11 proteins led to the formation of even more elongated JEPs than expression of EGFP–PEX11 fusions alone. Moreover, as exemplified in Fig. 5B, three-dimensional reconstructions illustrate that JEPs are composed of individual elongated peroxisomal structures. Thus, *h*Fis1 seems to be the limiting factor for continuing the proliferation process induced by ectopic expression of human PEX11 proteins. The effects of ectopic expression of yeast and plant PEX11 fusion proteins were counterbalanced by *h*Fis1, as well (Fig. 5A and supplementary material Fig. S2), which suggests that the molecular mechanism underlying peroxisome proliferation has been conserved through evolution. Although our experiments indicate that overexpression of *h*Fis1 restored peroxisome fission in PEX11-overexpressing cells we could not rule out the possibility that *h*Fis1 simply overrode PEX11-initiated peroxisome proliferation through another mechanism.

To distinguish between the two possibilities, we expressed the peroxisomal marker protein EGFP–Scp2 or PEX11 fusion proteins. When JEPs appeared in the latter (48 hours), cells were transfected a second time with plasmids coding for *h*Fis1, DRP1, or both, and grown for another 48 hours. Whereas DRP1 expression enhanced peroxisome elongation and JEP-formation (Fig. 5C), *h*Fis1 or *h*Fis1 and DRP1 expression led to well-separated and normal-sized peroxisomes regardless of the abundant presence of a yeast, plant or human PEX11 protein (Fig. 5). Obviously, proteins of the PEX11 family induce peroxisome proliferation, and *h*Fis1 function is essential to progress into fission and to finalize the proliferation process. The levels of *h*Fis1 do not vary upon PEX11 overexpression (supplementary material Fig. S1). To assess whether the *h*Fis1 levels are influential in the context of our experiments, we evaluated the effects of *h*Fis1 knockdown in human cells using siRNA. Cells expressing unspecific siRNA targeted to firefly luciferase (control) presented well-separated round-shaped peroxisomes. But, in cells expressing the *h*Fis1–siRNAs, peroxisomes were elongated and formed small JEPs. This effect was tremendously enhanced by the overexpression of *Hs*PEX11β (Fig. 5D). In that case, JEPs appeared much faster, which correlates with *h*Fis1 levels becoming limited. As a consequence, DRP1

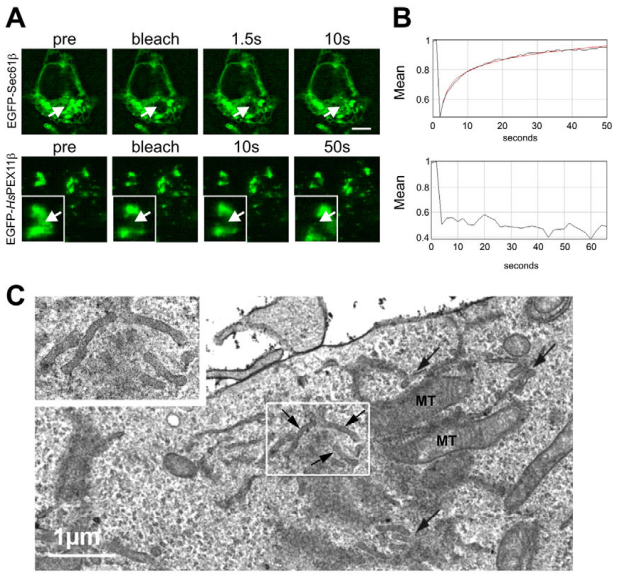

Fig. 4. FRAP experiments and electron microscopy images indicate that PEX11-derived clusters do not share a common membrane. (**A**) HEK293T cells were transfected with plasmids expressing either EGFP–Sec61β (an ER-membrane protein) or EGFP–*Hs*PEX11β and observed 30 hours after transfection. FRAP experiments were performed after two full pre-scans by bleaching a region of interest (arrows), and cells were subsequently imaged without delay to monitor recovery. Although the control expressing EGFP–Sec61β showed fast and full fluorescence recovery ($t_{1/2}$~7 seconds), the EGFP–*Hs*PEX11β did not show significant fluorescence recovery within 10 minutes (full time-frame not shown), indicating that the PEX11β-containing structures do not share a common membrane. Similar results were obtained in iFRAP experiments (data not shown). Scale bar: 10 μm. (**B**) The graphs show the measured fluorescent intensity in arbitrary units for either EGFP–Sec61β or EGFP–*Hs*PEX11β. The signal was normalized to the whole cell fluorescent intensity. The analysis was performed with FRAP Profiler, a MBF plugin (http://www.macbiophotonics.ca/imagej/index.htm) of ImageJ. (**C**) Representative electron micrograph of a HEK293T cell overexpressing EGFP–*Hs*PEX11γ. Tubular, smooth surfaced membrane profiles are visible (arrows); MT, mitochondria. They are characterized by rigid membranous structures with homogeneous electron-dense contents and a relative constant diameter of slightly greater than 80 nm (80–100 nm). The inset represents the central region magnified twofold.

cannot be properly recruited on peroxisomes and the fission process does not occur. Considering the difference between a knockdown and an overexpression, this result is consistent with the hypothesis that the PEX11 to hFis1 ratio determines the rate of peroxisome proliferation.

Interplay between components of the proliferation machinery in human cells

The yeast PEX11 is thought to act as a monomer (Marshall et al., 1996), whereas PEX11β seems to require homodimerization for its proliferating activity (Kobayashi et al., 2007). It has been reported that *Hs*PEX11α and *Hs*PEX11β could form homophilic complexes but that these two proteins could not interact with each other (Li and Gould, 2003), which suggests that they might be part of different pathways. Yet, only *Hs*Pex11β has been shown to be part of a ternary complex containing *h*Fis1 and DRP1 (Kobayashi et al., 2007). To reveal the interplay between PEX11 proteins and factors of the fission machinery, we performed a comprehensive molecular interaction study. Co-immunoprecipitations were performed on HEK293T cell lysates 48 hours after coexpression of: (1) *Hs*PEX11α–FLAG and EGFP–*Hs*PEX11α; (2) *Hs*PEX11α–FLAG and EGFP–*Hs*PEX11β; (3) *Hs*PEX11γ–FLAG and EGFP–*Hs*PEX11α; (4) *Hs*PEX11β–FLAG and EGFP–*Hs*PEX11β; (5) *Hs*PEX11γ–FLAG and EGFP–*Hs*PEX11β; and (6) *Hs*PEX11γ–FLAG and EGFP–*Hs*PEX11γ. In the presence of 0.2% digitonin, all pairwise interactions were detected, except between *Hs*PEX11α and *Hs*PEX11β (Fig. 6A) and, in addition, all three human PEX11 proteins co-precipitated with *h*Fis1 (Fig. 6B). Note that none of these interactions could be detected in the presence of 1% Triton X-100. It seems that the PEX11 proteins act as heteromeric pairs consisting of *Hs*PEX11α–*Hs*PEX11γ and *Hs*PEX11β–*Hs*PEX11γ, representing two separate proliferation pathways, which both require interaction with fission factors to fulfill their function. That immunoprecipitations can occur in the presence of digitonin but not of Triton X-100 suggests that the hydrophobic regions of each PEX11 protein and their membrane integration are required for interaction.

Microtubules and hydrophobic regions of PEX11 proteins are required for JEP formation

The oriented movement of JEPs during cell division suggests that microtubules and PEX11 proteins are involved in peroxisome inheritance (see supplementary material Movie 1). To test this, we treated cells expressing EGFP–*Hs*PEX11γ with Nocodazole, a chemical that blocks the self-assembly of tubulin leading to microtubule depolymerization. As shown in Fig. 7, a functional microtubule cytoskeleton is required for JEP formation.

To assess whether hydrophobic regions of PEX11 proteins are essential for the formation of JEPs, we expressed EGFP fusions of *Hs*PEX11α, *Hs*PEX11β or *Sc*PEX11 lacking their putative transmembrane regions in HEK293T cells (Fig. 8A). In cells overexpressing any one of the three proteins, the morphology and number of peroxisomes were indistinguishable from those in wild-type cells and no JEPs were formed. Interestingly, although EGFP expressing *Hs*PEX11α lacking its C-terminal hydrophobic region appeared predominantly soluble in the cytosol, a significant portion of the truncated EGFP–*Sc*PEX11 and EGFP–*Hs*PEX11β did localize to peroxisomes. We performed co-immunoprecipitations using these truncated PEX11 proteins. Here, $Hs\text{PEX11}\alpha^{\Delta 20}$ did not interact with the full-length *Hs*PEX11α and *Hs*PEX11γ. Although, $Hs\text{PEX11}\beta^{\Delta 26}$ was able to interact with the full-length *Hs*PEX11β, interaction with *Hs*PEX11γ or with *h*Fis1 could not be detected (Fig. 8B). Together, these data indicate that PEX11 proteins rendered less hydrophobic lose their function in peroxisome proliferation because they can neither form specific heterodimers nor bind *h*Fis1.

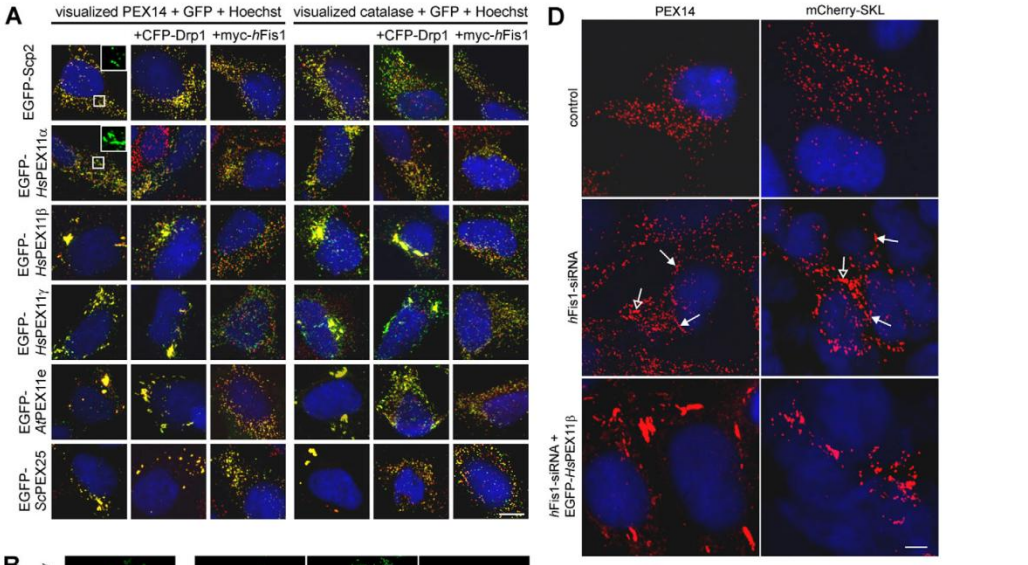

Fig. 5. *h*Fis1 overexpression is sufficient to separate PEX11-induced JEPs, and DRP1 enhances JEP formation. Images represent projected z-stacks acquired by CLSM. (**A**) Overexpression of EGFP–*Hs*PEX11α, EGFP–*Hs*PEX11β, EGFP–*Hs*PEX11γ, EGFP–*At*PEX11e or EGFP–*Sc*PEX25 (as indicated) led to elongation of peroxisomes and subsequent clustering as compared to control cells overexpressing EGFP–Scp2. Coexpression of CFP–DRP1 aggravated this tendency and led to the appearance of more elongated structures. Upon concomitant overexpression of myc-*h*Fis1, no more clustering could be observed. Counterstaining of peroxisomes was performed by immunofluorescence against PEX14 and catalase, respectively (Alexa Fluor 594, red channel). Nuclear structures were stained with Hoechst 33342, blue channel. Scale bar: 10 µm. Images in the inserts represent 3.5-fold magnifications of a single Z-layer in the delineated areas. (**B**) Images show a morphologic analysis of elongated structures derived from CFP–DRP1 overexpression in cells expressing EGFP–*Hs*PEX11γ or EGFP–*At*PEX11e (left panel). JEPs can be clearly visualized through deconvolution and three-dimensional reconstruction (right panels). The delineated regions (1–4) represent different magnifications and orientations (Huygens Professional). Scale bar: 5 µm. (**C**) HEK293T cells were first transfected with plasmids coding for EGFP-fusion proteins as indicated (1). After 48 hours, the cells were transfected with plasmids coding for either CFP–DRP1, myc-*h*Fis1, or both (2). Observation of peroxisomal structures reveals that the JEPs derived from overexpression of PEX11 further elongated upon ECFP–DRP1 overexpression, whereas they divided and appeared well-separated when myc-*h*Fis1 was expressed. GFP (green channel); Mitotracker IR (red channel). Scale bar: 5 µm. (**D**) HEK293T cells were transfected with siRNA against firefly luciferase (control) or with *h*Fis1–siRNA. After 24 hours, cells were transfected a second time with the siRNAs alone or with EGFP–*Hs*PEX11β (green channel) and analyzed after 48 hours. Peroxisomes were visualized either through expression of mCherry–SKL or through immunostaining with anti-PEX14 antibodies (red channel). Cells depleted for *h*Fis1 presented mostly elongated peroxisomes (closed arrows), and small JEPs could be observed (open arrows). The expression of EGFP–*Hs*PEX11β for 48 hours in these cells led to the formation of large JEPs. Note that in cells expressing the matrix marker mCherry–SKL, peroxisomes appear more round but that this did not alter the formation of JEPs. Scale bar: 5 µm.

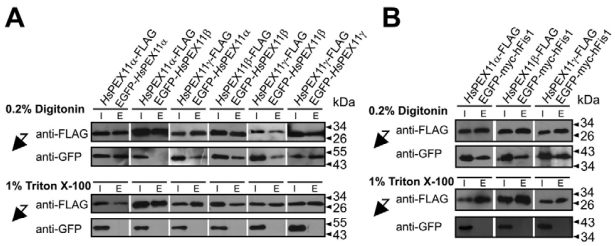

Fig. 6. *Hs*PEX11α and *Hs*PEX11β can both interact with *Hs*PEX11γ and with *h*Fis1. (A) HEK293T cells were transfected with plasmid pairs expressing *Hs*PEX11α–FLAG and EGFP–*Hs*PEX11α; *Hs*PEX11α–FLAG and EGFP–*Hs*PEX11β; *Hs*PEX11γ–FLAG and EGFP–*Hs*PEX11α; *Hs*PEX11β–FLAG and EGFP–*Hs*PEX11β; *Hs*PEX11γ–FLAG and EGFP–*Hs*PEX11β; or *Hs*PEX11γ–FLAG and EGFP–*Hs*PEX11γ and collected 48 hours after transfection. Cells were lysed in buffer containing either 0.2% digitonin or 1% Triton-X100. All immunoprecipitations were performed with equal cell fractions using anti-FLAG antibodies covalently attached to agarose beads and analyzed by western blotting. (B) HEK293T cells were transfected with plasmids expressing *Hs*PEX11α–FLAG and EGFP–myc-*h*Fis1; *Hs*PEX11β–FLAG and EGFP–myc-*h*Fis1; or *Hs*PEX11γ–FLAG and EGFP–myc-*h*Fis1 and collected 48 hours after transfection. Cell extracts and immunoprecipitations were prepared as described above. 3% of starting material (I) and 10% of eluate (E) obtained with excess of 3×FLAG peptides were loaded and separated on a 10% SDS-PAGE.

Discussion

Proteins of the PEX11 family are known to be essential regulators of peroxisome proliferation in all organisms studied. By shifting the delicate balance between proliferation factors we were able to observe intermediate stages of peroxisome proliferation. We show that ectopic expression of PEX11 proteins of yeast, plant and human induces a dynamic change in peroxisomal appearance in plant and human cells. Transiently expressed PEX11 proteins localize to peroxisomes and lead to the formation of membrane protrusions and to membrane elongation, which finally develop into JEP structures (Figs 1, 2). This model is supported by three-dimensional reconstructions of confocal images showing JEPs (Fig. 5B), which correspond in size and shape to the tubular membrane structures observed in electron micrographs (Fig. 4C). The size of these structures depends on which PEX11 protein is ectopically expressed (supplementary material Table S1). Similar changes in peroxisome appearance are induced upon a decrease in *h*Fis1 levels, implying that peroxisome fission is hampered in cells overexpressing either of the PEX11 protein fusions. This scenario is strengthened by the observation that an increase in *h*Fis1 fission factor levels leads to the dissolution of JEPs (Fig. 5). In addition, PEX11-driven peroxisome membrane elongation coincides with the segregation of PEX11 from matrix proteins, which suggests that PEX11 proteins are key factors that induce remodeling of the entire peroxisomal compartment.

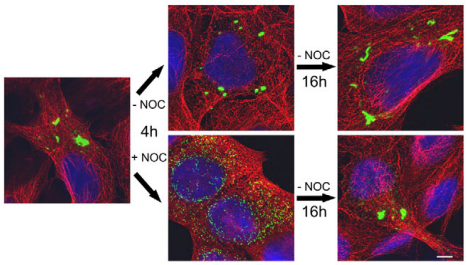

Fig. 7. The cytoskeletal tubulin network is involved in JEP formation. HEK293T cells expressing EGFP–*Hs*PEX11γ fusion protein (green channel) for 24 hours were treated with Nocodazole (NOC) for 4 hours to inhibit microtubule-polymerization. JEPs already present at this time-point (left panel) were greatly reduced after the treatment, and these cells presented more, smaller and elongated peroxisomes compared to control cells (DMSO-treated, middle panel). JEPs successfully reformed 16 hours after Nocodazole release (right panel). Microtubules were visualized through immunofluorescence against α-tubulin (Texas Red, red channel). Images from the red channel were deconvolved (Huygens Professional) to better perceive the structure of the microtubule network. Nuclear structures were stained with Hoechst 33342, blue channel. Scale bar: 5 μm.

Our data demonstrate that both the targeting and the function of PEX11 proteins have been conserved throughout evolution. Mammalian cells possess three PEX11 proteins, PEX11α, PEX11β and PEX11γ, and little is known regarding their precise function and interactions. We show that PEX11γ interacts with both PEX11α and PEX11β, but that these two proteins do not associate with each other. These results place PEX11γ at the crossroad of PEX11-induced peroxisome proliferation pathways. Using co-immunoprecipitation assays we confirmed the previously reported homodimerization of PEX11α and PEX11β (Li and Gould, 2003) and show that PEX11γ can similarly participate in homotypic interactions. A possible explanation for this finding is that the function of PEX11 proteins is regulated through the formation of homodimers. Indeed, homodimerization could constitute a molecular switch that allows PEX11 proteins to change from an active (monomer) to an inactive state (dimer), as already proposed for the yeast PEX11 protein (Marshall et al., 1996). Conversely, stimulus-driven PEX11 protein heterodimerization could allow the formation of PEX11-rich patches on the membrane, thereby promoting membrane protrusion and elongation at a distinct location on the peroxisomal surface. PEX11β might be needed for constitutive peroxisome proliferation, whereas PEX11α might be required for peroxisome proliferation in response to external stimuli. Our findings that overexpression of PEX11γ induces the early formation of JEPs (supplementary material Table S1) suggest that this protein either acts upstream of PEX11α and PEX11β or is the limiting factor, in agreement with the results of our quantitative RT-PCR. PEX11γ seems to recruit the other PEX11 proteins and position PEX11-rich patches on the peroxisomal membrane to facilitate further molecular associations. Our data suggest that PEX11γ is always required for

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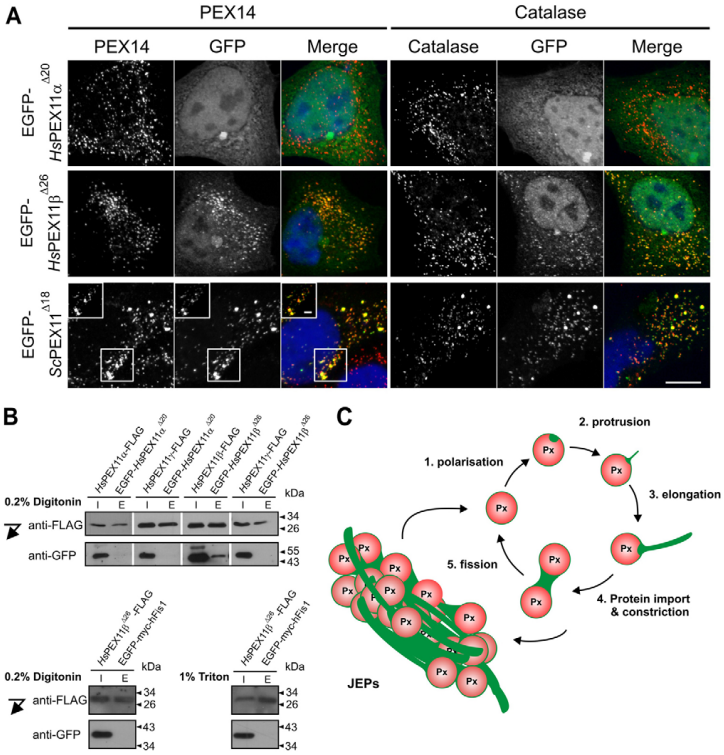

Fig. 8. PEX11 proteins lacking their hydrophobic region lose the ability to induce JEP formation and to interact with *Hs*PEX11γ. (**A**) Cells ectopically expressing EGFP–*Hs*PEX11α$^{\Delta20}$, EGFP–*Hs*PEX11β$^{\Delta26}$ or EGFP–*Sc*PEX11$^{\Delta18}$ (as indicated) were analyzed by immunofluorescence using antibodies directed to either PEX14 or catalase (Alexa Fluor 594, red channel). EGFP–*Hs*PEX11α$^{\Delta20}$ was only detectable in the cytosol (and nucleus), whereas EGFP–*Hs*PEX11β$^{\Delta26}$ partially localized to peroxisomes. Interestingly, expression of the latter truncation did not lead to a relevant effect on peroxisome morphology or number as did the full-length *Hs*PEX11β (see Fig. 1). Moreover, a significant portion of this protein mislocalized to the cytosol. By contrast, EGFP–*Sc*PEX11$^{\Delta18}$ showed peroxisomal staining but, similar to *Hs*PEX11β$^{\Delta26}$, seems to have lost the ability to influence peroxisome morphology and number. The intense staining of a few peroxisomes in cells expressing *Sc*PEX11$^{\Delta18}$ might correlate with an irregular distribution of this protein to peroxisomes. Nuclear structures were stained with Hoechst 33342 (blue channel). Images represent projected z-stacks. Insets represent a single confocal layer of the regions of interest. Scale bar: 10 µm; inset: 2 µm. (**B**) HEK293T cells were transfected with plasmid pairs expressing EGFP–*Hs*PEX11α$^{\Delta20}$ and *Hs*PEX11α–FLAG; EGFP–*Hs*PEX11β$^{\Delta26}$ and *Hs*PEX11β–FLAG; EGFP–*Hs*PEX11α$^{\Delta20}$ and *Hs*PEX11γ–FLAG; EGFP–*Hs*PEX11β$^{\Delta26}$ and *Hs*PEX11γ–FLAG; or *Hs*PEX11β$^{\Delta26}$–FLAG and EGFP–myc-*h*Fis1 and collected 48 hours after transfection and lysed in buffer containing 0.2% digitonin or 1% Triton-X100. All immunoprecipitations were performed with equal cell fractions using anti-FLAG antibodies covalently attached to agarose beads and analyzed by western blotting. *Hs*PEX11α truncated at its hydrophobic region could not interact with *Hs*PEX11α or *Hs*PEX11γ. By contrast, *Hs*PEX11β$^{\Delta26}$ showed weak interaction with *Hs*PEX11β, which might explain its partial localization to peroxisomes. However, *Hs*PEX11β$^{\Delta26}$ could no longer interact with *Hs*PEX11γ or *h*Fis1. 3% of starting material (I) and 10% of eluate (E) obtained with excess of 3×FLAG peptides were loaded and separated on a 10% SDS-PAGE. (**C**) Model for the action of PEX11 in membrane proliferation. PEX11 localizes to the peroxisomal membrane and upon activation assembles in patches at specific sites (1. polarization) stipulating the accumulation of phospholipids (2. protrusion). Further recruitment of lipids and membrane proteins (3. elongation), including the import machinery, allows the translocation of matrix proteins through the newly formed membrane, visualized as constricted membrane tubules (4. protein import and constriction). The PEX11 located in these constrictions recruits factors leading to membrane fission (5. fission). In excess of PEX11 proteins, fission factors become limiting, which results in the accumulation of proliferation intermediates. Microtubules might assist interorganellar interactions, e.g. via PEX11 leading to JEP formation. The red color represents peroxisomal matrix proteins and the green areas represent peroxisomal membranes loaded with PEX11 proteins.

elongation of the peroxisome membrane, and that PEX11α or PEX11β might support peroxisome proliferation and division only under inducing or non-inducing growth conditions, respectively.

PEX11γ was found to expose its N- and C-termini to the cytosol (Tanaka et al., 2003), and the same type of membrane topology was suggested for PEX11α and PEX11β (Abe and Fujiki, 1998; Passreiter et al., 1998; Schrader et al., 1998), implying that PEX11 proteins might possess a functional domain in their cytosol-oriented N- or C-terminal part. Two hybrid assays with PEX11β suggested that, whereas the presence of its N-terminus is important for dimerization,

its C-terminus might interact with *h*Fis1 and counteract homodimerization (Kobayashi et al., 2007). Here, we expanded this theme on other proteins of the PEX11 family and found that overexpression of PEX11 proteins, either untagged or tagged with EGFP at their N-termini, greatly affected peroxisomal structure (Figs 1, 2; supplementary material Fig. S4) but this effect was abolished when their hydrophobic domain was deleted (Fig. 8A). Interestingly, although C-terminal tagging with small tags such as haemagglutinin (HA) or FLAG did not hinder PEX11 function and JEPs were formed in the cells, large C-terminal tags, e.g., EGFP abolished the PEX11-driven effects. Thus, it is likely that the C-termini of PEX11 proteins play an essential role in promoting peroxisome membrane proliferation. In addition, the hydrophobic region close to the C-termini might contribute to trafficking events associated with their correct insertion into the peroxisomal membrane.

In lower eukaryotes, physiological levels of PEX11 are sufficient to cause fragmentation of peroxisomes, and peroxisomes are enlarged in cells lacking PEX11 (Erdmann and Blobel, 1995; Voncken et al., 2003). In human cells, high levels of PEX11 proteins lead to tubulation and elongation of the peroxisomal membrane (Figs 3, 4). Thus, PEX11 proteins might regulate the overall membrane curvature or associate with specific lipids to determine the correct composition of the peroxisomal membrane, which are two functions that might also be needed for de novo formation of peroxisomes.

Peroxisome movement has been reported to depend on microtubules in mammalian cells and on actin filaments in yeast and plants (Fagarasanu et al., 2006; Mathur et al., 2002; Rapp et al., 1996; Wiemer et al., 1997). The formation of PEX11-induced JEPs could, directly or indirectly, depend on the microtubular transport machinery. A study on the role of microtubules in peroxisome proliferation in fibroblasts from patients with Zellweger syndrome (*PEX1*-null) showed that the overexpression of PEX11β restored the alignment of peroxisomal structures along microtubules (Nguyen et al., 2006). Accordingly, we show that during cell division, JEPs have an ordered movement, are inherited (see supplementary material Movie 1) and their formation depends on the presence of intact microtubules (Fig. 7). Thus, an essential aspect of peroxisome proliferation could be the interaction of the organelle with the cytoskeleton, a process in which PEX11 proteins might fulfill a primordial function through association with microtubule binding proteins.

The known mitochondrial fission factors, dynamin-related-proteins and Fis1, also play a role in peroxisome fission in yeast, plant and mammalian cells (Kobayashi et al., 2007; Koch et al., 2005; Zhang and Hu, 2009). *Hs*PEX11β has previously been shown to bind *h*Fis1 (Kobayashi et al., 2007), raising the question of whether PEX11β is the sole factor involved in the recruitment of the fission factors to the peroxisomal membrane. Addressing this, we found that *h*Fis1 associates with all three human PEX11 proteins (Fig. 6B). Heteromeric PEX11 protein complexes, such as PEX11α–PEX11γ and PEX11β–PEX11γ dimers, might recruit *h*Fis1 and thereby initiate DRP1 self-assembly. This interaction cascade might induce constriction and scission of the peroxisomal membrane, as proposed for mitochondria (Fukushima et al., 2001). In yeast, it has been reported that the number of peroxisomes doubles shortly before cell division (Hoepfner et al., 2001). Similar to mitochondria (Taguchi et al., 2007), peroxisomes might become fragmented in early mitotic phase. Our results support the notion that proteins of the PEX11 family are essential for initiation of peroxisomal fission by anchoring DRP1 through *h*Fis1.

On the basis of our observation that PEX11 is unequally distributed in the peroxisomal membrane (Figs 1, 3; supplementary material Figs S2, S3), we suggest that this polarization represents a key step in the initiation of elongation of the membrane. We propose a model (Fig. 8C) for a conserved role of PEX11 proteins in peroxisome polarization, membrane protrusion, and elongation. In this model, PEX11γ initiates the proliferation by determining the site of protrusion through formation of PEX11-rich patches at the membrane. Consistent with this view, PEX11γ displays low constitutive expression level, interacts with both PEX11α and PEX11β and, upon ectopic expression, triggers the earliest appearance of JEPs. In the next stage, PEX11γ (with the help of PEX11α or PEX11β) initiates the formation of protrusions leading to elongation of the peroxisomal membrane. By these means, PEX11 proteins might coordinate peroxisome proliferation to the metabolic requirements of the cell. The final stages of membrane constriction and fission require additional factors and have to be coordinated with the delivery of matrix proteins and membranous components to the nascent organelle. Hence, by altering the composition of the PEX11 complex at the peroxisomal membrane, an effect on the import and distribution of peroxisomal matrix proteins can also be observed (Fig. 3). Our studies on PEX11 proteins provide new insights into the mechanism of peroxisome proliferation. We have revealed an intermediate morphological stage in the formation of JEPs, which became detectable as a consequence of a change in the equilibrium between PEX11 proteins and *h*Fis1.

Materials and Methods

Plasmids

All PEX11 proteins were N-terminally tagged with EGFP (human cells) or YFP (plant cells). The PCR fragments representing the cDNA of *Sc*PEX11, *Sc*PEX25 and *Sc*PEX27 (YOL147C, YPL112C, YOR193W) and *At*PEX11a *At*PEX11c (TAIR Acc. AT1G47750, AT3G47430, AT1G01820, AT2G45740 and AT3G61070) or *Hs*PEX11α, *Hs*PEX11β and *Hs*PEX11γ (Acc. NM_003847, NM_003846 and NM_080662) were cloned into pENTR4 (Invitrogen) using *Nco*I and *Xho*I restriction sites. The resulting pENTR4 PEX11 plasmids were sequenced and served as entry constructs for Gateway (Invitrogen) recombination-mediated cDNA transfer into the pDEST53 vector (Invitrogen), allowing for expression of EGFP PEX11 fusions under the control of the CMV promoter in human cell culture. Plasmids coding for EGFP *Hs*PEX11α$^{\Delta 20}$ (AA220 to AA239) and EGFP *Hs*PEX11β$^{\Delta 26}$ (AA230 to AA255) as well as EGFP *Sc*PEX11$^{\Delta 18}$ (AA215 to AA232) were engineered by PCR and cloned into pENTR4, followed by recombination as described above. *Hs*PEX11γ 3×FLAG in a pReceiverM14 was purchased from GeneCopoeia (Rockville, MD). *Hs*PEX11α, *Hs*PEX11β and *Hs*PEX11β$^{\Delta 26}$ were exchanged against *Hs*PEX11γ in the pReceiverM14 (*Kpn*I/*Nhe*I) to create *Hs*PEX11α 3×FLAG, *Hs*PEX11β 3×FLAG and *Hs*PEX11β$^{\Delta 26}$ 3×FLAG, respectively. For mammalian cells, pmCherry SKL was cloned via PCR by appending the tripeptide SKL to mCherry and replacing it against EYFP ER in the plasmid pEYFP ER (*Nhe*I/*Bgl*II, Clontech).

For plants, the coding sequences from the pENTR4 PEX11 plasmids were recombined into the binary plant expression vector pEarlyGate104 (Earley et al., 2006). The resulting vector allowed 35S promoter-driven in planta expression of N-terminally tagged YFP fusions. For estradiol-inducible expression of untagged *At*PEX11d, the according entry vector was recombined with pMDC7 (Curtis and Grossniklaus, 2003). The red fluorescent peroxisomal marker construct mCherry SKL cDNA was produced by PCR and transferred via a BP Gateway reaction into the pDONOR plasmid (Invitrogen), which served as template for an LR gateway recombination with the pMDC7 vector. For transient plant expression experiments, the according binary vectors were transferred by electroporation into the *A. tumefaciens* strain AGL1 (Lazo et al., 1991). To create the EGFP myc-*h*Fis1 expression plasmid, EGFP (*Nde*I-*Bgl*II) from pEGFP-C1 (Clontech) was inserted into the myc-*h*Fis1 (Yoon et al., 2003) expression vector. Plasmids expressing *Hs*PEX16 CFP (Brocard et al., 2005) and GFP Scp2 (Stanley et al., 2006) have been described before. For primers used in this study see supplementary material Table S2.

Cell culture, transfection, RNA INTERFERENCE and Immunofluorescence

Human embryonic kidney cells (HEK293T) were cultured in DMEM (+10% FCS, +1% penicillin/streptomycin; PAA Laboratories, Pasching, Austria) at 37°C (5% CO_2). Cells were transfected using FuGene6 (Roche) or nucleofected (Lonza). For microscopic analysis, cells were fixed with 3.7% formaldehyde in PBS (15 minutes) and embedded in Mowiol supplemented with 25 mg/ml DABCO (Carl Roth, Karlsruhe, Germany). Prior to immunofluorescence staining, cells were fixed,

permeabilized (0.1% Triton X-100 in PBS, 10 minutes) and blocked (2% BSA in PBS, 30 minutes). Subsequently, cells were incubated with the primary antibody, e.g. rabbit-anti-*Hs*PEX14 (1:400) or sheep-anti-catalase (1:250) for 1 hour, washed three times with PBS (10 minutes) and incubated with the appropriate secondary antibody (1:200) for 30 minutes followed by three washing steps (10 minutes) and embedded. Hoechst 33342 (1 μg/ml) was used for counterstaining the nuclei. For knockdown of *h*Fis1, siRNAs were transfected as described previously (Koch et al., 2005). Control experiments were performed using endoribonuclease-prepared siRNAs directed to firefly luciferase (FLuc) mRNAs (Sigma).

Transient expression in *N. benthamiana* leaves

Six-week old *Nicotiana benthamiana* plants, grown in the greenhouse at 22 25°C and 16 hours light, were used for leaf infiltration experiments with agrobacterial solutions harboring the relevant binary plasmids prepared as described (Winter et al., 2007). In short, for single expression or double expression studies, agrobacterial suspensions with an OD_{600} of 0.15 or 0.3 were infiltrated into leaves, respectively. For estradiol-induced expression, a final concentration of 10 μM estradiol (Sigma) was applied by addition from a 50 mM stock solution to the infiltration solution.

Immunoprecipitation

Cells expressing the appropriate proteins were processed 48 hours after transfection. Cells were washed in PBS, incubated in 1 ml lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA) containing either 1% Triton X-100 or 0.2% digitonin. The lysates were transferred onto columns containing anti-FLAG M2 affinity gel (Sigma Aldrich), incubated (2 hours, 4°C) and washed extensively (0.5 M Tris-HCl pH 7.4, 1.5 M NaCl). Immune-precipitates were eluted using 3×FLAG peptides (150 ng/μl; Sigma Aldrich) for 30 minutes at 4°C. Western blot analyses were performed on aliquots with anti-FLAG, anti-GFP or anti-myc antibodies. Signals were visualized using HRP-conjugated secondary antibodies and Super Signal West Pico chemiluminescence kit (Thermo Scientific).

Antibodies

Rabbit-anti-*Hs*PEX14 antibodies were a kind gift from Ralf Erdmann (Ruhr-Universität, Bochum, Germany). Sheep-anti-human catalase antibodies were purchased from The Binding Site (Heidelberg, Germany). Alexa Fluor 594 donkey-anti-sheep and Alexa Fluor 594 donkey-anti-rabbit antibodies were purchased from Molecular Probes (Invitrogen). Rabbit-anti-GFP antibodies were a kind gift from Michael Rout (The Rockefeller University, New York, NY). Rabbit-anti-calnexin antibodies were kindly provided by Erwin Ivessa (MFPL, Vienna, Austria). Rabbit-anti-*h*Fis1 antibodies were a kind gift from Jean-Claude Martinou (University of Geneva, Geneva, Switzerland). Anti-FLAG M2 monoclonal antibodies (HRP-conjugated) and the HRP-conjugated donkey-anti-sheep antibodies were purchased from Sigma-Aldrich. Mouse-anti-α-tubulin and Texas-Red-conjugated goat-anti-mouse antibodies were a kind gift from Gerhard Wiche (MFPL). HRP-conjugated sheep-anti-mouse and donkey-anti-rabbit antibodies were purchased from GE Healthcare.

Microscopy and statistical analysis

For human cells, confocal images were acquired on a LSM510META, Zeiss (Neofluar 100×1.45, pixel size 45×45 nm, *z*-stacks 200 nm, 1.6 μs pixel dwell time, 12-bit) using a 405 nm laser (BP420-480) for Hoechst staining, 488 nm laser (BP500-550) for GFP, 561 nm laser (LP585) for mCherry, and 633 nm laser (Meta 585-625) for mitotracker-IR. Cells were randomly chosen, and detector gain and amplifier offset were adjusted to avoid clipping.

Live-cell imaging was performed with an Olympus CellR unit (widefield) using appropriate filter sets for GFP (BP457-487 excitation; BP503-538 emission) and mCherry (BP510-550 excitation; LP590 emission).

FRAP experiments were performed on an LSM5Live DuoScan (Zeiss; Plan-Apochromat 63×1.4, pixel size 120×120 nm, 12-bit) using 489 nm laser (BP500-525) for GFP. Two pre-bleach images were recorded to ensure stable imaging conditions. Bleaching was performed with a 488 nm point laser (100 mW, 30%, pixel dwell time 6.25 μs). Post-bleach-images were recorded until the mean fluorescent intensity of the bleached region of interest (ROI) reached saturation.

Images were processed using ImageJ software (NIH, Bethesda, MD). Usually, images were filtered using a 3×3 Median Filter, stacks were projected along the *z*-axis (maximum intensity), and brightness and contrast were adjusted for each channel. Deconvolution (QMLE algorithm) and surface rendering was performed with Huygens Professional using an experimentally derived PSF. Figures were finally composed in CorelDrawX4.

For statistical analysis, established counting techniques (Kim et al., 2006) were used and expanded. Briefly, for each PEX11 expression, images were collected of at least 50 cells randomly chosen at 24 hours post-transfection. All images were taken in the widest focal plane of a cell. Images were filtered, converted to 8-bit, and a threshold was applied to highlight the peroxisomal fluorescence. Peroxisomes were counted using the Particle Analysis package of ImageJ. Herein, peroxisomes were separated into four categories according to their diameter in μm, I 0 0.35, II 0.36 0.66, III 0.67 0.95, IV 0.96 10.

For plant cells, confocal images were acquired on a Leica TCS SP equipped with a KrAr laser using the following settings. For YFP-tagged (green channel) and for cherry SKL (red channel), the laser emission was 476/568 nm and detection bandwidth was 500 535/600 635 nm, respectively. Chloroplast fluorescence (blue channel) was detected at 665 795 nm. The detector gain and amplifier offset were adjusted to avoid clipping, and the sequential imaging mode was used to ensure separated excitation and detection of the fluorescent proteins. To allow high resolution imaging of peroxisomes, the tissues were incubated in 500 μl 100 μM F-actin depolymerizing cytochalasin D (Sigma) for 30 minutes. This treatment led to immobile but otherwise normal peroxisomes (Mathur et al., 2002).

Electron microscopy

For ultrastructural analysis, cells grown on 12 mm Aclar discs (EMS, Hatfield, PA) were fixed in 3% glutaraldehyde (electron microscopy grade; Serva) in PBS for 60 minutes, osmicated in 2% veronal-acetate-buffered OsO_4 for 60 minutes, dehydrated in a sequential series of ethanol, and embedded in Epon. Sections of 80 nm were stained with uranyl acetate and lead citrate and examined in a Tecnai-20 electron microscope (FEI, Eindhoven, The Netherlands) at 80 kV; images were acquired with a slow-scan CCD camera (Gatan, MSC 794).

Note added in proof

During the reviewing process of this manuscript, the Schrader group (University of Aveiro, Portugal) reported a study on ectopic expression of a PEX11β–YFP fusion protein illustrating the formation of tubular peroxisomal accumulations (TPAs), in which matrix proteins are sequestered at the end of the peroxisomal tubules (Delille et al., 2010). The authors suggest that PEX11β–YFP affects the assembly of a functional fission complex, thereby inhibiting peroxisomal division, which explains the rapid kinetics of TPA formation as opposed to the slow process of JEP formation described in our study.

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http://jcs.biologists.org/cgi/content/full/123/19/3389/DC1

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6.4 Membrane elongation factors in organelle maintenance: the case of peroxisome proliferation

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Author contributions:

I performed all experiments described in the review, analyzed and interpreted the data. Especially, I designed and established the rFDAP experiment. I contributed to the writing the manuscript, and arranged the figures.

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Review

Membrane elongation factors in organelle maintenance: the case of peroxisome proliferation

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Abstract

Separation of metabolic pathways in organelles is critical for eukaryotic life. Accordingly, the number, morphology and function of organelles have to be maintained through processes linked with membrane remodeling events. Despite their acknowledged significance and intense study many questions remain about the molecular mechanisms by which organellar membranes proliferate. Here, using the example of peroxisome proliferation, we give an overview of how proteins elongate membranes. Subsequent membrane fission is achieved by dynamin-related proteins shared with mitochondria. We discuss basic criteria that membranes have to fulfill for these fission factors to complete the scission. Because peroxisome elongation is always associated with unequal distribution of matrix and membrane proteins, we propose peroxisomal division to be non-stochastic and asymmetric. We further show that these organelles need not be functional to carry on membrane elongation and present the most recent findings concerning members of the Pex11 protein family as membrane elongation factors. These factors, beside known proteins such as BAR-domain proteins, represent another family of proteins containing an amphipathic α-helix with membrane bending activity.

Keywords: amphipathic α-helix; DRP1/DLP1; FIS1; membrane remodeling; peroxisome proliferation; Pex11.

Introduction

Eukaryotic life relies on the arrangement of specialized intracellular microenvironments, the organelles, with several advantages including an increase in efficiency of metabolic activities. To ensure such functionality, processes exist that control the number, size and shape of organelles as well as their positioning during cell cycle progression. The molecular mechanisms triggering these events depend on specialized proteins, such as anchoring factors for the cytoskeleton, motor proteins or membrane shaping factors.

The above-mentioned processes share a common aspect: they require membrane remodeling and thus proteins that have the ability to shape the organelle. Proteins exist that affect membrane curvature, their specialized domain bends the phospholipid bilayer, thereby stabilizing the charged concave surface of the membrane. In the absence of such morphogenic factors, the endoplasmic reticulum (ER) would be misshaped, mitochondria or peroxisomes would be unable to divide and vesicular trafficking, endocytosis or neuronal function would not be possible.

Evidently, the field of membrane remodeling is very broad and we are unable to cover it entirely in only few pages. Therefore, we point at excellent overviews on endocytosis and vesicular trafficking involving factors such as BAR proteins (1–7). Here, we focus on processes that ensure proper maintenance of peroxisomes for cellular homeostasis. We elaborate particularly on proteins involved in the elongation of the peroxisomal membrane.

The peroxisome, a dynamically shaped organelle

Peroxisomes integrate into the organellar system in all eukaryotic organisms to perform a variety of tasks mostly associated with lipid metabolism, e.g., β-oxidation in *S. cerevisiae*, methanol oxidation in *Y. lipolytica* as well as α- and β-oxidation of very long chain fatty acids or plasmalogen synthesis in mammals, and detoxification of reactive oxygen species (ROS) (8–11). A role for peroxisomes in ageing and inflammation response has also been suggested (12–14). Consequently, the absence of functional peroxisomes causes severe diseases eventually leading to early death, e.g., Zellweger spectrum diseases such as the Zellweger syndrome, neonatal adrenoleukodystrophy or infantile Refsum disease (15–17). Similarly, yeast mutant cells lacking peroxisomes are unable to grow on media containing fatty acids as the sole carbon source, but they can easily ferment if the culture medium is supplemented with sugars such as glucose (18, 19).

To perform their wide-ranging tasks, peroxisomes are adaptable organelles. Indeed, they exchange material with the endoplasmic reticulum (ER) and the mitochondria (20–24). They also adjust their size, shape, number and even their protein content according to the organism, the tissue or the environmental conditions (8, 25). To ensure such high versatility the maintenance of the peroxisomal compartment must be precisely regulated. Regulatory steps include the selective degradation of superfluous or elderly peroxisomes

via micro and macropexophagy, a mechanism conserved throughout kingdoms (26, 27). In addition, tight regulation of peroxisome inheritance during cell division was shown to occur in yeast through the function of specialized proteins controlling peroxisome positioning in the mother cell or in the bud (28). Furthermore, when their function is required peroxisomes can proliferate. Their propagation is either constitutive during cell cycle progression or inducible upon environmental pressure, e.g., growth of yeasts on fatty acids; fibrate supply for rodents or UV-light, high-levels of ROS, and xenobiotics in mammals (8, 11, 29, 30).

Biogenesis of peroxisomes, a need for membrane proliferation

What is the origin of the peroxisomal compartment? The peroxisome field has been highly studied and debated over the last decades but the mechanistics of peroxisome biogenesis and proliferation still requires investigation. However, owing to the characterization of mutant cells, the use of GFP- or photoactivatable GFP-fused proteins *in vivo*, it is now clear that two main routes lead to peroxisome formation: (i) *de novo* biogenesis from the ER and (ii) growth and division from existing peroxisomes (19, 31–39).

Studies either report on *de novo* biogenesis or on growth and division, yet focusing on only one side of peroxisome proliferation. However, the two pathways leading to formation of peroxisomes might not be controlled by completely independent mechanisms. How could the growth and division model possibly work without membrane recruitment? Although a role for the ER in the import of peroxisomal membrane proteins has been suggested (40, 41), little is known on how peroxisomes exchange material with the ER or acquire their membrane lipids.

Generally, most proteins involved in peroxisome biogenesis and proliferation belong to the group of *PEX* genes-encoded peroxins, most of which act as part of the peroxisomal matrix protein import machinery (42). Only a subset of peroxins, to which the Pex11-protein family belongs, controls the size, shape and number of peroxisomes. Conceptually, peroxisome proliferation can be divided into five steps: (i) organellar polarization, (ii) membrane protrusion, (iii) membrane elongation, (iv) protein import and (v) membrane scission (43). While the Pex11 proteins have been suggested to control the first steps (44), the actual peroxisomal membrane scission is performed by factors also known to operate in mitochondrial fission (45–47).

The Pex11 protein was first identified in the yeast *S. cerevisiae*. Deletion of the *PEX11* gene led to the occurrence of fewer and enlarged peroxisomes and upon overexpression of Pex11p, the cells contained more and smaller peroxisomes than wild type cells (48). Homologues of *Sc*Pex11p are known in most eukaryotic organisms and these usually contain more than one Pex11 protein (44, 49–60). Depending on the species, up to three proteins of the Pex11 family were identified in yeasts, e.g., Pex11p, Pex25p and Pex27p in *S. cerevisiae*; Pex11p, Pex11Cp and Pex25p in *H. polymorpha*; Pex11p and Pex11Cp in *Y. lipolytica*; and Pex11p in *P. pastoris* (61). Plants typically contain five Pex11 proteins, PEX11a to -e, whereas mammals harbor three, namely PEX11α, PEX11β and PEX11γ. Noteworthy, PEX11α, PEX11β and PEX11γ are related to *Sc*Pex11p only, and no homolog has been identified for *Sc*Pex25p or *Sc*Pex27p in mammals, so far.

Dynamin-related proteins are involved in mitochondrial and peroxisomal fission

The role of Pex11 proteins in peroxisome proliferation was strengthened by the results of several studies notably showing that human PEX11β was able to interact with hFis1, a component of the peroxisomal fission machinery (43, 62). In human, the peroxisomal fission apparatus consists of hFis1, a tail-anchored recruitment factor, and the dynamin-related protein DRP1/DLP1, the actual scission factor (62–67). Recently, a new protein, Mff (mitochondrial fission factor), has been identified that acts in both, mitochondrial and peroxisomal fission processes (68). Furthermore, in knockdown studies *Mff* RNAi seemed to have a stronger effect than *hFis1* RNAi. Similar to DRP1 knockdown, they induced tubulation of peroxisomes suggesting that Mff is an important player in the process of peroxisome proliferation (68, 69).

Similarly, in plant proteins of the dynamin family, DRP3A/B and DRP5, were identified as proliferation factors and shown to be accountable for peroxisome fission (70–73). Again, these are recruited to the peroxisomal membrane by FIS1 proteins, homologues of the mammalian hFis1 (70, 72, 74). In biomolecular fluorescence complementation assays with split YFP, FIS1B interacted with all five plant Pex11 proteins (70).

In yeasts, the dynamin-like protein Dnm1p was identified as the peroxisomal membrane scission factor. Dnm1p is recruited by Fis1p through adaptor proteins, either Mdv1p or its paralogue Caf4p (45, 75–79). In addition, a second and apparently independent pathway was identified relying on the function of another dynamin-related protein, Vps1p, as fission factor (80–82). In contrast to plant or human, no interaction between the scission factors and Pex11 proteins could be established in yeast, so far. Yet, a very recent study in *S. cerevisiae* reported the characterization of peroxin 34, a peroxisomal membrane protein (83). Its interaction with the Pex11 proteins as well as with Fis1p was illustrated in yeast-two-hybrid assays establishing the first link between Pex11 proteins and the fission machinery in yeast. Noteworthy, Pex34p seems to only exist in yeasts and no homolog could be identified in higher eukaryotes (83).

Together with the fission machinery, the cytoskeleton also plays a crucial role in organellar maintenance. Indeed, peroxisomes attach to the cytoskeleton and move along cytoskeletal tracks i.e., microtubules in human (84, 85) or actin in plant (86) and yeast (87). Additionally, it has been shown that organellar fission depends on the cytoskeleton as exemplified by Dnm1p-dependent scission of mitochondria in

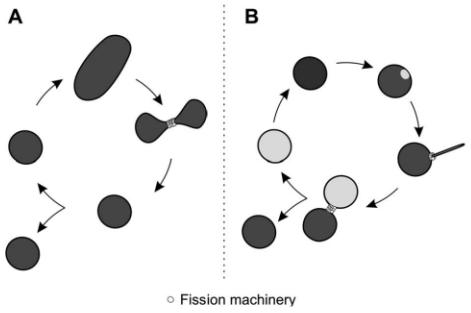

Figure 1 Stochastic versus asymmetric peroxisome proliferation. (A) In a simplified model for peroxisome proliferation, a peroxisome grows and elongates and, upon a critical size, the membrane is constricted and divides through fission. Herein, the inheritance of membranes and proteins is stochastic. (B) In asymmetric proliferation, the peroxisome becomes polarized and its membrane elongates at a specific site reorganizing membrane proteins. The fission machinery assembles at the site of membrane protrusion and import of new matrix proteins assembles a daughter peroxisome which separates from the mother organelle through membrane scission.

S. pombe (88). In human cells, functional microtubules and dynein motors were shown to be essential for peroxisome biogenesis (89).

Interactions of Pex11 proteins with fission factors give some insight into a molecular mechanism for peroxisomal proliferation; however, many questions remain unanswered. How does the peroxisomal membrane arrange for scission? What are the factors involved in the membrane remodeling process? Do proteins of the Pex11 family organize this whole process and why do most organisms contain more than one Pex11 protein? In the following sections we integrate the most recent findings that tackle these questions.

Mechanistic aspects of peroxisome division, Pex11 steering membrane elongation

Although several modes of proliferation are possible for organelles, the peroxisome relies on an apparently simple growth and division process. A simplified model depicts a single round-shaped peroxisome starting to elongate (Figure 1A). Once a critical size is reached, the membrane tightens and constricts until scission occurs through the action of the fission machinery. This leads to stochastic distribution of lipids and proteins between the two newly formed organelles. Evidently, this model is questionable: how does a typically round-shaped organelle start to elongate and what are the factors that squeeze the membrane and generate the constriction? In a more realistic model, extension of the peroxisome would be controlled in a concerted manner such that both, membrane elongation and assembly of the fission machinery take place at the site of membrane protrusion. Then, scission would occur across the axe of elongation generating a new daughter organelle (Figure 1B). Here, two alternatives can be foreseen, namely (i) non-polarized elongation equally dividing the peroxisomal matrix content or (ii) polarized elongation of the membrane followed by protein import at the site of membrane outgrowth. In such a model the peroxisome does not require a constriction factor *per se* since the thin membrane protrusion already fulfills the criteria for scission i.e., suitable membrane diameter to adapt the fission factors. Nevertheless, in both models proposed the membrane must elongate and factors are required to initiate its outgrowth. The findings that the Pex11 proteins interact with the fission machinery in plant and mammal suggest that they act as recruitment factor for the fission apparatus. But, this does not explain how the peroxisomal membrane arranges for fission.

Assessment of the information known about the fission machinery, especially proteins of the dynamin family, might allow for mechanistic assumptions. Dynamin proteins, including DRP1, are self-assembling and self-activating large GTPases. They typically carry three distinct domains, an N-terminal GTPase domain, a middle domain and a GTPase effector domain (GED) at their C-terminus (90). These three domains arrange into an evolutionary conserved structure: the middle domain and the GED region form a neck and a trunk, respectively, whereas the GTPase domain lies on the top. All dynamin-related proteins dimerize along their GTPase domain, further stabilized by their GED region (91, 92). This dimerization step seems to correlate with nucleotide binding and was proposed to arrange the catalytic machinery for GTP hydrolysis (93, 94). Recent structural data however, suggest that the dynamin dimers build spirals around the membrane in its GDP-bound form, which implies that GTP hydrolysis is not the trigger for membrane fission (95). The exact structure of the dynamin spiral is still a matter of discussion. Nonetheless, it creates such high curvature and instability in the membrane that the sudden breakdown of the spiral through GDP dissociation is ultimately resolved by membrane fission (96, 97). Electron microscopy analyses showed that Dnm1p-spirals are exactly fitting mitochondrial constriction sites exhibiting a diameter of about 110 nm. *In vitro*, high non-physiological levels of Dnm1p were able to elongate liposomes (1 μm in diameter) to 110 nm wide tubules (98). Elegant experiments making use of giant unilamellar vesicles (GUVs) demonstrated that dynamin polymerization requires high membrane curvature. The authors demonstrated that adsorption of dynamin monomers to the bare tubes did not significantly affect curvature of the membrane, however, clusters of dynamins occurred by pulling tubes from these GUVs thereby decreasing the tube radius (99). In agreement, at physiological concentrations, dynamin proteins were shown to only assemble and function on already curved membranes (100, 101). In fact, BAR-domain proteins were reported to prepare the membrane and target the function of dynamin such as amphiphysin in the scission of clathrin coated vesicles (101). No BAR-domain protein has been identified that acts on the peroxisomal membrane.

The conformation of dynamin proteins appears to be regulated through GTP hydrolysis performed by the intrinsic GED region thought to function as internal GTPase-activat-

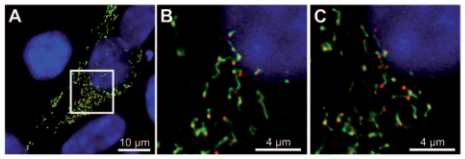

Figure 2 Pex11 induces peroxisome elongation.
(A) Maximum intensity projection of a confocal microscopic image showing the effect of ectopic expression of EGFP–HsPEX11β (green channel) on peroxisomes in human embryonic kidney cells (HEK293T). The elongated peroxisomal membrane shows segregation of the matrix marker, mCherry–Px (red channel). (B, C) Single z-layers from the insert region indicated in panel (A).

ing protein (GAP). However, Lee et al. reported a role for phospholipase D as external GAP for dynamin increasing its GTPase activity in a more effective manner than the inherent GED. The molecular mechanism appears similar to that of other GAPs based on the positioning of an arginine finger (102). Interestingly, Erdmann and colleagues showed in *S. cerevisiae* that Lpx1p, a phospholipase, is targeted to peroxisomes (103). Although this enzyme was suggested to have a metabolic function, the authors report drastic changes in peroxisome morphology including membrane invaginations and formation of intra-peroxisomal vesicles in mutant cells lacking *LPX1*. It is thus tempting to speculate that besides its metabolic activities Lpx1p influences the remodeling of the peroxisomal membrane during proliferation.

Several studies connected the function of Pex11 proteins not only to the recruitment of the fission machinery, but also to peroxisomal membrane remodeling, elongation, prior to fission (52, 104, 105). In previous studies we showed that overexpression of Pex11 proteins from yeast, plant and human resulted in elongation and thereafter clustering of peroxisomes in human cells (43). Peroxisome clustering had already been reported for *Hs*PEX11γ only (60). Close analysis of the peroxisomal clusters in 3D-reconstitutions and electron microscopy revealed that these are composed of individual, elongated peroxisomes that intertwined in a superstructure that we called juxtaposed elongated peroxisomes (JEP). Fluorescence recovery after photobleaching (FRAP) experiments demonstrated that the membranes of the individual peroxisomes in JEPs did not share components (43). Furthermore, we observed an evident separation of matrix and membrane proteins, with the matrix proteins accumulating at one or both extremities of the tubular peroxisomes (Figure 2). In parallel, Schrader and coworkers described the formation of tubular peroxisome accumulations after overexpression of PEX11β tagged with YFP at its extreme C-terminus (106). The authors also state the separation between matrix proteins in the tubular peroxisomes and report the differential localization of some peroxisomal membrane proteins. Interestingly, the early peroxisome biogenesis factors, PEX3, PEX16 and PEX19, were rather found on the stretched and elongated part of the peroxisome, whereas other membrane proteins, e.g., PMP70, PMP22 localized to the globular part. A very recent study in *H. polymorpha* on differential localization of various peroxisomal membrane proteins during membrane elongation showed that the spatiotemporal dynamic of membrane proteins ultimately depends on Pex11p function (107).

Asymmetric division of peroxisomes – segregation of the matrix protein content

The finding that upon Pex11 overexpression matrix proteins were unequally distributed alongside JEP cast some doubts about the current view that peroxisome division is stochastic. The observation could be merely due to a dilution effect with low amounts of matrix proteins in the elongated structures being below the detection limit in fluorescence microscopy. Alternatively, during the process of membrane protrusion matrix proteins could be sequestered leading to their exclusion from the thin tubular elongation. To differentiate between the two possibilities we measured repetitive fluorescence decay after photoactivation (rFDAP) of photoactivatable-GFP targeted either to mitochondria (paGFP-Mito) or to peroxisomes (paGFP-Px). A small region in mitochondria was photoactivated and the GFP signal was monitored in living mammalian cells. Mitochondria constantly fuse and divide giving them a network-like appearance. Hence, the paGFP-Mito signal could quickly diffuse through the mitochondrial network (Figure 3A, B). In contrast, in cells co-expressing mRFP-*Hs*PEX11β and paGFP-Px the activated GFP signal did not decline with time suggesting that paGFP-Px remained static and sequestered at one side of the elongated peroxisomal membrane (Figure 3C, D). In agreement with this observation, using the HALO-tag, Delille et al. demonstrated that the matrix content in the globular part of the elongated peroxisomes was present before membrane elongation occurred (106). In summary, under the effect of PEX11 peroxisomes elongate in a polarized fashion leaving their matrix content trapped at its original position although we cannot exclude that limited diffusion of small amounts of matrix content occurs during the elongation process. Hence, elongation of the peroxisomal membrane seems to create a matrix protein gradient, thereby segregating the 'old' matrix from the 'new' membrane. Segregation of matrix proteins during peroxisome elongation could ensure that old and possibly damaged proteins do not populate the new organelle. New matrix proteins would then target to the tip of the new membrane thereby inflating the new peroxisome and modeling the membrane constriction required for fission.

The observations by Delille et al. upon expression of a PEX11β-YFP suggest that the chimera inhibits peroxisomal fission while allowing their elongation. We showed that PEX11-driven peroxisomal elongation and even JEPs could be dissolved by providing high amounts of hFis1 to the cells (43). Interestingly, overexpression of the dynamin protein, DRP1, led to the appearance of elongated peroxisomes or to an increase in JEP size in cells expressing PEX11 proteins rather than to fission. These findings place hFis1 as limiting factor in the process of peroxisomal fission and highlight the importance of PEX11 as recruitment factor.

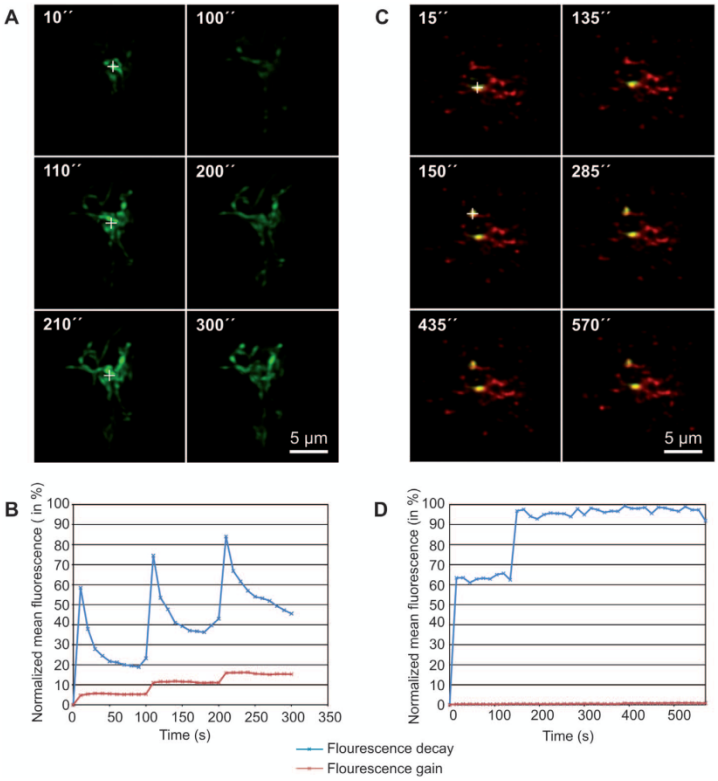

Figure 3 Peroxisomal matrix proteins are kept back during peroxisome elongation.
HEK293T cells expressing either the mitochondrial matrix marker, paGFP-mito (A, B), or the peroxisomal matrix marker, paGFP-Px and mRFP-*Hs*PEX11γ (C, D) were analyzed 48 h after transfection. Diffusion of matrix proteins was analyzed in repetitive fluorescence decay after photoactivation (rFDAP) experiments. paGFP was activated in a small area and fluorescence was monitored for decay along with measurement of fluorescence gain in the rest of the cell. (A) For mitochondria, repetitive activation of a single area (white crosses) led to rapid diffusion of the paGFP signal throughout the mitochondrial network. (B) Quantification of (A) showing fluorescence decay in the activated region (blue line) and gain of fluorescence in the non-activated region (red line). (C) paGFP was activated in JEPs caused by overexpression of mRFP-*Hs*PEX11γ. Since no decay was measured in the activated region (white cross 15″), a second area was activated (white cross 150″). (D) Quantification clearly shows that no signal was lost during acquisition and no diffusion took place. Image acquisition parameters: LSM DuoScan (ApoChromat 63x1.4; settings: paGFP (489 nm, MBS490, BP 500–525), mRFP (532 nm, MBS 535, BP 560–675); activation: 405 nm).

Recent experiments on mitochondrial fission described that Mff, another tail-anchored protein, is the ultimate recruiter of hFis1 for membrane fission (69). Although this latter study focused on mitochondrial fission, it had been shown earlier that Mff also played a role in peroxisome proliferation (68). Indeed, mammalian cells transfected with *Mff* RNAi presented peroxisomes that were more elongated than peroxisomes in cells depleted for hFis1. In consequence, assuming that the interplay between Mff, hFis1 and DRP1 is comparable in mitochondrial and peroxisomal fission, hFis1 might rather modulate DRP1 function than act as recruitment factor. In the light of these new observations it would be intriguing to test whether Pex11 proteins interact with Mff. Interaction with hFis1 only would suggest that Pex11 proteins act as membrane elongation factors, which stimulate the fission machinery. But, interaction with both, Mff and hFis1, would strengthen the role of Pex11 proteins in powering fission of the peroxisomal membrane.

Pex11 proteins elongate membranes *in vitro*

All these findings strongly point at the involvement of the Pex11 proteins in the membrane elongation event. A first

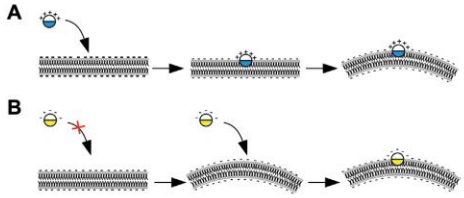

Figure 4 Amphipathic helices as membrane curvature sensors or inducers.
(A) A positively charged amphipathic helix leads to membrane bending upon insertion into one leaflet of the lipid bilayer. The energy cost for helix insertion can be compensated through electrostatic interactions. (B) If the amphipathic helix displays a negatively charged surface, it cannot deform the membrane, and acts as membrane curvature sensor.

hint about the molecular function of Pex11p was presented by Opalinski et al. (2010). The authors report the presence of an amphipathic α-helix at the N-terminus of several Pex11 proteins from yeast to mammal (108). Incubation of peptides containing the Pex11 amphipathic region with small unilamellar vesicles (SUVs) clearly showed an ability to restructure membranes. The initially round SUVs elongated and formed tubules in the presence of the Pex11 peptides. Similar results were obtained using the purified first 95 amino acids of *P. chrysogenum* Pex11p. The size and shape of the elongated SUVs could be altered by introducing bulky tryptophan residues in the amphipathic peptide. Changes in the peptide composition, such as introduction of negative charges or proline residues, annihilated the effect on membrane elongation. *In vivo*, expression of a mutated Pex11p protein lacking this alpha-helix was unable to protrude the peroxisomal membrane suggesting a mechanistic role for this helical structure in membrane elongation.

Amphipathic helices have been reported in a variety of proteins, well-known examples being the BAR proteins (1, 109–112). It has been suggested that two types of amphipathic helices exist namely, curvature sensors or inducers (113). Upon insertion of the helix into one leaflet of the lipid bilayer, the space requirement of this leaflet increases with respect to the other, which leads to membrane bending (Figure 4). This insertion requires the lipids to be pushed aside. If the energy cost is compensated by the presence of positively charged amino acids, it favors interaction between the charged head groups of the lipids and the polar face of the amphipathic helix, the helix can actively curve the membrane (Figure 4A). Alternatively, the helix contains mainly negatively charged residues, which hinder its insertion into a flat membrane. Hence, such helices are unable to induce membrane curvature and require a membrane already curved to insert. These amphipathic helices are membrane curvature sensors (Figure 4B). Evidently, this mechanism depends on the nature of the membrane including its lipid composition and local enrichment in specific lipids. Indeed, the often neglected physical properties of membrane lipids might determine the limits in which proteins can act (114). A well-studied example of curvature sensors is the ArfGAP1 lipid packing sensor (ALPS) motif, which contains numerous serine and threonine residues that favor its adsorption onto membranes with strong positive curvature (113). Curvature inducers are for instance the BAR domain proteins. The N-BAR domain e.g., in endophilin adopts a banana-wedge shape that bends the membrane to give it a curved form. Interestingly, mathematic modeling suggests that induction of membrane curvature relies on the sole property of the amphipathic helix and not on the entire N–BAR domain (115).

Consequently, amphipathic helices play pivotal roles in a plethora of intracellular processes and their presence in Pex11 proteins seems to be crucial for proliferation of the peroxisomal membrane. The generation of high curvature in the peroxisomal membrane could explain the redistribution of peroxisomal membrane proteins along the peroxisome tubules. Recent quantitative fluorescence microscopy analyses showed that membrane curvature as such can account for redistribution of integral or membrane anchored proteins (116). In the context of peroxisome proliferation such reorganization could lead to (i) attraction of the fission machinery and (ii) redistribution of membrane proteins including the import machinery to ensure efficient transport of matrix proteins into the newly formed peroxisome. Because the polar face of the Pex11 amphipathic helix contains lysine and arginine residues, it seems to rather induce membrane curvature. However, membrane curvature still needs to be tightly regulated. No polarized outgrowth would occur if all Pex11 amphipathic helices equally distributed and inserted into the peroxisomal membrane. Therefore, spatiotemporally confined protrusion has to be established to ensure elongation of the peroxisomal membrane. Thus, a strict control is required for Pex11 protein positioning on the membrane or for molecular interactions. This could arise through post-translational modifications. A study in the yeast *S. cerevisiae* showed that Pex11p is modified through phosphorylation. Cells expressing a phospho-mimicry mutant of Pex11p displayed more and smaller (S→D, 'phosphorylated') or less and bigger (S→A, 'non-phosphorylated') peroxisomes than wild type cells (117).

Several Pex11 proteins interact to orchestrate peroxisome proliferation

The interplay of the various Pex11 proteins in organisms that contain more than one Pex11 protein remains to be elucidated. Earlier studies showed the homodimerization properties of several Pex11 proteins including the human PEX11β and *Sc*Pex11p (62, 118). In addition to *Sc*Pex11p homodimerization, yeast-2-hybrid analyses showed homo-dimerization of *Sc*Pex25p and *Sc*Pex27p, respectively, but no hetero-oligomerization (56). In human cells, all three Pex11 proteins homo-oligomerized and both, PEX11α and PEX11β were shown to interact with PEX11γ. Co-immunoprecipitation experiments also revealed that the three proteins interacted with the fission machinery (43). *In vitro* binding

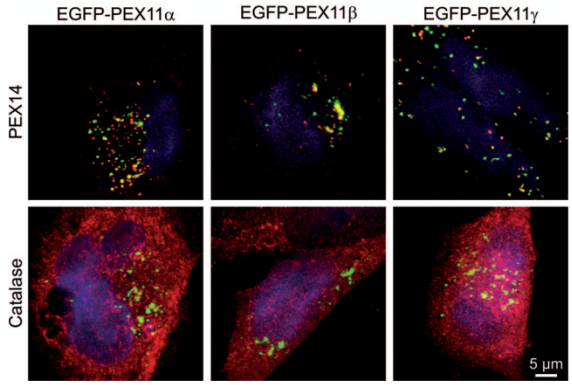

Figure 5 Pex11 membrane elongation factors do not require peroxisomal matrix content to function.
Analysis of mutant fibroblast cells with mutated PEX5 containing empty peroxisomal membranes for the effect of ectopic expression of GFP-tagged human Pex11 proteins. Pex11 proteins elongated the peroxisomal membrane in the absence of matrix content as demonstrated by immunofluorescent stainings for the peroxisomal membrane protein PEX14 (red channel, upper panel) and the matrix protein catalase (red channel, lower panel). Images represent maximum intensity projection of confocal images acquired on a LSM510META [objective 100×1.45; settings: EGFP (488 nm, MBS 488, BP 500–525), AlexaFluor594 (561 nm, MBS 561, LP 585)].

assays demonstrated a direct interaction between *Hs*PEX11β and hFis1 (62). Importantly, all these experiments were performed using digitonin, a mild detergent that preserves lipid environment, and the addition of Triton X-100 abolished interactions. This implies the requirement of membrane lipids for interactions. The orientation of several Pex11 proteins has been studied based on differential cell permeabilization with digitonin or protease accessibility of their extreme termini (50, 52, 60, 105, 119) however, their exact topology in the membrane remains to be elucidated. Hence, such information would be important to comprehend the mutual influence of Pex11 proteins and the fission machinery.

It is still unclear whether all Pex11 proteins are equally important for peroxisome proliferation. In yeast, the absence of Pex11p resulted in reduced growth of the cells on oleic acid (48) and in the abscence of Pex11p, Pex25p and Pex27p cells were unable to grow on oleate-containing medium. Interestingly, Pex25p alone was able to rescue the oleate non-utilizing phenotype of the *pex11Δpex25Δpex27Δ* mutant cells (56). In mammal, while PEX11α expression is inducible, PEX11β is constitutively present in the cell (49, 50, 120, 121). Knockout mouse models showed that in the absence of PEX11β mice developed pathologies similar to those of Zellweger patients and the number of peroxisomes per cell was significantly decreased (121, 122). Deletion of *PEX11α* did not have a phenotype neither did it worsen the condition in PEX11$\alpha^{-/-}$/$\beta^{-/-}$ mice (120). These data suggest that in mammal, two routes exist for peroxisome proliferation, one inducible and one constitutive, driven by either PEX11α or PEX11β, respectively. Both ways might require the function of PEX11γ. Homodimerization, interaction with PEX11γ or both could allow for recruitment of the fission machinery. Analysis of PEX11β suggested that its C-terminus was required to interact with hFis1 (62). Proteineaceous interactions were proposed to depend on one of the tetratricopeptide repeat (TPR) regions of hFis1 (123). Peptide-scan analyses demonstrated that proline-rich peptides efficiently bind hFis1, specifically in the TPR region (124). Interestingly, plant and human Pex11 proteins contain proline-rich regions, among which some resemble a Fis1 binding site. In contrast, none of the *S. cerevisiae* Pex11 family member contains such motif suggesting that in this species Pex11 proteins might not directly recruit Fis1 to peroxisomes.

Oligomerization of Pex11 proteins could regulate their activity. In *S. cerevisiae*, dimerization of Pex11p was suggested to act as molecular switch. Considering that *Sc*Pex11p was localized to the inner surface of the peroxisomal membrane it could easily be influenced by the peroxisomal redox state. Hence, redox-sensitive dimerization of Pex11p could represent a signal for proliferation (118). A redox-sensitive dimerization of Pex11 proteins has not been reported in mammalian cells. However, a recent study investigated the mammalian peroxisomes redox-balance using a redox-sensitive variant of EGFP and an artificial light-triggered ROS-induction protein. The authors demonstrate that although peroxisomes resist to an oxidative stress produced elsewhere in the cell, the intraperoxisomal redox status is strongly affected by the environmental growth conditions. Interestingly, the redox state of peroxisomes did not correlate with their age (125).

To address whether the peroxisomal matrix content exerts an influence on the function of Pex11 proteins we assessed whether PEX11 could act on empty peroxisomal membranes (remnants) in cells expressing a mutated PEX5, a receptor for peroxisomal matrix proteins (126). Most peroxisome remnants elongated and formed JEPs upon overexpression of either of the human Pex11 proteins (Figure 5). This observation points to an independent mode of regulation for per-

oxisome function and proliferation. Nevertheless, the expression of some Pex11 proteins is tightly regulated, which allows for coordination of the proliferation machinery and the metabolic state of peroxisomes. Alternatively, matrix proteins could affect the properties of the peroxisomal membrane thereby modulating proliferation of the organelle as already suggested for the peroxisomal enzyme acyl-CoA oxidase in *Yarrowia lipolytica* (127).

Perspectives

Recent reports placed the Pex11 proteins as key actors in the process of peroxisomal membrane remodeling. These proteins elongate the peroxisomal membrane. It will be important to test how their positioning selects the site of membrane protrusion, and how they interact with the fission machinery to coordinate membrane scission. Future experiments will be required to determine whether Pex11 proteins represent a new family of amphipathic alpha-helix-containing proteins with membrane bending activities.

Furthermore, evidence exists that peroxisome elongation is polarized. Asymmetric division of the matrix protein content during membrane elongation might allow for import of new material at the site of membrane growth. We propose this mechanism to ensure selective retention instead of dilution of old matrix content. Whether the selective degradation of peroxisomes via pexophagy is specifically targeted to old organelles is an attractive question.

Although the distribution of matrix proteins seems to be highly regulated, the action of the Pex11 proteins does not depend on the functionality or maturity of the peroxisomes. As shown in our experiments, overexpressed Pex11 acts on the membrane obviously without requiring feedback from the matrix. It remains to be elucidated whether the function of the Pex11 proteins is directly or indirectly influenced by the metabolic state of the cell.

The Pex11 interactome was shown to require the integrity of the peroxisomal membrane. Thus, understanding the membrane topology of Pex11 proteins is important in order to gain insight in its role as membrane elongation factor. Eventually, structural studies will deliver the missing elements to understand how these proteins act at the molecular level.

In conclusion, the two pathways leading to peroxisome formation, *de novo* biogenesis and growth and division, are presumably connected at the stage of membrane uptake. Consequently, with Pex11 proteins as membrane shaping factors, it would not be surprising that some of these proteins also contribute to *de novo* peroxisome biogenesis from the ER. Interestingly, a very recent study on the identification of peroxisome biogenesis factors in the yeast *H. polymorpha* revealed the importance of Pex25p for the reintroduction of peroxisomes in mutant cells lacking these organelles (128). Noteworthy, an interaction between the rat PEX11 and Arf1/coatomer has been reported and coatomer inhibition in temperature sensitive CHO-mutant cells correlated with the occurrence of tubular peroxisomes (119).

Alterations in peroxisomal metabolism and peroxisome proliferation cause neurodegenerative diseases and might also represent a trigger for cellular ageing. Understanding how peroxisomes proliferate and, more specifically, generate membrane protrusion to facilitate scission, will have a major impact on understanding the dynamics of biological membranes. The concept of organelle polarization and asymmetric membrane growth and division might engage the re-investigation of the proliferation of other organellar membranes.

Highlights

- Most organisms contain more than one Pex11 protein and all Pex11 proteins act on the peroxisomal membrane
- Pex11 proteins are regulated at transcriptional, and post-translational levels through modifications as well as homo- and heterodimerization
- Pex11 proteins influence the shape of the peroxisomal membrane
- Pex11 proteins coordinate the fission machinery shared between peroxisomes and mitochondria
- Pex11 proteins contain an amphipathic alpha-helix suggested to bend the peroxisomal membrane
- Pex11 proteins act as membrane elongation factors regardless of whether peroxisomes are functional
- Asymmetric inheritance of peroxisomal matrix proteins during peroxisome proliferation might lead to rejuvenation of the peroxisome pool in the cell

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6.5 PEX11 proteins attract Mff and hFis1 to coordinate peroxisomal fission

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I contributed to the experimental setup, and performed all experiments described in the study including the analysis and interpretation of the data. Moreover, I contributed to the writing the manuscript and arranged the figures.

PEX11 proteins attract Mff and hFis1 to coordinate peroxisomal fission

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SUMMARY

Fission of membrane-bound organelles requires membrane remodeling processes to enable and facilitate the assembly of the scission machinery. Proteins of the PEX11 family were shown to act as membrane elongation factors during peroxisome proliferation. Furthermore, through interaction with fission factors these proteins coordinate progression of membrane scission. Using a biochemical approach, we determined the membrane topology of PEX11γ, one of the three human PEX11 proteins. Analysis of mutated PEX11γ versions, which localize to peroxisomes revealed essential domains for membrane elongation including an amphipathic region and regulatory sequences thereof. Through pegylation assays and in vivo studies, we establish that the PEX11γ sequence encloses two membrane anchored domains, which dock an amphipathic region onto the peroxisomal membrane thereby regulating its elongation. The interaction profile of PEX11γ and mutated versions reveals a rearrangement between homo- and heterodimerization and association with fission factors. We also demonstrate the presence of the mitochondrial fission factor Mff on peroxisomes and its interaction with PEX11 proteins. Our data allow for assumptions on a molecular mechanism for the process of peroxisome proliferation in mammalian cells, that i) PEX11γ is required and acts in coordination with at least one of the other PEX11 proteins to protrude the peroxisomal membrane, ii) PEX11 proteins attract both, Mff and hFis1 to their site of action and, iii) the concerted interaction of PEX11 proteins provides spatiotemporal control for growth and division of peroxisomes.

INTRODUCTION

Most eukaryotic cells contain peroxisomes, small organelles essential for several cellular functions mainly associated with the metabolism of lipids. These round-shaped organelles harbor a crucial detoxifying function in response to stress assaults. Their function has also been associated with the process of ageing and in antiviral innate immunity (Angermuller et al., 2009; Dixit et al., 2010; Koepke et al., 2007; Wanders and Waterham, 2006). Accordingly, the peroxisomal compartment adapts to changes in cellular microenvironments through proliferation and specific degradation (Fagarasanu et al., 2007; Oku and Sakai, 2010). Studies on peroxisome turnover in mammalian cells revealed a half-life of about two days (de Duve et al., 1974; Huybrechts et al., 2009). To guarantee the maintenance of peroxisomes under varying conditions, mechanisms exist that insure their steady-state either by growth and division of pre-existing organelles or via de novo biogenesis from the endoplasmic reticulum (Geuze et al., 2003; Motley and Hettema, 2007; Toro et al., 2007; Toro et al., 2009). Both pathways seem to continually replenish the pool of peroxisomes in human cells. In vivo analyses using an engineered photoactivatable peroxisomal membrane protein suggested that peroxisomes prevalently arose de novo (Kim et al., 2006). However, the underlying mechanisms and regulations are only poorly understood. Proteins have been characterized that participate in the assembly and function of peroxisomes. These proteins, called peroxins, are encoded by *PEX* genes (Distel et al., 1996). Mutations in several *PEX* genes have been associated with the development of lethal genetic diseases characterized as peroxisomal biogenesis disorders or PBDs e.g., the Zellweger syndrome (Steinberg et al., 2006).

The peroxisome growth and division model can be divided into several steps including, i) peroxisome polarization, ii) membrane protrusion followed by iii) membrane elongation, iv) import of membrane and matrix proteins and a final v) membrane scission step. This process gives rise to the formation a new daughter organelle (Delille et al., 2010; Fagarasanu et al., 2007; Koch and Brocard, 2011; Koch et al., 2010). Within the factors involved in this process, proteins of the PEX11 family and fission factors shared with mitochondria play essential roles. The mammalian genome codes for three different PEX11 proteins, PEX11α, PEX11β and PEX11γ, which exhibit different expression patterns (Abe and Fujiki, 1998; Abe et al., 1998; Li et al., 2002a; Li and Gould, 2002; Schrader et al., 1998; Tanaka et al., 2003). Knock-out mice models have been studied that lack PEX11α and

PEX11β (Li et al., 2002a; Li et al., 2002b; Li and Gould, 2002). Taken together, the expression of PEX11α is inducible through variations of the environment and this protein is not essential for the formation of functional peroxisomes. In contrast, absence of the otherwise constitutively expressed PEX11β leads to the development of Zellweger-like symptoms in mice.

Interestingly, proteins of the mitochondrial fission machinery, the tail-anchored protein hFis1 as well as the dynamin-related protein DRP1, also execute their function at the peroxisomal membrane (Gandre-Babbe and van der Bliek, 2008; Koch et al., 2003; Koch et al., 2005). Protein interaction analyses revealed the association of PEX11 proteins and hFis1 suggesting that PEX11 participates in the recruitment of the membrane fission machinery onto peroxisomes (Kobayashi et al., 2007; Koch et al., 2010). This interpretation was challenged by the finding that the mitochondrial fission factor, Mff acted as efficient DRP1 recruitment factor, assigning hFis1 a rather regulatory function on DRP1 (Otera et al., 2010). Indeed, the fission machinery recruited to peroxisomes must act in tight coordination with membrane elongation factors to facilitate membrane constriction and division. We previously showed that proteins of the PEX11 family represent such a class of proteins (Koch et al., 2010). Their overexpression affected the morphology of peroxisomes in cultured human cells causing their elongation. Ultimately, excess of PEX11 proteins in the cells leads to the formation of structures composed of juxtaposed elongated peroxisomes (JEPs) and to the disappearance of distinct round-shaped peroxisomes. Thus, following the formation of JEPs in cells represents a useful tool to analyze the details of the molecular mechanism involved in peroxisome proliferation.

Mechanistic insights into peroxisomal membrane elongation were recently provided through the finding that yeast PEX11 proteins and mammalian PEX11α and PEX11β harbor an amphipathic alpha-helix. In vitro assays on liposomes with peptides from *Sc*Pex11p, *Hp*Pex11p and *Hs*PEX11α showed their ability to elongate membranes (Opalinski et al., 2010). In the absence of its amphipathic helix, *Hp*Pex11p was unable to protrude the peroxisomal membrane in the yeast Hansenula polymorpha.

Most organisms contain several PEX11 proteins. This implies that either each protein plays a different role in the proliferation process or that these represent redundant factors. Recent studies on PEX11 proteins from different yeast species demonstrated that each member of the PEX11 family is involved in a different pathway that leads to the formation of

peroxisomes. While Pex11p is involved in growth and division of pre-existing peroxisomes, Pex25p regulates de novo biogenesis from the ER, as *pex3Δpex25Δ* mutant yeast cells expressing plasmid-born Pex3p were unable to generate peroxisomes (Huber et al., 2011; Saraya et al., 2011). In contrast to the yeast proteins Pex11p and Pex25p, the sequences of the three mammalian PEX11 proteins are closely related. Indeed, they share high amino acid sequence homology (83%) and altogether they are more closely related to Pex11p than to Pex25p. This raises the question how three independent but similar proteins act in concert or influence each other during peroxisome proliferation in mammalian cells.

Using a biochemical approach, we determined the detailed topology of PEX11γ at the peroxisomal membrane and identified functional motifs in its sequence including an amphipathic domain. We demonstrate the requirement for this region for membrane elongation and show that modulating the activity of the amphipathic domain directly influences elongation of the peroxisomal membrane in human cells. Based on peroxisomal targeting of PEX11γ mutated versions, we further elucidate how this protein interacts with the other PEX11 proteins and with members of the fission machinery. Especially, we establish the interaction of Mff with the PEX11 proteins and its localization at the peroxisomal membrane. We propose a mechanistic model for peroxisome proliferation in mammalian cells.

RESULTS

PEX11γ inserts into the peroxisomal membrane

PEX11 proteins have been identified in most eukaryotic organisms and all proteins studied were shown to localize at the peroxisomal membrane (Abe and Fujiki, 1998; Abe et al., 1998; Schrader et al., 1998; Tanaka et al., 2003). However, depending on the organism various topologies were proposed for PEX11 proteins. While *Sc*Pex11p was suggested to localize in the inner side of the peroxisomal membrane (Marshall et al., 1996), differential permeabilization experiments showed that the mammalian PEX11 proteins exposed their both termini to the cytosol (Abe et al., 1998; Schrader et al., 1998; Tanaka et al., 2003). Their exact topology in the peroxisomal membrane, however, remains to be elucidated. To tackle this issue, we first performed in silico analysis of PEX11γ revealing two hydrophobic stretches (aa 133-155 and aa 215-233, Fig. 1A). Then, to determine whether it integrated into the peroxisomal membrane or only attached to it through protein-protein interactions, we carried out a carbonate-extraction on human cell lysates expressing PEX11γ-FLAG. Similar to the well-studied peroxisomal membrane protein PEX14 (Will et al., 1999), PEX11γ was not extractable with sodium carbonate and was exclusively detected in the membrane pellet, indicating that it is a true integral membrane protein (Fig. 1B). This raises the question, whether the two hydrophobic stretches identified represent domains that entirely cross the membrane or whether they stand as anchors for PEX11γ, which are only buried into the phospholipid bilayer.

To analyze the membrane topology of PEX11γ, we employed a biochemical approach based on the property of PEG-maleimide (mPEG) to selectively react with reduced cysteins, thereby leading to a mobility shift of proteins in SDS-PAGE. As hydrophilic substance, mPEG cannot cross intact lipid membranes. Notably, wild type PEX11γ contains six cysteins, all present in the N-terminal moiety (Fig. 2A), allowing the analysis of the membrane topology of this region and the contribution of these cysteins to the function of PEX11γ. To establish the method, an engineered peroxisomal matrix marker containing four accessible cysteins (EGFP-C4-Px) was expressed in human cells and tested for pegylation (Fig. 2). EGFP-C4-Px was entirely imported into the peroxisomal matrix, as confirmed by microscopic analysis and

colocalization with the peroxisomal protein PEX14 (Fig. S1). Hence, it was only pegylated when TritonX-100 was added to the protein extracts (Fig. 2B). The small portion of modified EGFP-C4-Px in the absence of detergent might indicate the minor peroxisomal leakage that occurs during the preparation of the cell lysates or small amounts of cytosolic proteins en route to peroxisomes. Further, to insure the reliability of our assay with regard to the study of peroxisomal membrane proteins, we tested the genuine integral membrane protein PEX14. Accessibility experiments showed that its C-terminus faces the cytosol (Oliveira et al., 2002; Will et al., 1999). Accordingly, in our assay its single cystein (C362) was almost fully pegylated without addition of detergent demonstrating the accessibility of this part of PEX14 from the cytosol (Fig. 2B). With PEX11γ, despite the presence of six cysteins along the N-terminal half, we observed only one clear band-shift after pegylation, indicating that when peroxisomes were kept intact a single cystein was substrate for mPEG (Fig. 2C). To identify the accessible cystein, we altered the sequence of the PEX11γ protein by individually replacing all cysteins to alanines and performed new pegylation assays. We reasoned that, if the modifiable cystein was mutated, no pegylation should be visualized. While mutations at positions 27/28, 59, 91 and 106 did not influence the pegylation state of PEX11γ, mutation C39A hardly showed pegylation (Fig. 2C). This could be due either to steric hindrance or to involvement of these cystein residues in reversible disulfide bridges. Note that a small fraction of the protein was modified, suggesting that although not fully accessible to mPEG all cysteins might face the cytosol. Although mutation C39A led to a significant increase in non-pegylated PEX11γ species, obviously another cystein was at least partially accessible for mPEG.

To rule out compensatory effects, we engineered a version of PEX11γ devoid of cystein residues, PEX11γ^{C0} as well as several PEX11γ forms each containing a single cystein. Expectedly, PEX11γ^{C0} was not pegylated, and each mutant with a single cystein was modified in the presence of detergent (Fig. 2D). Yet again, PEX11γ^{C39} was the only version that was fully pegylated in the absence of Triton X-100 as confirmed through the absence of the non-modified PEX11γ band in this lane. These observations validate that cystein 39 is freely accessible from the cytosol. Although these results demonstrate that the N-terminus of PEX11γ lies on the cytosolic face of the peroxisomal membrane all other cysteins seem to be only partially accessible for mPEG modification.

An explanation for the differential pegylation pattern in the N-terminal half of PEX11γ could be that the cysteins influence the stability or localization of PEX11γ. We previously showed that human PEX11 proteins act as membrane elongation factors and their overexpression were always associated with excessive elongation and clustering of peroxisomes eventually leading to the formation of juxtaposed elongated peroxisomes (JEPs; Koch et al., 2010). Consequently, we reasoned that functional PEX11γ should be able to localize to peroxisomes, induce strong elongation of the peroxisomal membrane and form JEPs. We assessed this property for EGFP-PEX11γ^{C0}, EGFP-PEX11γ^{C39A} and EGFP-PEX11γ^{C59A}, in comparison to wild type EGFP-PEX11γ using immunofluorescence staining for PEX14. All versions of PEX11γ clearly localized to peroxisomes, and influenced the shape of the peroxisomal membrane inducing elongation and JEP formation (Fig. 2E). Interestingly, in contrast to cells over-expressing wild type PEX11γ, cells expressing the C59A and C0 mutations also presented many small peroxisomes suggesting a role for PEX11γ N-terminal region in peroxisome proliferation. However, all tested mutations behaved like wild type with regard to the formation of JEP structures. We conclude that PEX11γ N-terminus resides in the cytosol and that none of the cysteins influence the membrane-elongation properties of PEX11γ. Consequently, membrane elongation and JEP formation must be allied to the function of PEX11γ C-terminal half. Alternatively, PEX11γ could trigger membrane elongation through activation of another protein. We therefore, sought to analyze the C-terminus of PEX11γ in more details.

Membrane-buried regions in PEX11γ dock its amphipathic domain onto peroxisomes

The C-terminal half of PEX11γ is highly hydrophobic and contains three predicted helical regions, two of which fulfill the requirements for membrane spanning domains (Fig. 3A). To determine the exact membrane topology of this region, we introduced cystein residues in or between the hydrophobic helices and performed pegylation assays (Fig. 3A,B).

As shown in Figure 3B, cysteins introduced at positions 134 (A134C) and 219 (T219C) were inaccessible for pegylation, which demonstrates that these parts of the protein were protected and inserted into the membrane as suggested from the in silico prediction (Fig. 1A). Because the C-terminus of PEX11γ presents highly ordered secondary structures two of which being hydrophobic, absence of pegylation due to steric hindrance can be ruled out (Fig. 1). Interestingly, for all other PEX11γ mutant versions, namely, A160C, A206C and A238C, an additional band of lowed electrophoretic mobility was observed as compared to wild type

showing that these were pegylated, and accessible, in the absence of detergent (Fig. 3B). These results imply that the two hydrophobic alpha-helices do indeed represent membrane-buried regions, suggesting anchoring of the protein in the cytosolic face of the peroxisomal membrane. Our in silico prediction different in silico approaches (see Material and Methods) identified another alpha-helical domain between these two hydrophobic regions (aa 176-192, Fig. 4A). Close inspection of this domain predicts the presence of a strong amphipathic α-helix. Such amphipathic regions have already been identified in some PEX11 proteins including, *Sc*Pex11p, *Hp*Pex11p and *Hs*PEX11α and *Hs*PEX11β (Opalinski et al., 2010). Those were shown to play a role in membrane elongation in vitro and deletion of this region in *Hp*Pex11p led to the absence of peroxisome elongation in the yeast H. polymorpha; however, such effect has never been shown for the mammalian PEX11 proteins. In vitro studies using PEX11α peptides only showed the ability of the peptides to elongate neutral small unilamellar vesicles (Opalinski et al., 2010). In contrast to the regions described for PEX11α, the predicted amphipathic α-helix of PEX11γ features a large distinct hydrophobic face (Fig. 4B), and its polar face consists of several charged residues. Visualization through 3D rendering clearly shows that the negatively charged residues are concentrated in the middle of the helix, whereas the positive amino acids arrange at the ends of the helix.

The predicted PEX11γ amphipathic alpha-helical region is required for elongation of the peroxisomal membrane

To study the potential function of this amphipathic domain on the regulation of PEX11γ, we introduced a proline at position 182, which breaks the helical structure (PEX11γ^{A182P}), and analyzed the effect of this mutation on peroxisome morphology (Fig. 4B). Upon ectopic expression in HEK293T cells, EGFP- PEX11γ^{A182P} localized to peroxisomes as shown using immunofluorescence staining for PEX14 (Fig. 5A). Remarkably, cells expressing this latter mutation did not display significant peroxisome elongation and did not form JEPs. Instead, peroxisomes presented only faint elongations. In contrast, as we reported previously (Koch et al., 2010), expression of EGFP-PEX11γ typically induced strong membrane elongation and formation of JEPs. These results outline the mechanistic importance of the amphipathic region for PEX11γ function and, for the first time, strongly point to the idea that PEX11γ is capable of protruding the peroxisomal membrane.

Interestingly, we identified a region containing four prolines out of 10 amino acid residues a few residues upstream of the alpha-helical domain (Fig. 4A). The existence of such proline-

rich motif might indicate a regulatory function. Indeed, prolines exist in two isoforms, cis or trans, the latter being the more prominent isoform in natural proteins. Proline isomerization has been suggested to play a role in protein folding and in determining the tertiary structure of proteins as for instance in caveolin-1 (Aoki et al., 2010). Especially, such isomerization can constitute a switch to change the overall protein structure to either activate/inactivate the protein itself or modulate its interactions with other factors (Feng et al., 2011; Sarkar et al., 2011). The isomerization is prevalently achieved through the action of peptidyl-prolyl-cis/trans-isomerases (PPIs), enzymes that catalyze this isomerization step (Lu et al., 2007). Overall, this proline-rich motif might play a pivotal role to regulate the amphipathic region and we sought to modify this motif by mutating the proline at position 158 to an alanine and test the resulting version, PEX11γ^{P158A}, for its ability to affect peroxisome morphology.

Similarly to EGFP-PEX11γ^{A182P}, EGFP-PEX11γ^{P158A} localized to peroxisomes (Fig. 5B). In this case, however, peroxisome elongation was not abolished but only postponed. Up to 72 hours after transfection individual round-shape peroxisomes were present in the cells, but the number of peroxisomes was highly increased as compared to overexpression of EGFP-PEX11γ. At later time points, the cells presented elongated peroxisomes and JEPs formed which correlated with a decrease in the number of individual peroxisomes. This confirms that PEX11γ^{P158A} can trigger elongation of the peroxisomal membrane. Yet, the kinetics seem to be off-balance suggesting that the proline-rich motif might indeed be involved in the regulation of the amphipathic region. Cells expressing EGFP-PEX11$\gamma^{P152A,A182P}$ neither showed strong peroxisome elongation nor elevated number of peroxisomes. This demonstrates the dominant effect of mutation A182P (helix break) and confirms the key function of the amphipathic region of PEX11γ in the molecular mechanism leading to elongation of the peroxisomal membrane (Fig. 5B).

Two prolines in the identified proline-rich motif, P165/167, resemble a motif, "PLP", recently identified as binding sequence for hFis1 (Serasinghe et al., 2010), a factor of the peroxisomal fission machinery already shown to interact with the PEX11 proteins (Kobayashi et al., 2007; Koch et al., 2010). To decipher whether this tripeptide is involved in the regulation of PEX11γ, we chose to study the effect of its deletion from the PEX11γ sequence in vivo (Fig. 4A).

EGFP-PEX11γ lacking aa 165-167, EGFP-PEX11^{noPLP}, localized to peroxisomes and led to effects on the peroxisomal membrane that were similar to those of EGFP-PEX11γ (Fig. 5C).

In fact, JEP formation seemed to be slightly enhanced and the structures usually appeared more interconnected and bigger. We previously reported that the dramatic elongation of peroxisomes was due to the out-titration of the fission machinery (Koch et al., 2010). Accordingly, JEPs could be dissolved by overexpression of hFis1 and compensated for the high amounts of PEX11 proteins in over-expressing cells. If the PLP-motif of PEX11γ is involved in hFis1 recruitment then, over-expression of PEX11γ^{noPLP} protein should immediately lead to extensive JEP formation. However, such observation is prone to subjective interpretation and thus we sought to perform a reverse experiment.

Rather than following the activity of the mutated PEX11γ version through analysis of peroxisome elongation, we focused on the dissolution of the JEP structures already present in the cell. We co-expressed myc-hFis1 and either wild type EGFP-PEX11γ or EGFP-PEX11γ^{noPLP} and evaluated the peroxisome morphology 24 and 48 h after transfection (Fig. 5D). While cells co-expressing EGFP-PEX11γ and myc-hFis1 presented many small, round-shaped peroxisomes as expected, the simultaneous expression of myc-hFis1 and EGFP-PEX11γ^{noPLP} led to the appearance of significantly elongated peroxisomes (Fig. 5D). Obviously, in this case the concomitant expression of hFis1 did not have high impact on peroxisome morphology, which could be due to weakened interaction between the two proteins. These results suggest that the PLP-motif in PEX11γ might be involved in hFis1 binding.

A complex interaction network around PEX11γ regulates peroxisome proliferation

The factors involved in peroxisome proliferation in human cells, PEX11 proteins and fission factors, act together to ensure that the number of peroxisomes adapts to the metabolic requirement of the cells. In vitro experiments showed that PEX11β directly interacts with hFis1 (Kobayashi et al., 2007). We reported previously that beside its homodimerization, PEX11γ interacts with both, PEX11α and PEX11β. However, the latter two proteins did not co-precipitate. Moreover, in affinity purification all three PEX11 proteins co-purified with hFis1 (Koch et al 2010). This strongly favors the idea that PEX11 proteins act in concert to execute their functions.

To analyze whether the introduced mutations in PEX11γ, namely A182P, P158A and C0 affected the ability of the protein to interact with hFis1, PEX11β or wild type PEX11γ, we

performed affinity purifications and tested for co-precipitating proteins (Fig. 6B,C). HEK293T cells expressing the appropriate plasmid pairs were lyzed in buffer containing 0.2% digitonin. The protein hFis1 co-precipitated with all tested PEX11γ mutations. Yet, the amounts of EGFP-myc-hFis1 proteins obtained using PEX11γ^{A182P}-FLAG as bait, were significantly higher than those yielded with wild type PEX11γ (Fig. 6B). Interestingly, the amounts of hFis1 obtained with PEX11γ^{P158A} were undistinguishable from those gained with non-mutated PEX11γ indicating that this proline is not required for hFis1 binding per se. Alternatively, another PEX11 protein could influence hFis1 binding. To differentiate between these possibilities, we analyzed the binding of PEX11γ mutants with wild type versions of either PEX11β or PEX11γ. We identified EGFP-PEX11β and EGFP-PEX11γ in affinity purifications with all mutated versions of PEX11γ (Fig. 6C). Although EGFP-PEX11γ co-precipitated with PEX11γ^{A182P}-FLAG to a degree similar to the wild type PEX11γ-FLAG, the amounts of EGFP-PEX11β identified were much lower as visualized by western blotting. This finding suggests that co-precipitation of hFis1 and PEX11γ is not due to association via PEX11β but rather that PEX11γ directly interacts with hFis1.

We re-evaluated this finding through immunoprecipitations from lysates originating from cells co-expressing three proteins, namely, a mutated or non-mutated version of PEX11γ-FLAG, EGFP-myc-hFis1 and either EGFP-PEX11β or EGFP-PEX11γ (Fig. S2). Because they have a different molecular weight, the EGFP-tagged proteins can be visualized as two distinct bands through western blot analysis with anti-GFP antibodies. Our results show that upon over-expression of EGFP-PEX11β, little amounts of hFis1 co-precipitated with wild type PEX11γ but these were increased upon expression of PEX11γ^{A182P}. In contrast, when EGFP-PEX11γ was expressed more hFis1 was visualized in the affinity purified fractions. However, in the presence of EGFP-PEX11γ, PEX11γ^{A182P}-FLAG co-precipitated hFis1 in amounts similar to the non-mutated PEX11γ-FLAG (Fig. S2). These data demonstrate that hFis1 indeed directly interacts with PEX11γ and that this interaction strongly depends on the interaction of PEX11γ with other PEX11 proteins.

Previous studies on yeast Pex11p have suggested that its homodimerization depends on the presence of a disulfide bridge through cystein at position 3 (Marshall et al., 1996). In contrast, a version of human PEX11γ lacking cystein residues (C0) co-precipitated hFis1, PEX11β and wild type PEX11γ showing that these interactions did not require the formation of disulfide

bridges (Fig. 6B,C). Hence, a mechanism similar to that proposed for *Sc*Pex11p is unlikely for the human PEX11γ.

In vivo as well as in vitro analyses established that the mitochondrial fission factor, Mff, recruits DRP1 to the mitochondrial outer membrane (Otera et al., 2010). Knock-down experiments also showed that peroxisomes elongated in the absence of Mff (Gandre-Babbe and van der Bliek, 2008). However, whether Mff localizes to peroxisomes and cooperates with PEX11 proteins has not been studied. Hence, we tested whether the PEX11 proteins also interacted with Mff to coordinate peroxisomal fission. Our pull-down experiments show that Mff co-precipitated with both, PEX11β and PEX11γ, however, the co-purified amounts were higher with PEX11β (Fig. 6D). When PEX11γ^{A182P} was used as bait, equal amounts of Mff as compared to PEX11γ could be visualized in the affinity purifications (Fig. 6D). This is in opposite to the results obtained with hFis1 (Fig. 6B). To unambiguously determine whether a portion of Mff resides at the peroxisomal membrane as expected from the co-immunoprecipitation experiments, we co-expressed EGFP-Mff and a peroxisomal matrix marker, mCherry-Px, and stained the mitochondria with Mitotracker (Fig. 6E). Most EGFP signal localized to mitochondria that appeared fragmented as previously reported for over-expression of Mff (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010). In addition, a significant portion was present exclusively on peroxisomes. This finding was confirmed through 3D object analyses. To avoid unclear co-localization due to an overlap between the peroxisomal and mitochondrial signals, we first removed all Mff signals that also contained mitochondrial staining. Then, intersections between Mff and peroxisomes were made visible (Fig. 6E). Although we cannot exclude that the peroxisomes present near mitochondria also contain Mff, for our co-localization study we only considered isolated peroxisomes. Strikingly, peroxisomes were slightly elongated in cells expressing Mff. Interestingly, similar to hFis1, Mff was able to dissolve PEX11γ-induced JEPs (Fig. 6F). Moreover, in contrast to hFis1, Mff completely dissolved elongated peroxisomes invoked by the expression of PEX11γ^{noPLP} (Fig. 5D and 6G). This suggests that upon over-expression of a single PEX11 protein, the effect observed on peroxisomal morphology does not only lie in inefficient recruitment of hFis1 but of the whole fission machinery. Overall, these findings confirm the involvement of Mff in PEX11-driven peroxisomal fission and suggest a slightly different role for PEX11β and PEX11γ.

Because concomitant expression of PEX11β and PEX11γ seemed to differentially influence the binding capabilities of PEX11γ to the fission machinery, we analyzed the effects of their

co-expression on peroxisomes. It has been shown that overproduction of PEX11β alone first leads to an increased number of small and round-shaped peroxisomes and, at a later time point, to the formation of peroxisome clusters similar to PEX11γ (Delille et al., 2010; Koch et al., 2010). Here, upon over-expression of mRFP-PEX11β and EGFP-PEX11γ, elongated peroxisomes and JEPs were observed even 24 h after transfection (Fig. 7A). Remarkably, in addition to JEPs the cells contained many small peroxisomes, indicating that the activities of PEX11β and PEX11γ on peroxisome proliferations are different and that these both proteins play an important role in this process. In contrast, concomitant expression of mRFP-PEX11β and EGFP-PEX11γ^{A182P} did neither lead to strong peroxisome elongation nor to the formation of JEPs (Fig. 7A). Rather, we observed a dramatic increase in peroxisome number, which was not the case when PEX11γ^{A182P} was expressed alone (Fig. 5A). The manifestation of a high number of peroxisomes was also observed 72 h after transfection a time point at which overexpression of PEX11β alone led to the formation of JEPs. Although unable to protrude the peroxisomal membrane, PEX11γ lacking its proline at position 182 (PEX11γ^{A182P}) interacted with hFis1 suggesting the requirement for a subtle interplay between members of the peroxisome proliferation machinery for proper maintenance of the organelle. A regulated cascade of molecular interactions between members of the PEX11 protein family and the fission machinery seems to coordinate the sequence of events leading to peroxisome proliferation.

DISCUSSION

The number of peroxisomes per cell is rigorously maintained through the coordination of proliferation via de novo biogenesis from the ER (Kim et al., 2006; Toro et al., 2009), growth and division from pre-existing peroxisomes (Purdue and Lazarow, 2001) and degradation of the organelles via pexophagy (Oku and Sakai, 2010). It has been shown that the various members of the PEX11 protein family are involved in pathways that lead to the formation of peroxisomes in yeast, plant and human cells (Koch et al., 2010; Lingard and Trelease, 2006; Rottensteiner et al., 2003). Besides their interaction with the fission machinery, in some PEX11 proteins, an amphipathic α-helix was suggested to provide a mechanistic for their mode of action on the peroxisomal membrane (Opalinski et al., 2010).

Here, we present a detailed analysis of PEX11γ topology at the peroxisomal membrane, confirming that both, N- and C-termini face the cytosol. Using mPEG, a cystein-selective reagent, we mapped the accessibility of all six cysteins of PEX11γ along its N-terminal half (Fig. 2). Interestingly, only cystein at position 39 seemed to be freely accessible for pegylation, the others might be involved in a tertiary structure protecting them from pegylation. As control, we engineered PEX11γ^{C0} devoid of cysteins. This mutated version still localized to peroxisomes and acted on the peroxisomal membranes similar to PEX11γ (Fig. 2E). Thus, our finding implies that the region required for membrane elongation resides in the C-terminal half of the protein. Nevertheless, cysteins in the N-terminal moiety could be involved in maintaining the structure of the protein similar to *Sc*Pex11p which was proposed to homodimerize via a disulfide bridge at cystein 3 (Marshall et al., 1996). However, our immunoprecipitation experiments confirmed that PEX11β, wild type PEX11γ and hFis1 co-purified with PEX11γ^{C0} (Fig. 6B). Ultimately, this shows that no covalent cystein bond is required for protein-protein interactions which had already been implied by the necessity to use the mild, membrane-preserving detergent digitonin during immunoprecipitations. Indeed, in previous experiments we showed that the hydrophobic regions of PEX11β and also PEX11α were required for interaction with PEX11γ and hFis1 (Koch et al., 2010). If at all, the cysteins of PEX11γ might rather stabilize the overall structure of the cytosolic N-terminal part of the protein or strengthen protein interactions through transient disulfide bridges.

Close inspection of the C-terminal half of PEX11γ revealed two membrane-inserted helices that anchor and span an amphipathic helical region in the peroxisomal membrane (Figs. 3, 4, 5A, 6A). Herein, the amphipathic helix is inserted from the cytosol to generate positive membrane curvature necessary for protrusion and subsequent fission of the peroxisomal membrane. Noteworthy, in contrast to other PEX11 proteins the amphipathic region of PEX11γ is located at its C-terminus. Besides, the membrane-anchored PEX11γ (Fig. 1) stands out within members of the PEX11 protein family because it features two membrane-buried segments, whereas in PEX11α and PEX11β a single hydrophobic region was predicted, and shown to be required for their proper localization (Koch et al., 2010). The two membrane-bound regions of PEX11γ might enable the protein to properly position its amphipathic region and maintain protein-protein interaction spatially controlled in the membrane. Here we show that the amphipathic domain of PEX11γ is indeed necessary for both, i) membrane elongation and ii) interaction with PEX11β and hFis1 (Figs. 5A,B, 6B,C). It might be regulated by a proline-rich motif, where cis/trans isomerization could represent a plausible regulatory mechanism. In fact, mutation of the proline at position 158 of PEX11γ led to delayed peroxisome elongation and correlated with an increase in peroxisome number at early time points (Fig. 5B). Moreover, deletion of the PLP-motif in PEX11γ strongly affected the properties of JEPs with regard to their susceptibility to fission factors (Fig. 5C,D). Our findings that co-production of PEX11γ and PEX11β led to a dual phenotype suggest that these two proteins act at different levels in the process of peroxisome proliferation (Fig. 7A). While PEX11β increases the number of peroxisomes PEX11γ promotes elongation of the peroxisomal membrane. However, these two mechanisms are not mutually exclusive and most likely influence each other to ensure adequate regulation.

Obviously, two cases can be distinguished: i) PEX11γ homodimerizes, and cannot interact with hFis1, or ii) PEX11γ heterodimerizes, and efficiently binds hFis1. In the first case, PEX11γ would be involved in membrane elongation, in the second case PEX11γ would rather act on the fission machinery. Such explanation is plausible since the events of membrane elongation and fission, must be coordinated and are unlikely to occur simultaneously. Differential di- or even oligomerization and interaction with hFis1 synchronized with steric adjustment of membrane-bending motifs might represent a mechanism to control the progress of peroxisomal proliferation.

Additionally, a third player of the fission machinery, Mff, should now be included in the scheme of PEX11-controlled peroxisomal fission. Obviously, PEX11 proteins do not only interact with hFis1, but also with Mff which we clearly found to be present on peroxisomes (Fig. 6D,E). Furthermore, Mff dissolved JEPs induced by wild type PEX11γ and mutated PEX11γ^{noPLP} (Fig. 6F,G), suggesting a role in peroxisome proliferation similar to that in mitochondrial proliferation as recruitment factor for DRP1 (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010). In contrast to hFis1, Mff is the first protein of the fission machinery that showed different interaction properties for the various PEX11 proteins suggesting a stronger interaction with PEX11β. Thus, it is conceivable that PEX11β recruits or positions the fission machinery whose action would then be triggered by PEX11γ once the peroxisomal membrane is properly remodeled and protruded for fission (Fig. 7B).

In this work, we have established new tools, namely PEX11 proteins that target to peroxisomes but lose their ability to perform their task, that help decipher the cascade of molecular interactions leading to i) polarization and elongation of the peroxisomal membrane and ii) fission of the organelle. Our studies on mutated versions of PEX11γ show that this protein has an uncharacteristic topology at the peroxisomal membrane (Fig. 6A).

The exact mechanism by which DRP1 severs organellar membranes in vivo and the function of hFis1 and Mff in this process are poorly understood. Resolving these issues will be crucial for a deeper understanding of both, peroxisomal and mitochondrial proliferation. With regard to the proliferation of peroxisomes several questions remain especially on the mode of lipid recruitment during proliferation of the organelles. Presumably, growth/division and de novo biogenesis of peroxisomes integrate at the stage of lipid uptake. Whether PEX11 proteins are involved in this process remains to be elucidated.

Interestingly, a correlation has been noted in some mammalian systems between the high number of peroxisomes in hippocampal neurons and protection against neurodegeneration (Santos et al., 2005). Peroxisomes may indeed play a primordial role in the protection against accumulation of ß-amyloid peptides and their function might reduce the pathological development of Alzheimer´s disease (Kou et al., 2011). For instance, similar to increased mitochondrial fission during apoptosis, peroxisome elongation might represent a good assessment for the pathogenesis of neurological disorders. Knowing the factors involved in this process and the effects caused by their malfunction could lead to the development of new diagnostic targets to differentiate between peroxisomal and mitochondrial disorders.

MATERIALS and METHODS

Plasmids

Plasmids coding for EGFP-*Hs*PEX11γ, *Hs*PEX11γ-FLAG, EGFP-myc-hFis1, myc-hFis1 and mCherry-Px were described before (Koch et al., 2010). EGFP-Mff was purchased from GeneCopoeia. EGPF-C4-SKL was engineered by insertion of a linker (annealed oligonucleotides CB396/ 397) in the EGFP-C1 (Clontech, BglII/ EcoRI). EGFP-Scp2 was described before (Stanley et al., 2006). For mRFP-*Hs*PEX11β and -PEX11γ, the coding sequence of PEX11β and PEX11γ were amplified and inserted into pcDNA3.1-mRFP (EcoRI/ XhoI) obtained from Jeffrey Gerst (Weizmann Institute of Science, Israel). Mutations were introduced in the original EGFP-*Hs*PEX11γ encoding plasmid through site-directed mutagenesis via PCR with oligonucleotide pairs harboring the respective mutation (see Table S1), followed by DpnI digestion. All mutations were controlled via sequencing of the obtained plasmids.

In silico analysis

TMPred was used for predicting the hydrophobic segments (http://www.ch.embnet.org/software/TMPRED_form.html). The α-helical content was predicted using different algorithms, JPred (Cole et al., 2008), NetSurfP (Petersen et al., 2009), JUFO (Meiler and Baker, 2003) and PSIPred (Jones, 1999).

Cell culture, transfection, and immunofluorescence and -precipitation

Human embryonic kidney cells (HEK293T) were cultured in DMEM (+10% FCS, +1% penicillin/streptomycin; PAA Laboratories, Pasching, Austria) at 37°C (5% CO_2). Cells were transfected using FuGene6 (Roche) or nucleofected (Amaxa). For microscopic analysis, cells were fixed with 3.7% formaldehyde in PBS (15 min) and embedded in Mowiol supplemented with 25 mg/ml DABCO (Carl Roth, Karlsruhe, Germany). Immunofluorescence and immunoprecipitation were carried out as described previously (Koch et al., 2010). Mitochondria were stained using Mitotracker Deep Red FM (Molecular Probes). Nuclei were counterstained with Hoechst 33342 (Molecular Probes).

Pegylation assay

HEK293T cells expressing the appropriate proteins were harvested 48 h after transfection, washed in PBS, and resuspended in pegylation buffer (Antonenkov et al., 2004 without DTT). Cells were lyzed with a Potter-Elvehjem (1500 rpm, 10 strokes) and cell debris were pelleted via centrifugation (800 g, 10 min). Equal fractions of the supernatant were incubated with either 4 mM mPEG (O-2(-maleimidoethyl)-O'-methylpolyethylene glycol 5.000, Sigma-Aldrich), 0.1% v/v Triton X-100 or both for one hour at 4°C. In control experiments, DTT (1 mg/ml) was added (not shown). Pegylation was stopped by addition of 1 µl β-mercaptoethanol to the samples. Equal fractions were loaded onto an SDS-gel and analyzed by western-blotting.

Carbonate extraction

HEK293T cells expressing the appropriate protein were harvested 48 h after transfection, washed in PBS and resuspended in lysis buffer (10 mM Tris HCl pH 7.4, 1 mM EDTA) with complete protease inhibitors (Roche). An aliquot of these crude extracts (IP) was removed and stored. Cells were homogenized using a Potter-Elvehjem (1500 rpm, 10 strokes) and centrifuged (100.000 g, 60 min). The supernatant (S1) was stored and the pellet was resuspended in high salt buffer (10 mM Tris pH 7.4, 0.5 M KCl) and mixed for 30 min. After centrifugation (100.000 g, 60 min), the supernatant (S2) was stored and the pellet was resuspended in carbonate buffer (100 mM Na_2CO_3). After mixing on a rotating wheel for 30 min and centrifugation (100.000 g, 60 min), the supernatant (S3) and final membrane pellet (MP) were stored. Equal fraction of IP, S1, S2, S3 and MP were loaded onto an SDS-gel for western-blot analysis.

Antibodies

Rabbit-anti-*Hs*PEX14 antibodies were a kind gift from Ralf Erdmann (Ruhr University, Bochum, Germany). Alexa Fluor 594 donkey-anti-rabbit antibodies were purchased from Molecular Probes (Invitrogen). Rabbit-anti-GFP antibodies were a kind gift from Michael Rout (The Rockefeller University, New York, USA). Deep Red donkey-anti-mouse antibodies were purchased from Jackson Laboratories. Anti-FLAG M2 monoclonal antibodies (HRP-conjugated) was purchased from Sigma-Aldrich. HRP-conjugated sheep-anti-mouse and donkey-anti-rabbit antibodies were purchased from GE Healthcare.

Microscopy and Image Analysis

Confocal images were acquired on a LSM510META, Zeiss (Neofluar 100x1.45, pixel size 45x45 nm, z-stacks 200 nm, 1.6 µs pixel dwell time, 12-bit) using a 405 nm laser (BP420-480) for Hoechst staining, 488 nm laser (BP500-550) for GFP, 561 nm laser (LP585 or BP 575-615) for mCherry/ mRFP and 633 nm laser (LP650) for deep red dyes. Cells were randomly chosen, and detector gain and amplifier offset were adjusted to avoid clipping. All images were deconvolved using the QMLE algorithm of Huygens Professional (SVI, The Netherlands), projected (maximum intensity) and adjusted in ImageJ. Object colocalization analysis was performed in Huygens Professional. Briefly, objects were created for peroxisomes (mCherry-Px), mitochondria (Mitotracker Deep Red FM) and Mff (EGFP-Mff). Then each Mff-object that also contained mitochondrial staining was removed and excluded from further analysis. Intersections of the residual Mff objects with peroxisomal objects were calculated.

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FIGURE LEGENDS

Figure 1: PEX11γ is an integral membrane protein harboring two hydrophobic segments.

(A) In silico analysis of PEX11γ using the TMpred algorithm reveals two hydrophobic segments ranging from aa 133-155 and aa 215-233, respectively. The rather low score suggests that these regions represent membrane-buried domains because true transmembrane domains would yield a higher score.

(B) Carbonate extraction was performed on HEK293T cells expressing PEX11γ-FLAG and EGFP-Px, a peroxisomal matrix marker. Equal fractions of input (I), low-salt supernatant (S1), high-salt supernatant (S2), carbonate supernatant (S3) and membrane pellet (MP) were loaded. PEX11γ showed the same distribution as PEX14, an integral membrane protein of peroxisomes. In contrast, EGFP-Px was already present in high-salt buffer indicating the rupture of peroxisomes.

Figure 2: The cystein-rich N-terminus of PEX11γ is exposed to the cytosol.

(A) PEX11γ N-terminus contains six cysteins as indicated. The C-terminal half carries three predicted helices two of which represent the hydrophobic regions and a third helical domain in between.

(B) Pegylation assays were established to study peroxisomal proteins with EGFP-C4-Px, an artificial peroxisome matrix marker, and the genuine PEX14 in HEK293T cells. For addition of each mPEG moiety the modified proteins showed a mobility-shift on SDS-PAGE (lane 2/4) as compared to the non-modified protein (arrowhead). Addition of Triton X-100 allowed the hydrophilic mPEG to access the cystein residues in the peroxisomal matrix or membrane (lane 4). The modified EGFP-C4-Px without detergent (lane 2) indicates minor peroxisome leakage or proteins on their way to peroxisomes. PEX14 exposes its single cystein to the cytosol.

(C) PEX11γ-FLAG expressed in HEK293T cells was analyzed using pegylation. Wild-type PEX11γ contains six cysteins, one of which was accessible for pegylation. PEX11γ-FLAG^{C39A} showed reduced pegylation as compared to the other mutations (red asterisk).

(D) To confirm accessibility of the cysteins in PEX11γ, all six cysteins were mutated to alanines (PEX11γ-FLAG^{C0}). Upon individual reintroduction of each cystein, only PEX11γ-FLAG^{C39} showed mPEG-derived modification in the absence of detergent (red asterisk).

(E) Selected PEX11γ variants (green channel) co-localized with the genuine peroxisomal protein PEX14 (red channel). Cells expressing EGFP-tagged versions of PEX11γ as indicated were subjected to immunofluorescence 1, 2, 3 or 9 days after transfection. All PEX11γ mutations affected peroxisome morphology similar to non-mutated PEX11γ. Nuclei (blue channel). Bar: 5 μm

Figure 3: Two membrane-buried domains anchor PEX11γ in the peroxisome membrane.

(A) Schematic representation of the PEX11γ sequence as outlined in Figure 2A, showing the two helical hydrophobic regions (red) separated by a predicted amphipathic helix (blue).

(B) To determine the topology of the C-terminal region of PEX11γ-FLAG via pegylation, mutations were introduced as indicated. Cysteins introduced at positions 160, 206 and 238 were accessible for pegylation as additional bands with an electrophoretic mobility shift were observed without addition of Triton X-100 indicating that these residues face the cytosol. The amino acids at position 134 and 219 were only modified upon addition of Triton X-100 suggesting that these residues are buried in the membrane.

Figure 4: PEX11γ contains an amphipathic region preceded by a proline-rich motif.

(A) Schematic view of the PEX11γ sequence as outlined in Figs. 2A, 3A showing the two helical hydrophobic regions in red, and the single predicted amphipathic helix in blue. The proline-rich motif is indicated. The two proline residues analyzed in Fig. 4B and Fig. 5A,B are highlighted (green stars). The PLP-motif analyzed in Fig. 5C,D is indicated in red.

(B) The amino acids 176-193 of PEX11γ assemble into a helical region, illustrated as wheel (left). The hydrophobic side is marked in yellow. Pymol rendered cartoons show the wild type predicted amphipathic helix of PEX11γ (middle) whereas a mutated version in which a proline was introduced at position 182 breaks the helical structure (right).

Figure 5: Intact amphipathic region is required for PEX11γ to elongate the peroxisomal membrane and a proline-rich motif influences its function.

(A) EGFP-tagged PEX11γ or PEX11γ^{A182P} (green channel) localized to peroxisomes in HEK293T cells as shown through co-staining with the genuine peroxisomal protein PEX14 (red channel) 1, 2, 3 and 9 days after transfection. Cells expressing non-mutated PEX11γ showed typical peroxisome elongation eventually leading to the formation of JEPs, whereas PEX11γ^{A182P} only induced slight elongation of peroxisomes (arrowheads). Nuclei (blue channel). Bar: 5 µm

(B) EGFP-tagged PEX11γ mutated in its proline-rich-motif, PEX11γ^{P158A} and the binary mutant PEX11γ$^{P158A/A182P}$ (green channel) were analyzed for their ability to induce peroxisome elongation. Peroxisomes were visualized with anti-PEX14 antibodies (red channel). Cells expressing PEX11γ^{P158A} showed delayed elongation, whereas PEX11γ$^{P158A/A182P}$ did not induce elongation. Nuclei (blue channel). Bar: 5 µm

(C) EGFP-tagged PEX11γ lacking its PLP motif, PEX11γ^{noPLP}, was analyzed along with wild type EGFP-PEX11γ (green channel) for their time-dependent influence on the peroxisomal morphology. Peroxisomes were visualized with antibodies against PEX14 (red channel). Expression of EGFP-PEX11γ^{noPLP} led to peroxisome elongation similar to wild type PEX11γ. At early time points, JEP formation was enhanced upon expression of EGFP-PEX11γ^{noPLP} as compared to EGFP-PEX11γ. Nuclei (blue channel). Bar: 5 µm

(D) HEK293T cells co-expressing EGFP-PEX11γ (green channel) and myc-hFis1 did not present elongated peroxisomes. In contrast, cells simultaneously expressing myc-hFis1 and EGFP-PEX11γ^{noPLP} (green channel) presented significantly elongated peroxisomes. Peroxisomes were visualized with antibodies against PEX14 (red channel). Nuclei (blue channel). Bar: 5 µm

Figure 6: A complex interaction network around PEX11γ determines the rate of peroxisome division through assembly of the fission factors.

(A) Summary showing the topology of PEX11γ as demonstrated through carbonate extraction and pegylation assays. Cysteins accessible from the cytosol are shown in green, whereas buried residues are depicted in red. PEX11γ inserts its hydrophobic regions (brown) into the

peroxisomal membrane from the cytosolic side. The predicted amphipathic alpha-helix (blue helix) is anchored in the outer leaflet of the membrane, thereby promoting bending and elongation of the peroxisomal membrane.

(B) HEK293T cells expressing the indicated proteins were lyzed 48h after transfection in buffer containing 0.2% digitonin. Immunoprecipitations were performed using anti-FLAG antibodies. 2% of starting material (I) and 5% of the eluates obtained with excess of 3xFLAG peptides (E) were separated via SDS-PAGE. Higher amounts of EGFP-myc-hFis1 were yielded with PEX11γ^{A182P} as compared to wild type PEX11γ or with other mutated PEX11γ versions.

(C) Co-immunoprecipitations were performed as described in Figure 6B. All analyzed versions of PEX11γ-FLAG co-precipitated both, EGFP-PEX11β and EGFP-PEX11γ. However, using PEX11γ^{A182P}-FLAG smaller amounts of EGFP-PEX11β were recovered in the precipitates.

(D) Co-immunoprecipitations were performed as described in Figure 6B. PEX11β-FLAG, PEX11γ-FLAG and PEX11γ^{A182P}-FLAG precipitated EGFP-Mff. While for PEX11γ and PEX11γ^{A182P} comparable amounts of Mff were visualized in the affinity purifications, PEX11β precipitated significantly higher amounts of Mff.

(E) Cells co-expressing mCherry-Px (white channel) and EGFP-Mff (green channel) were stained for mitochondria using Mitotracker (red channel). Nuclei (blue channel). EGFP-Mff predominantly localized to mitochondria, which appear fragmented. However, a significant portion of EGFP-Mff localized exclusively to peroxisomes (arrowheads), some of which appeared slightly elongated. A 3D object analysis was performed to unambiguously identify peroxisomes decorated with Mff. The image shows EGFP-Mff in green, mCherry-Px in red, both as objects, and the mitochondrial stain as maximum intensity volume in gray. Bar: 5 µm

(F) HEK293T cells co-transfected with plasmids coding for mRFP-PEX11γ (red channel) and EGFP, EGFP-myc-hFis1 or EGFP-Mff (green channel) were analyzed for changes in peroxisome morphology. Typically, expression of mRFP-PEX11γ led to elongated and clustered peroxisomes. As expected, cells expressing both, PEX11γ and hFis1 displayed normally shaped peroxisomes and no significant elongation or clustering of peroxisomes was observed in these cells. Likewise, cells co-expressing Mff and PEX11γ presented round-

shaped peroxisomes indicating that Mff also counteracts PEX11γ-induced peroxisome elongation.

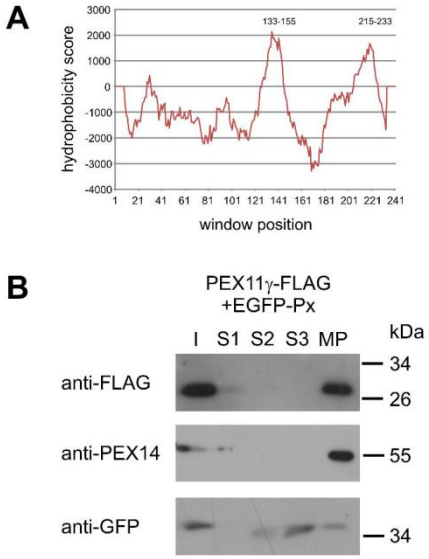
(G) Cells co-expressing PEX11γ-FLAG or PEX11γ^{noPLP}-FLAG and EGFP-Mff were analyzed for changes in peroxisome morphology. Cells were immuno-stained using either anti-PEX14 or anti-FLAG antibodies (red channel). Cells over-expressing EGFP-Mff (green channel) displayed round-shaped peroxisomes regardless of whether wild type PEX11γ-FLAG or PEX11γ^{noPLP}-FLAG was expressed in these cells. Bar: 5µm

Figure 7: Interplay between PEX11 proteins and the fission machinery during the process of peroxisome proliferation.

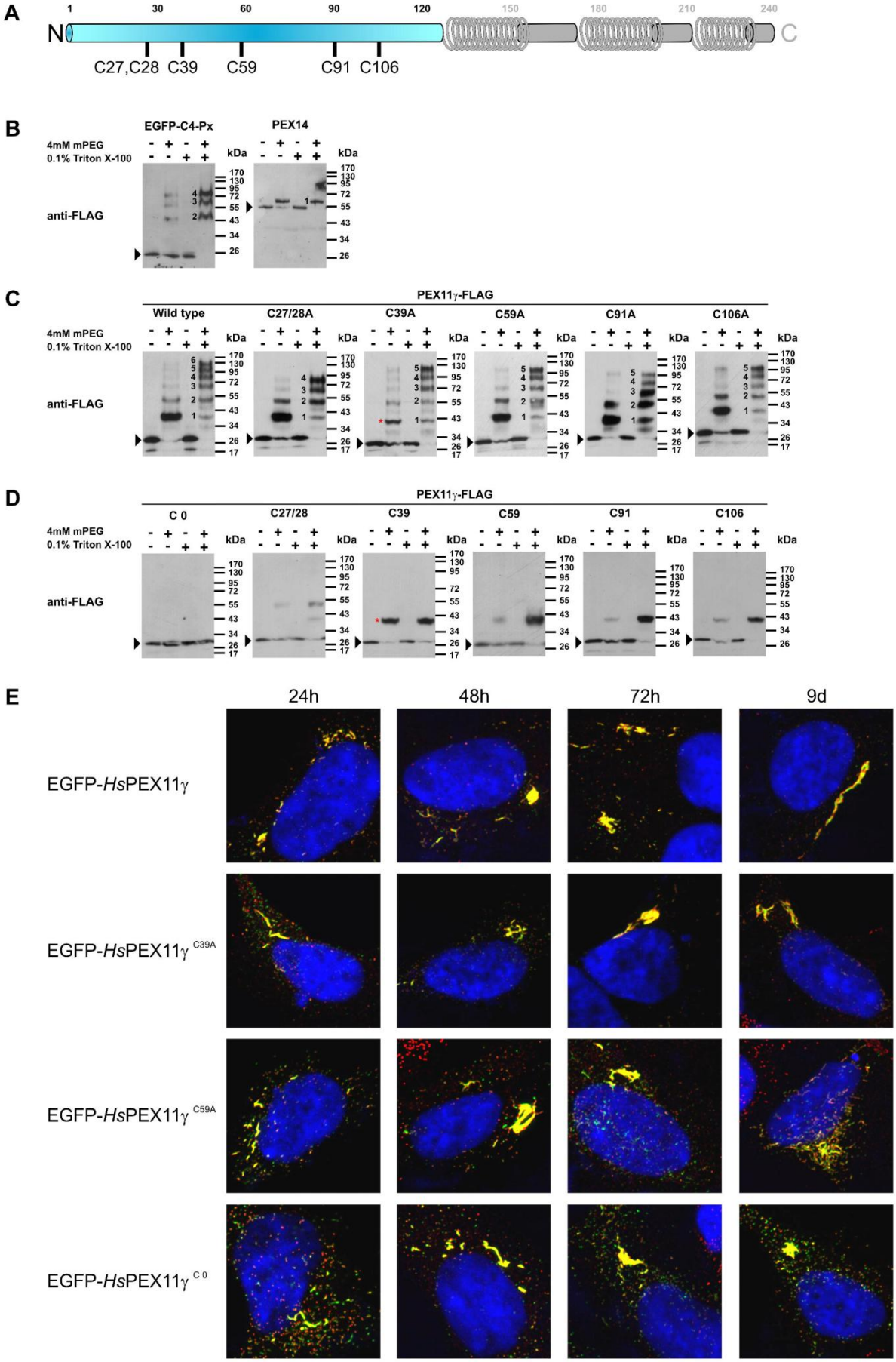
(A) Cells co-expressing mRFP-PEX11β (red channel) and either EGFP-PEX11γ or EGFP-PEX11γ^{A182P} (green channel) were analyzed for the effects on peroxisomes. Cells expressing mRFP-PEX11β and EGFP-PEX11γ displayed an increased number of peroxisomes and peroxisome elongation including JEPs. Upon expression of EGFP-PEX11γ^{A182P} the number of peroxisomes drastically increased and cells did not form JEPs despite co-expression of mRFP-PEX11β. Nuclei (blue channel). Bar: 5 µm.

(B) In our model, PEX11γ acts together with PEX11ß to protrude the peroxisomal membrane and remodel it for fission (1). The fission machinery, including hFis1 and Mff, accumulates at the site of membrane elongation allowing recruitment of the dynamin-related protein DRP1 (2). The peroxisomal membrane elongates and DRP1 assembles for fission (3). Self-activated DRP1 polymerizes and proceeds to scission of the peroxisomal membrane (4).

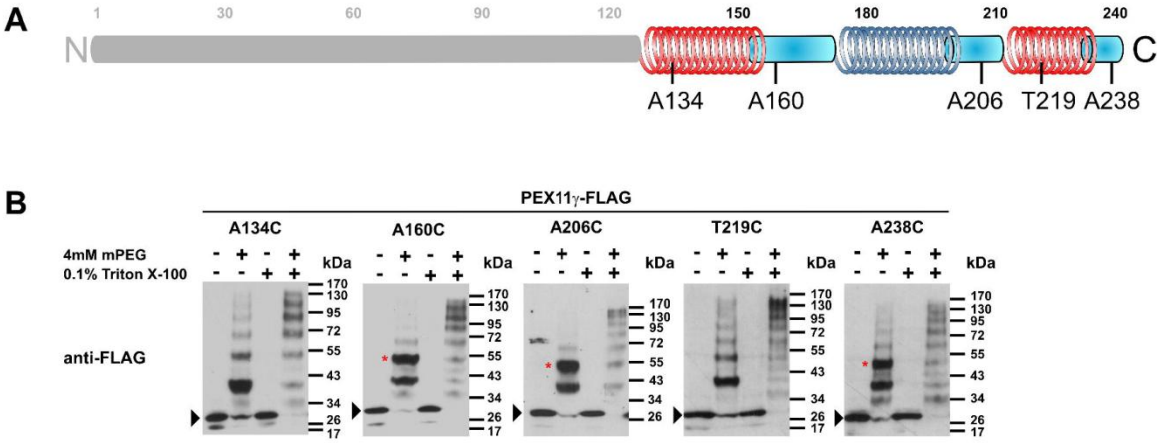
Koch and Brocard, Figure 1



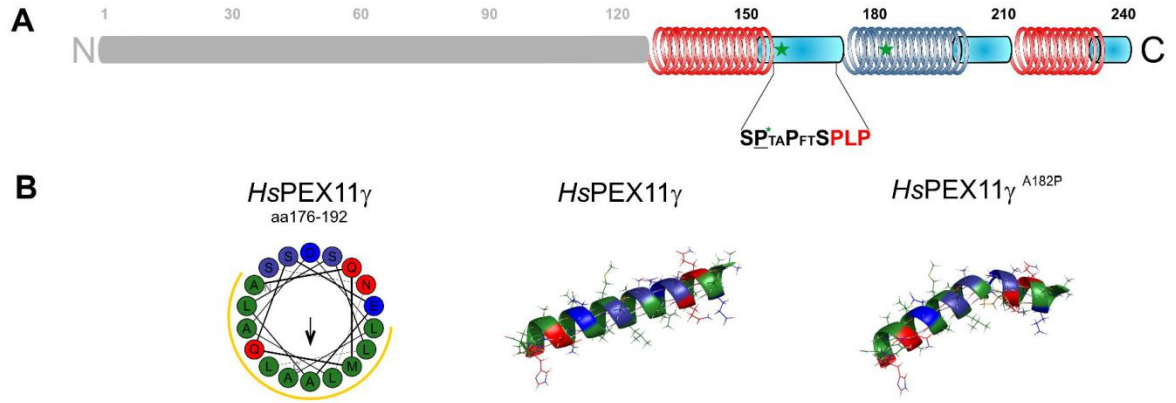
Koch and Brocard, Figure 2



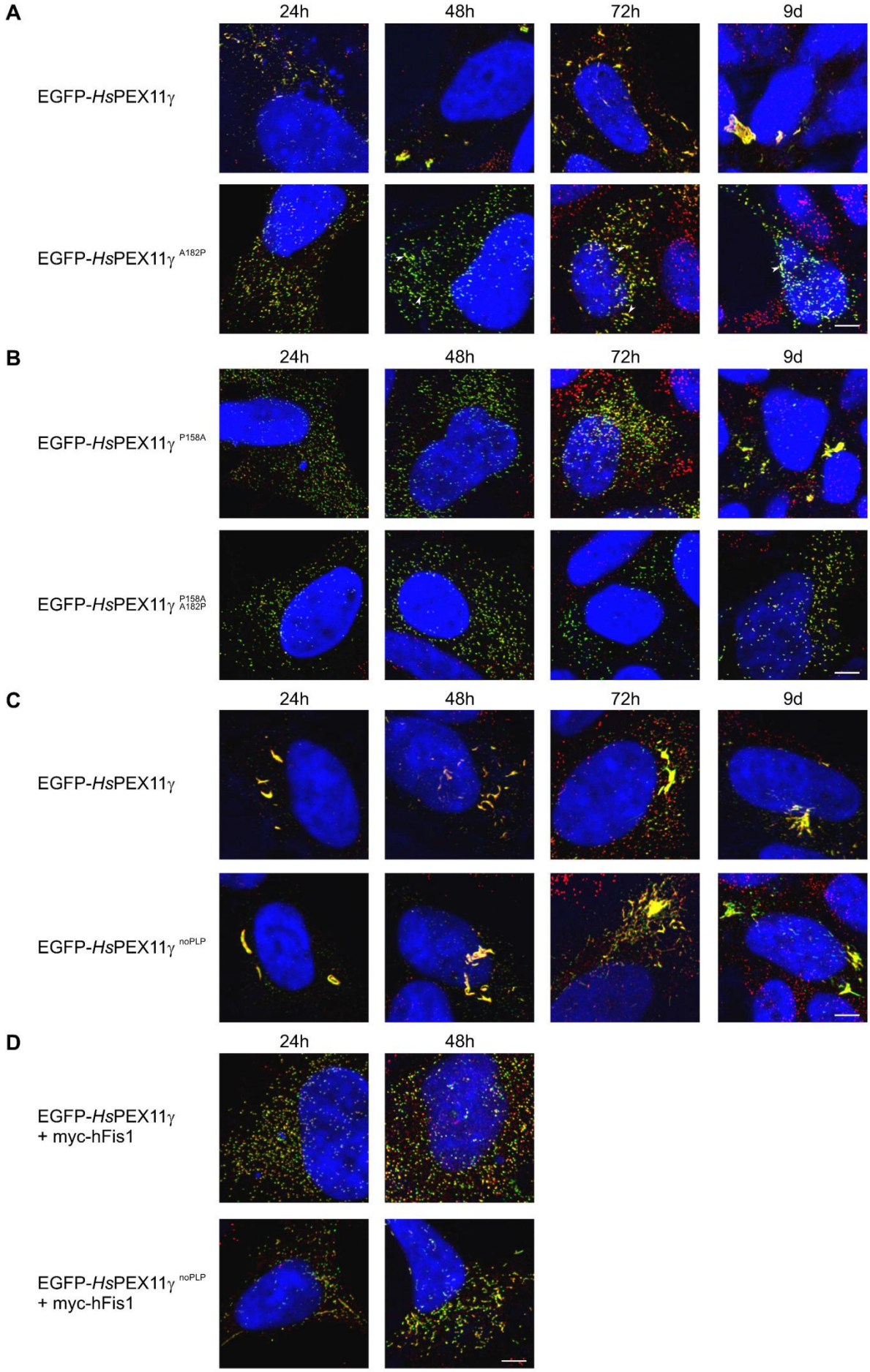
Koch and Brocard, Figure 3



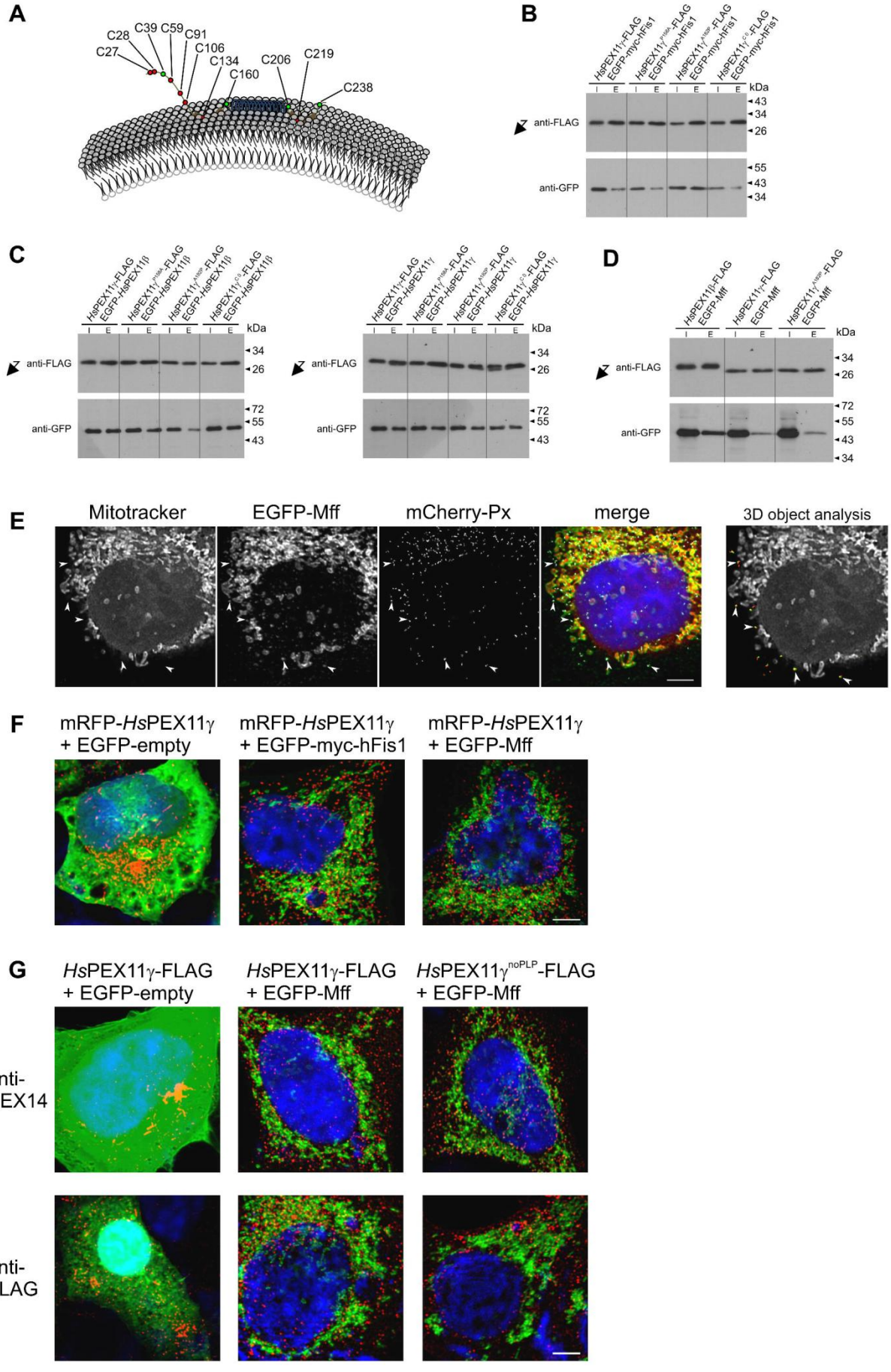
Koch and Brocard, Figure 4



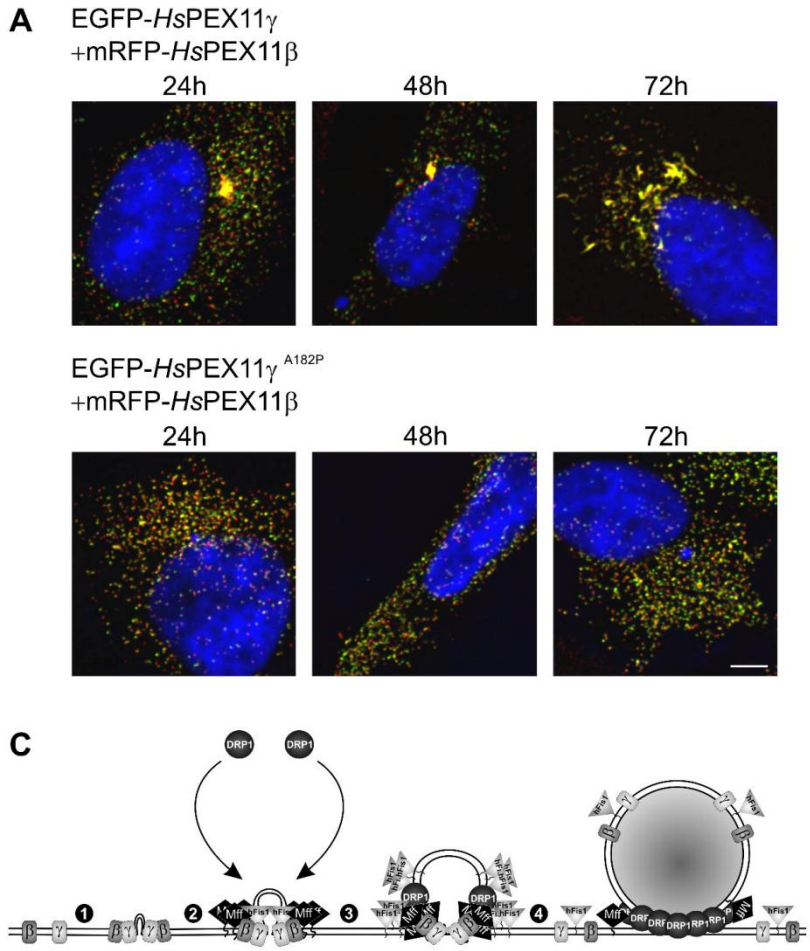
Koch and Brocard, Figure 5



Koch and Brocard, Figure 6



Koch and Brocard, Figure 7



7 Appendix

7.1 Summary

Peroxisomes are essential organelles present in every eukaryotic cell. They participate in many metabolic processes including lipid metabolism and ROS detoxification. Their absence correlates with the occurrence of severe cerebrohepatorenal diseases, such as the Zellweger syndrome, leading to death early after birth. Thus, understanding the molecular mechanisms by which cells maintain their pool of peroxisomes is critical.

Peroxisomes multiply by growth and division of pre-existing peroxisomes, and they can be generated *de novo* from the ER. The proteins involved in the proliferation of peroxisomes are called peroxins (PEX).

This work investigated the molecular mechanisms governing peroxisome biogenesis in yeast and human cells. We analyze proteins of the PEX11 and the PEX30 family, both responsible for regulating peroxisomal number and shape. The two mechanisms of peroxisome formation are not independent. Tight regulation must exist to ensure proper transfer of lipids and membrane proteins from the ER to peroxisomes. Based on our studies of the Pex30p protein in *S. cerevisiae* we propose that large membrane protein complexes including Pex30p act as hub for peroxisomes to the ER and, together with ER morphogenic proteins, designate specific ER sites for both, *de novo* formation and contact sites for existing peroxisomes.

Furthermore, we analyzed proteins of the PEX11 family in *S. cerevisiae* and showed that while Pex11p is involved in the fission of pre-existing peroxisomes, Pex25p is required for the reintroduction of peroxisomes from the ER. Here, we also demonstrated that Pex30p acted downstream of Pex25p and independently of Pex11p.

In further studies in yeast, plant and human cells, we established the PEX11 proteins as membrane elongation factors that coordinate peroxisome fission by protruding the peroxisomal membrane. We showed that the matrix protein content segregated during peroxisome fission and proposed this as quality control mechanism. Finally, based on our detailed analysis of the interplay between the human PEX11 proteins and factors of the fission machinery, we suggested a mechanism for peroxisome fission in human cells.

Our data on multiple aspects of peroxisome proliferation contribute to the understanding of ER-to-peroxisome crosstalk and provide new insights on peroxisomal maintenance at the molecular level.

7.2 Zusammenfassung

Peroxisomen sind essentielle Organellen in eukaryontischen Zellen. Sie nehmen an vielen metabolischen Prozessen teil, vor allem im Lipidstoffwechsel und in der Entgiftung von ROS. Ihr Verlust führt zu schweren cerebrohepatorenalen Krankheiten, wie zB dem Zellweger Syndrom, die bereits kurz nach der Geburt zum Tod führen. Daher ist das Verständnis der molekularen Mechanismen, mit denen Zellen ihre Peroxisomen erhalten, notwendig.

Peroxisomen vermehren sich durch Wachstum und Teilung von existierenden Peroxisomen, und können auch *de novo* vom ER generiert werden. Die in der Proliferation involvierten Proteine heißen Peroxine (PEX).

Diese Arbeit untersucht die molekularen Mechanismen der Peroxisomen-Biogenese in Hefe und Humanzellen. Wir analysierten Protein der PEX11 und PEX30 Familie, die beide für die Regulation der Anzahl und Form der Peroxisomen verantwortlich sind. Die beiden Mechanismen der Peroxisomenbildung sind nicht unabhängig voneinander. Strenge Regulation muss existieren, um einen ordnungsgemäßen Transfer von Lipiden und Membranproteinen vom ER zu Peroxisomen zu gewährleisten. Basierend auf unseren Studien am Pex30p Protein in S. cerevisiae schlagen wir vor, dass ein großer Membranproteinkomplex inklusive Pex30p als Andockstelle für Peroxisomen am ER dient, und zusammen mit ER-morphogenen Proteinen, spezifische Stellen am ER markiert sowohl für de novo Generierung und als Kontaktstelle für existierende Peroxisomes markiert.

Weiters analysierten wir die PEX11 Protein Familie in *S. cerevisiae* und zeigen, dass während Pex11p eher in der Teilung von existierenden Peroxisomen involviert ist, Pex25p für die Wiedereinführung von Peroxisomen vom ER verantwortlich ist. Hierbei arbeitet Pex30p unterhalb von Pex25p und unabhängig von Pex11p.

In unseren Studien in humanen, Hefe- und Pflanzenzellen etablierten wir die PEX11 Proteine als Membranelongationsfaktoren, die die peroxisomale Teilung durch ein Herausstülpen der peroxisomalen Membran koordinieren. Wir zeigten, dass der Matrixproteininhalt während der Peroxisomenteilung segregiert und schlagen dies als Qualitätskontrollmechanismus vor. Schließlich, basierend auf unseren detaillierten Analysen des Zusammenspiels der humanen PEX11 Proteine und Faktoren der Teilungsmaschinerie, zeigten wir einen möglichen Mechanismus der peroxisomal Teilung in humanen Zellen.

Unsere Daten über verschiedene Aspekte der peroxisomalen Proliferation tragen zum Verständnis des Wechselspiels zwischen ER und Peroxisomen bei, und ergeben neue Sichtweisen auf den molekularen Mechanismus der Peroxisomenerhaltung.

7.3 Curriculum Vitae

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education

2010	visiting student at the Weizmann Institute of Science, Israel, Prof. Jeffrey Gerst (3-months)
2008	start of doctoral studies, molecular biology, MFPL, University of Vienna
2008	graduation summa cum laude, diploma in Chemistry, specialization in Biochemistry
2008-2003	studies of chemistry at the University of Vienna, additional studies: Economics and Business Administration, Universtiy of Economics, Vienna
2002	graduation (Matura) cum laude, Schottengymnasium, 1010 Wien Scholarly research paper in chemistry, awarded with the 1st prize by the "Gesellschaft österreichische Chemiker"

Employment

2011-2005	University of Vienna, Institute of Biochemistry, teaching
2007	Baxter AG, Orth a. d. Donau, In-Process-Control, GLP, internship
2008-2005	Peek & Cloppenburg GmbH & Co KG, Wien, cashier
2005	Austrian Research Center Seibersdorf, Systems Research GmbH, internship
2004	G.L. Pharma, Vienna, quality control, GLP, internship
2003-2002	G.L. Pharma, Vienna, assistent to the production manager, internship
2001	Vemap.Com, Vienna, internship
2000	McDonalds, Klosterneuburg, internship

Skills

languages	English (excellent), French (moderate), Spanish (basic)
computer	MS Windows, MS Office (Word, Excel, Powerpoint, Access), different image analysis and quantification programs

driving license B

Invited Talks

Open European Peroxisome Meeting, Lunteren, NL, 2010, Oct20-22, “Evidence for the participiation of PEX11 proteins in peroxisome polarization and membrane elongation during proliferation”.

International Meeting on Peroxisome Research, Seattle, USA, 2009, Nov20-22, “Pex11-induced membrane dynamics in peroxisome proliferation”.

Congress Participations

3rd EMBO meeting, Vienna, AT, Sep11-13.
Open European Peroxisome Meeting, Lunteren, NL, 2010, Oct20-22.
Symposium on Trafficking of the Israeli Cell Biology Society, Herzilja, IL, 2010, May13.
Meeting on Intracellular Trafficking Processes, Rehovot, IL, 2010, May3-4.
International Meeting on Peroxisome Research, Seattle, USA, 2009, Nov20-22.
ImageJ conference, Luxembourg, LUX, 2008, Nov7-8.
FEBS-IUBMB, Athens, GR, 2008, Jun28-Jul3.

Grants

2009 EMBO short term fellowship (ASTF 502-2009, Weizmann Institute, Jeffrey Gerst, Rehovot, Israel)

2008 “Leistungsstipendium aus den Mitteln des Bundesministeriums”of the University of Vienna

2008 FEBS- bursary grant for FEBS congress 2008 in Athens, GR

2006 “Leistungsstipendium aus den Sondervermögen” as well as “Leistungsstipendium aus den Mitteln des Bundesministeriums”of the University of Vienna