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Peroxisomal membrane protein degradation in yeast

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Peroxisomal membrane protein degradation in yeast

SRISHTI DEVARAJAN

The studies presented in this thesis were performed in the research group Cell Biochemistry of the Groningen Biomolecular Sciences and Biotechnology (GBB) Institute of the University of Groningen, The Netherlands.

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university of
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Peroxisomal membrane protein degradation in yeast

PhD thesis

to obtain the degree of PhD at the
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on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

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Friday 18 September 2020 at 12.45 hours

by

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Aim and outline

Aim and outline

Peroxisomes are present in nearly all eukaryotic cells and they perform a wide variety of metabolic functions. Two major peroxisomal functions include β -oxidation of fatty acids and hydrogen peroxide removal however, other many functions exist and they can vary depending on the species, cell type and environmental conditions. Peroxisomal functions are determined by the peroxisomal matrix and peroxisomal membrane proteins (PMPs) that are present in peroxisomes. Therefore, peroxisomal protein homeostasis - protein synthesis, targeting and degradation - plays an important role in regulating peroxisome function. Since peroxisomes are devoid of protein synthesis machinery, all peroxisomal proteins are encoded by nuclear genes, synthesized in the cytosol and post-translationally imported to peroxisomes. While much effort has been placed on studying the targeting of PMPs to peroxisomes, little is currently known about how and why PMP degradation occurs.

The aim of this thesis is to shed light on PMP degradation by identifying which PMPs are targeted for degradation as well as the factors involved in their degradation. Furthermore, since protein degradation can occur for quality control purposes or to regulate organelle functions, we aimed to investigate why PMPs are targeted for degradation, in order to understand the importance of PMP degradation in peroxisome function. Hence, the research presented in this thesis utilizes various approaches to investigate some of the underlying mechanisms and cellular functions of PMP degradation in yeast.

In **Chapter 1**, we present an overview of various factors that regulate membrane protein degradation, focusing on the ubiquitin proteasome system (UPS) and its role in degrading membrane proteins from various organelles, including peroxisomes.

Previous findings reported that the PMPs Pex3p and Pex13p in the yeast *H. polymorpha* and Pex15p in the yeast *S. cerevisiae* are degraded in a UPS-dependent manner. In **Chapter 2**, we aimed to identify additional PMP substrates of the degradation pathway using *S. cerevisiae* as model organism. In our study, we utilized a tandem fluorescent timer (tFT) consisting of sfGFP and mCherry, to provide insights into PMP turnover. We observed that individual PMP-tFT fusions in cells display varied stability profiles, establishing that a tFT can be used to report on the stability of individual PMPs. Furthermore, we show that Pex13-tFT and Pxa1-tFT are the two most unstable proteins which are degraded rapidly under peroxisome inducing conditions. These PMP-tFT fusions were observed to be well-induced and displayed peroxisome localization in cells grown on oleate. This work demonstrates that a tFT is a valid and robust approach to examine PMP stability and identify unstable PMPs that have a fast turnover.

We observed that the tFT tag attached to Pxa1p affects the function of Pxa1p, indicating that Pxa1-tFT could be degraded for quality control purposes, due to reduced functionality.

Because PMPs are involved in peroxisomal biogenesis, any faulty PMPs present in the peroxisomal membrane need to be removed in order to maintain peroxisome homeostasis. Currently next to nothing is known about PMP quality control. In **Chapter 3**, we aimed to study PMP quality control in the yeast *S. cerevisiae* using Pxa1p, a peroxisomal fatty acid transporter, as a substrate. Using a mutant form of Pxa1p (Pxa1^{MUT}) fused to mGFP, we show that Pxa1^{MUT}-mGFP targets to peroxisomes and is rapidly degraded by a process that is dependent on proteasome. Unlike Pxa1^{MUT}-mGFP, we observed mGFP tagged Pxa1p to be stable, establishing a role for quality control in Pxa1^{MUT}-mGFP turnover. By using the tFT approach in combination with synthetic genetic array, we show that Ufd4p, a cytosolic E3 ligase, is required for Pxa1^{MUT}-mGFP degradation while we also show that inhibiting Pxa1^{MUT}-mGFP degradation, by the deletion of UFD4 partially restores Pxa1p function and thereby peroxisomal β -oxidation *in vivo*. These findings establish that faulty PMPs can undergo proteasome-mediated degradation while also demonstrating that cytosolic proteins involved in general protein quality control can impact on peroxisome function.

The docking complex PMP Pex13p in *H. polymorpha* was reported to undergo degradation by a process that is dependent on Pex2p and Pex4p. Similar to *H. polymorpha*, our tFT analysis show that tFT tagged Pex13p in *S. cerevisiae* is rapidly degraded (**Chapter 2**), indicating that Pex13p degradation may be a general feature of peroxisomes. In **Chapter 4**, we aimed to examine whether Pex13p degradation is a conserved process across yeast species and identify factors that are required for *S. cerevisiae* Pex13p degradation. Our data reveal that *S. cerevisiae* Pex13p fused to mGFP (Pex13-mGFP) undergoes degradation, likely via a similar mechanism to that in *H. polymorpha*. As in **Chapter 3**, we utilized a tFT approach to identify which additional factors other than peroxisomal proteins are involved in Pex13-mGFP degradation. Our data indicate that cytosolic proteins such as Ubc4p, Ufd4p and Ubr2p may play a role in Pex13-mGFP turnover. Furthermore, inactivation of the AAA protein Cdc48p, which plays a role in extracting membrane proteins from the ER and mitochondria, does not result in stabilization of Pex13-mGFP, establishing that Pex13-mGFP degradation probably occurs via a different mechanism to that of other organellar membrane proteins. Together, these data provide further evidence that Pex13p degradation is a conserved process while also uncovering novel components of the UPS that play a role in Pex13p degradation.

1

Chapter 1

The knowns and unknowns of peroxisomal membrane protein degradation

Srishti Devarajan and Chris Williams

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Author contributions:

SD wrote the manuscript and CW edited the manuscript

1. Introduction

Proteins perform many functions in the cell. Some proteins provide structural support and regulate movement of organelles and cells (Sweeney and Houdusse, 2010). Others function in the transport of ions or small molecules across membranes (Wilkens, 2015), transmission of extracellular signals to the cell interior (DeWire et al., 2007) while still others catalyse various metabolic reactions (Poirier et al., 2006). For the cellular systems and metabolic pathways to function efficiently, protein abundance needs to be tightly controlled, through protein synthesis and degradation (Eagle et al., 1959; Fonseca et al., 2006). Generally, protein degradation play two vital roles in the cell; firstly, to remove redundant proteins that function in regulatory processes such as cell cycle progression and transcriptional activation (Lee and Yaffe, 2016) and secondly, to remove faulty or damaged proteins that are dysfunctional, a process referred to as protein quality control (Sherman and Goldberg, 2001).

Since proteins are involved in almost every biological process, inefficient degradation of abnormal proteins or regulatory proteins could lead to severe consequences. Accumulating evidence indicates that reduced or accelerated degradation of proteins represents a prevalent pathogenic mechanism underlying a plethora of diseases (Ciechanover and Schwartz, 2004). For instance, cystic fibrosis (CF) and rare metabolic disorders including Adrenoleukodystrophy (ALD) occur as a result of increased protein degradation. The mutations in cystic fibrosis transmembrane conductance regulator (CFTR) result in protein misfolding and rapid degradation of CFTR mutants, leading to decreased chloride channel activity and impaired ion transport across the epithelial membrane (Du et al., 2005). In most cases, ALD patients harbour mutations in the peroxisomal fatty acid transporter Adrenoleukodystrophy protein (ALDP), which often result in ALDP degradation. As a result, very-long chain fatty acids are not transported to- and metabolized within- the peroxisomes but instead accumulate in the brain and other tissues. (Engelen et al., 2014; Morita et al., 2019).

Neurodegenerative disorders on the other hand can be caused due to reduced protein degradation and can be characterized by the accumulation of protein aggregates. These include β -amyloid peptide and hyper phosphorylated tau protein aggregates in Alzheimer's disease (Hardy and Selkoe, 2002; Ward et al., 2012), aggregation of α -Synuclein (α -Syn) in Parkinson's disease (Martin et al., 2011; Uversky, 2007) and mutant huntingtin in Huntington's disease (Tsoi et al., 2012; Williams et al., 2008a). The intracellular inclusions detected in the brains of patients with neurodegenerative diseases are often ubiquitin-positive and contain misfolded disease-specific proteins, indicating that misfolded proteins, although ubiquitinated, are not effectively removed by the degradation machinery, proteasome. (Ross and Poirier, 2004; Soto, 2003). Moreover, repeated attempts to remove protein aggregates or abnormal proteins could saturate the capacity or clog the entire proteasome, hindering its ability to degrade other proteins that are critical for regulating cellular activities. Aggregated proteins

that are resistant to degradation may also spread from one cell or brain area to another and function as seeds to instigate protein misfolding and aggregation in previously unaffected areas (Walker and LeVine, 2012). These examples demonstrate that protein degradation plays a vital role in cellular health.

Here we present an overview of membrane protein degradation, with emphasis on peroxisomal membrane protein (PMP) degradation. We first describe the functions of protein degradation and outline the mechanism of degradation by the ubiquitin proteasome system (UPS), the major protein degradation pathway in eukaryotic cells. Next, we discuss the challenges associated with removing proteins from membranes. Furthermore, we present the current knowledge on membrane protein degradation from other organelles such as the ER and mitochondria. We then introduce the topic of peroxisomes, protein targeting into peroxisomes and peroxisomal protein degradation. Finally, we discuss the current knowledge on PMP degradation, including known and putative PMP substrates of the degradation pathway and we also discuss possible roles for peroxisomal and other UPS factors in PMP degradation.

Keywords: Protein degradation, protein quality control, regulated protein degradation, ubiquitin proteasome system, membrane protein removal, peroxisomes, peroxisomal membrane protein degradation

2. Functions of protein degradation

As mentioned above, proteins can undergo degradation either to remove faulty proteins by quality control pathway (2.1) or to regulate cellular activities (discussed in 2.2)

2.1 Protein quality control: Elimination of faulty proteins

Faulty proteins can appear in the cell due to mutations in the gene sequence, protein synthesis errors, inefficient protein folding, faulty post-translational modifications or damage caused by exposure to for example environmental stress (Drummond and Wilke, 2008; Mandelkow and Mandelkow, 2012; Radi, 2013; Sitte et al., 2000). Abnormal proteins produced by any of these methods are dysfunctional, resulting in loss of protein function. However, build-up of abnormal proteins results in a gain-of-toxic function that is unrelated to the protein's function and which has become increasingly relevant to human disease (Winklhofer et al., 2008). Generally, aberrant proteins tend to expose hydrophobic regions to the cytosol that are normally buried within the core of the protein (Gibson and Ellory, 2002). Non-native proteins exposing hydrophobic patches can take part in unwanted protein-protein interactions and may ultimately culminate in protein aggregation that eliminates the activity of other proteins (Dobson, 2004). To minimize potentially harmful effects caused by the build-up of abnormal proteins, cell employs protein quality control systems such as molecular chaperones (Hartl et al., 2011; Stirling et al., 2003) and protein degradation systems (Fredrickson and Gardner, 2012; He and Klionsky, 2009; Hershko and Ciechanover, 1998). Along with protein folding, chaperones also

function in refolding misfolded proteins, acting as a first line of defence against the formation of protein aggregates (Muchowski and Wacker, 2005). Nevertheless, some aberrant proteins undergo rapid degradation due to the inability of chaperones to refold them to their native state.

2.2 Protein degradation in regulation of cellular activities

In addition to the role in quality control, protein degradation helps maintain the appropriate cellular level of proteins and hence their activities, as well as allowing the rapid downregulation of protein levels to occur, to help cells respond to changing conditions (Varshavsky, 2005). Effectors of the cell cycle, developmental regulators, homeotic proteins, transcription factors, protein kinases and oncogene products can all be targeted for degradation, in order to maintain the appropriate protein levels or as response to changing environmental conditions (Bassermann et al., 2014; Ee and Lehming, 2012). For instance, the selective and timely degradation of cell-cycle regulatory proteins, such as cyclins, inhibitors of cyclin-dependent kinase and anaphase inhibitors are critical for cell-cycle progression (Bassermann et al., 2014). Cell growth and proliferation are further controlled by the degradation of tumour suppressors such as p53 and proto-oncogenes including β -catenin (Kitagawa et al., 2009). The rapid degradation of numerous transcriptional regulators is involved in controlling various signal transduction processes and responses to environmental cues (Conaway et al., 2002). The output of many signalling pathways is the transcription of genes that encode proteins necessary for the desired cellular response.

3. Ubiquitin Proteasome System and protein degradation

The two main pathways involved in the degradation of proteins are the ubiquitin-proteasome system (UPS) and autophagy (He and Klionsky, 2009; Hershko and Ciechanover, 1998). While UPS is primarily responsible for rapid protein turnover, including the selective removal of abnormal and misfolded proteins (Ciechanover et al., 2000), autophagy mostly targets long-lived proteins and large structures like protein aggregates or organelles (Goldberg, 2003; Seglen and Bohley, 1992). The ability of cells to degrade proteins by the UPS was first demonstrated more than 40 years ago (Goldberg, 1972). In general, degradation of aberrant or regulatory proteins by the UPS involves two major steps 1) ubiquitination of the target protein and 2) degradation of the ubiquitinated protein by the proteasome. Ubiquitin (Ub), a highly conserved globular protein with 76 amino acids, is covalently attached to the lysine residue of a substrate protein (Komander and Rape, 2012; Swatek and Komander, 2016). Conjugation of ubiquitin to the substrate involves multiple steps catalysed by three enzymes- an Ub-activating enzyme (E1), an Ub-conjugating enzyme (E2) and an Ub ligase (E3) (Figure 1) (Scheffner et al., 1995). Ub is first activated by the formation of a thioester bond between the C-terminal Gly76 of the Ub molecule and the active site cysteine (Cys) of the E1 (Figure 1) (Schulman and Harper, 2009). Activated Ub is then passed on from E1 to the active site cysteine residue of the E2.

Often, E2 enzymes directly transfer Ub to a target protein, typically with the help of E3s such as really interesting new gene (RING) domain containing ligases, also called as RING E3s (Figure 1, point 1) (Ozkan et al., 2005). However in some cases, the Ub is first transferred from the E2 to the active site of a specific class of E3 (Figure 1, point 2), which possess homologous to the E6-AP carboxyl terminus (HECT) domain and these HECT E3s are responsible for attaching Ub to the lysine residue of the target protein (Metzger et al., 2012; Petroski et al., 2006). A third class of E3 ligase, distinct from the RING and HECT classes, are referred to as RING-in-between-RING (RBR) E3s (Figure 1, point 3) and they often contain a RING domain, followed by an RBR domain and finally a RING-like domain. These RBR E3s recruit an E2, which transfers ubiquitin to a cysteine in the RING-like domain before transferring to the substrate (Figure 1, point 3) (Wenzel et al., 2011).

Figure 1: Schematic overview of the enzyme cascade catalyzing protein ubiquitination and degradation. Ubiquitin (Ub) is first activated by a ubiquitin activating enzyme (E1) with ATP hydrolysis. Next, the E1 transfers the Ub to a ubiquitin conjugating enzyme (E2), which is eventually transferred to a substrate with the aid of a ubiquitin ligase (E3). (1) RING-E3 serve as a bridge to enable Ub to be passed directly from the E2 to the substrate while (2) HECT-E3s accept Ub from the E2 onto their own active site before attaching it to a substrate.

RBR-E3 ligases (3) bind a Ub conjugated E2 and Ub is transferred from the E2 to the E3, after which Ub is then transferred to the substrate. Substrate proteins with single Ub can undergo several rounds of Ub chain elongation. Poly-ubiquitinated substrates are then targeted to the proteasome for degradation.

Cells possess one or two E1s, several E2s (~11 in yeast and ~37 in humans) and multiple E3s (~50 E3s in yeast and ~1000/thousand in humans) (Finley et al., 2012; Li et al., 2008; Pickart, 2001; Pickart and Eddins, 2004). E3s selectively recognize and mediate the ubiquitination of substrate proteins dependent on E1 and E2 enzymes (Pickart, 2001). Several lines of evidence show that efficient ubiquitination of certain substrates require additional conjugation factors, termed as E4 enzymes. Examples of this class include cullins and E4s such as UFD2 and they work in association with E1, E2 and E3 (Hoppe, 2005).

Proteins can be modified either with a single Ub molecule either at a single site (mono-ubiquitination) or on multiple sites (multiple mono-ubiquitination) (Pickart and Eddins, 2004). Furthermore, because Ub itself possess seven lysine residues, poly-ubiquitin chains can be formed by attachment of other Ub molecules to one of these internal lysine residues (Ikeda and Dikic, 2008) or to the N-terminal methionine of the previously attached Ub (Kirisako et al., 2006). The fate of poly-ubiquitinated proteins depends on the lysine chain specific ubiquitination (Pickart, 2000). Some poly-ubiquitin chains, particularly the lysine 48 (K48) and to a lesser extent (K29), act a potent signals for destruction by the proteasome (Yau and Rape, 2016), while other poly-ubiquitin linkages (e.g. K6, K11, K27 and K63), as well as mono-ubiquitination, are known to mediate non-proteolytic cellular process, including DNA repair, cell-cycle regulation, signal transduction and protein-protein interactions (Hicke, 2001; Husnjak and Dikic, 2012; Komander and Rape, 2012).

As a rule, a chain of four or more Ub molecules, linked through K48, is required to allow protein degradation by the 26S proteasome (Adams, 2003; Tanaka, 2009; Thrower et al., 2000). although mono-ubiquitination was also shown to be sufficient for the degradation of certain substrates (Braten et al., 2016; Kravtsova-Ivantsiv et al., 2011). While protein degradation by the proteasome mostly depends on Ub conjugation, there are a number of proteins that can be degraded in a process independent of Ub but still requiring the proteasome (Baugh et al., 2009). Several examples of ubiquitin-independent proteasomal degradation include the cyclin dependent kinase inhibitor p21Cip1 and Ornithine decarboxylase (ODC), an enzyme involved in polyamine biosynthesis (Bercovich et al., 1989; Sheaff et al., 2000).

Proteasomes have an ancient origin and all eukaryotes, archaea as well as some bacteria possess proteasomes of varying complexity (Dahlmann et al., 1989; Gille et al., 2003). In eukaryotes, the 26S proteasomes are located mainly in the nucleus and cytosol as free or ER attached. Approximately 80% of proteasomes in the yeast *Saccharomyces cerevisiae* cluster at the ER, compared to around 20% in mammalian cells (Enenkel et al., 1998). The 26S proteasome is a cylindrical complex comprising of a 20S catalytic core capped at the both ends

by a 19S regulatory subunit (Coux et al., 1996). The 19S regulatory particle consist of at least 19 subunits, including six ATPases, referred to as Rpt proteins, de-ubiquitinating enzymes (DUB) and polyubiquitin-binding subunits (Finley, 2009). The 20S particle harbours 28 subunits arranged in four, seven-membered stacked rings in which proteolysis of the substrate protein takes place. Of the four rings, the two inner rings are composed of β subunits (β 1- β 7) and the β 1, β 5 and β 7 subunits contain caspase-like, trypsin-like and chymotrypsin-like activities, respectively (Groll et al., 1997). Proteasomes contain a large number of proteins that recognize ubiquitinated cargos, including two 19S regulatory subunits (Rpn10p and Rpn13p) (Husnjak et al., 2008) and three proteins (Rad23p, Dsk2p and Ddi1p) which temporarily associate with the proteasome only during substrate delivery (Elsasser et al., 2004; Funakoshi et al., 2002; Kleijnen et al., 2003; Madura, 2004). The latter three proteins interact with the proteasome and ubiquitinated cargos through their Ubiquitin-like (UBL) domain and one or more Ubiquitin-associated (UBA) domains, respectively (Hartmann-Petersen and Gordon, 2004; Rao and Sastry, 2002; Wilkinson et al., 2001). Unlike the UBL/UBA proteins, which can bind substrates and recruit them to the proteasome for destruction, 19S subunits recognize only substrates that are in close proximity to the proteasome (Kleijnen et al., 2000; Madura, 2004). Followed by binding to Ub-receptors, substrates are unfolded by the Rpt proteins of the 19S subunit in an ATP-dependent manner and the unfolded polypeptide chain is fed into the chamber of the 20S particle (Groll et al., 2000; Navon and Goldberg, 2001). During this process, the 20S particle and 19S subunit remove the Ub molecules from the substrate, with the help of de-ubiquitinating enzymes (DUBs), to recycle the Ub (Guterman and Glickman, 2004; Lee et al., 2016; Peth et al., 2013). The substrates passing further through the 20S particle are cleaved into small peptides by the β 1, β 5 and β 7 subunits. Association of the 20S subunit with a 19S subunit in the cell is a dynamic process and requires ATP-hydrolysis (Glickman and Raveh, 2005; Lecker et al., 2006). While the 26S proteasome (consisting of 19S and 20S particles) can degrade natively folded proteins, mediated by the Ub linkage on the substrate protein (Raynes et al., 2016), 20S proteasomal subunits can only degrade unfolded proteins independent of Ub attachment and this accounts for 20% of the substrate proteins (Baugh et al., 2009; Ben-Nissan and Sharon, 2014; Pickering and Davies, 2012).

4. The challenges associated with membrane protein degradation

The list of substrate proteins that undergo UPS-dependent degradation is continually growing and we have a detailed mechanistic understanding of how many cytosolic proteins are degraded by the proteasome. However, this is generally not the case for the degradation of membrane proteins. Since membrane proteins constitute around 30% of the proteome and perform essential cellular activities, including transport of molecules across membranes and organelle biogenesis (Krogh et al., 2001), timely removal of membrane proteins is critical to maintain membrane protein homeostasis and cell vitality (Ng et al., 2012). Considering the complex nature of a typical membrane protein that harbours multiple transmembrane domains (TMDs),

which are embedded in the lipid layer (Fiedler et al., 2010), one of the major challenges in membrane protein degradation is to pull the TMDs out of the membrane (Bagola et al., 2011). Pathways that perform such functions require a significant input of energy, to extract membrane embedded proteins and target them to the proteolytic machinery. Another challenge is to specifically recognize the target protein, amongst other membrane proteins, to attach a Ub molecule to mark the substrate for degradation via the proteasome. These challenges have also made it difficult to understand the mechanism by which membrane proteins are degraded and therefore, a detailed understanding of how a given membrane protein is degraded via the UPS is an exception rather than a rule.

How proteins are recognized for degradation by the UPS has been of high interest since the discovery of the UPS degradation pathway (Ciechanover et al., 1978). This interest has led to the identification and characterization of multiple degradation signals, termed as degrons (Varshavsky, 1991). The majority of proteins residing in the cytosol, nucleus and the ER possess inherent or hydrophobic degrons that allow their specific recognition and effective proteolysis (Ravid and Hochstrasser, 2008). Inherent degrons can be specific amino acid sequences permanently present in the proteins and examples of this class include the destruction box of cyclins (Barford, 2011) or N- and C-terminal amino acids corresponding to the N-degron and the C-degron pathways (Varshavsky, 2019). Acquired degrons, on the other hand, are transient elements that are obtained by the post-translational modification of specific amino acids within the substrate proteins, such as phosphorylation, oxidation, the ligation of Small Ubiquitin-like Modifier (SUMOylation) and hydroxylation (Ella et al., 2019; Ravid and Hochstrasser, 2008; Sriramachandran and Dohmen, 2014). For instance, transient phosphorylation at serine or tyrosine residues facilitates rapid turnover of proteins such as G1 cyclins, which are involved in cell-cycle progression (Skowyra et al., 1997; Willems et al., 1996). A large area of hydrophobic residues that are normally buried in the protein core or within interaction surfaces between subunits of protein complexes may also act as an acquired degron, when this area becomes exposed because the protein unfolds or fails to fold properly or when the protein fails to assemble into a complex (Furth et al., 2011; Scazzari et al., 2015; Sung et al., 2016). The exposure of such hydrophobic regions can often result from mutations introduced into the protein, through protein translocation errors or mutations in the gene encoding for the protein or through protein damage caused by external stress. Therefore, the detection of such hydrophobic regions are an essential aspect of the cellular quality control systems. For example, San1 (Sir antagonist 1), an Ub ligase involved in nuclear protein quality control in yeast binds to hydrophobic domains of misfolded proteins and targets them for UPS-mediated degradation (Rosenbaum et al., 2011).

While the above-mentioned examples concern soluble proteins, our knowledge on the degradation signals or molecular factors that aid in the recognition of membrane proteins is far from complete. Generally, the biosynthesis of membrane proteins involves the integration of

the hydrophobic TMD into the lipid bilayer, folding of soluble domains on both sides (cytosol and lumen) of the membrane and in the case of multi-spanning proteins, assembly of TMDs within the bilayer (Christis et al., 2008; Fiedler et al., 2010). These events pose complex challenges to the quality control machinery and thereby necessitate that quality control systems be present in distinct compartments (membrane, cytosol and lumen) to monitor the folding status of the protein and degrade membrane proteins that are not only incorrectly folded but also redundant, damaged or non-functional. In line with this consideration, one could speculate that a membrane protein degron would need to be location-specific. For example, errors in the cytosolic or luminal domain of a membrane protein may lead to the exposure of normally buried residues which could serve as a recognition signal for degradation. This has been observed for non-native or mutant form of plasma membrane transporters such as Pma1p (Pma1-D378N) and Ste6* (Ste6 with C-terminal truncation) both of which exposes amphipathic or hydrophobic helical segment that facilitates recognition by their cognate E3 ligases (Ravid et al., 2006). However, a degron for the TMD would need to display different properties. Since the proper folding of TMD is achieved through a series of inter- and intra-helical hydrogen bonds and salt bridges (Bowie, 2011), misfolding or mutations in the TMD could lead to misaligned helices. This would result in the exposure of polar residues, that are normally involved in hydrogen bonding, to the hydrophobic environment of the membrane. Detection of these such residues could be accomplished by interaction with an E3 ligase, as has been showed for the Tul1p ubiquitin ligase, which recognizes misfolded TMD containing proteins in the Golgi-complex through interactions via hydrophilic residues in the TMD (Reggiori and Pelham, 2002).

As mentioned earlier, not only the recognition step is challenging but also the extraction of the TMD containing proteins that are stably anchored in the lipid bilayer presents many challenges that need to be overcome. This process requires a significant energy input to transfer the membrane-spanning segments from a hydrophobic to a hydrophilic environment and facilitate the accompanying unfolding process and solvation of TM helices (Cymer et al., 2015). A class of proteins that mediates this type of reaction is the AAA proteins or ATPase Associated with cellular Activities, which provide energy by ATP hydrolysis. Such proteins provide the mechanical force required to remove a substrate molecule from the membrane (Ogura and Wilkinson, 2001). A highly conserved and widely studied AAA protein is p97, also known as VCP (mammals), or CDC48 (yeast) is located mainly in the cytosol and functions in unfolding protein substrates, disassembling protein–protein complexes and dislocation of proteins from various organelle membranes (Jarosch et al., 2002; Ye et al., 2001). Most AAA-ATPases form oligomeric complexes with each of the monomers consisting of either one (type I) or two (type II) functional ATPase domains (named D1 and D2) accompanied by a regulatory N-terminal domain. Upon ATP binding and hydrolysis, Walker A (P-loop) and B motifs present in the ATPase domains undergo conformational changes that can be propagated to act upon a

target substrate, leading to their removal from the membrane. An alternative to ATP dependent membrane protein extraction could be clipping of transmembrane segments from cellular membranes by intramembrane proteases (Lemberg, 2011). These proteases function by initially unwinding the helical structure of a TM segment in the substrate, cleavage the unfolded segments and subsequently release of the cleaved products to either side of the membrane (Langosch et al., 2015). Although proteases are capable of clearing an entire membrane protein, including the TMD, this mode of action has only been observed for membrane proteins with one or two TMDs (Langosch et al., 2015). This, together with the observation that such proteases have not been identified in the membranes of key organelles such as mitochondria and peroxisomes, suggests that AAA-ATPase dependent extraction may well be the major mode of membrane protein extraction.

In the following two sections, we present an overview of UPS-dependent degradation pathways that remove membrane proteins from the Endoplasmic Reticulum (ER) and mitochondria. We discuss how membrane proteins from these organelles are recognized by the degradation machinery. Furthermore, we also outline various modes by which membrane proteins are extracted from these organellar membranes, in order to facilitate their degradation by the proteasome.

5. Membrane protein degradation from the ER

The ER-associated degradation (ERAD) pathway is one of the most well studied degradation pathways that targets membrane proteins. Since the ER is the major site for the synthesis and assembly of membrane proteins, the ER plays a vital role in membrane protein homeostasis. However, this folding process is not infallible, hence the need for a pathway to ensure that terminally misfolded or unassembled membrane proteins do not accumulate at the ER but are instead degraded (Ng et al., 2012). In addition, ERAD also participates in regulating the levels of correctly folded ER membrane proteins such as Pca1p and Hmg2p in response to cellular signals (Adle et al., 2007; Adle et al., 2009; Hampton et al., 1996). ERAD begins with the recognition of substrate proteins followed by ubiquitination, ATP dependent extraction from the ER and proteasomal degradation (Christianson and Ye, 2014). The detection of substrate proteins depends principally on an E3 ligase complex, which recognizes degradation signals in substrates and stimulates Ub transfer from the E2 to the target protein (Ruggiano et al., 2014). In yeast, two membrane embedded E3 ligase complexes exist; HRD and DOA (Figure 2). The HRD complex, consisting of the RING E3 ligase Hrd1p and its co-factor Hrd3p, catalyses the ubiquitination of several membrane protein substrates, working together with the E2 enzymes Ubc7p, Ubc1p and, less frequently, Ubc6p (Figure 2, right) (Bays et al., 2001; Deak and Wolf, 2001). The DOA complex, on the other hand, ubiquitinates membrane proteins through the concerted action of Doa10p (ER embedded RING E3 ligase) and the E2 enzymes Ubc6p and Ubc7p (Figure 2, left) (Swanson et al., 2001; Vashist and Ng, 2004). While the yeast ERAD

machinery is in itself complex, that of the mammalian machinery is more so. The number of mammalian E3 ligases involved in ERAD includes ER embedded synoviolin/HRD1 (Kikkert et al., 2004) and GP78 (Fang et al., 2001), both of which are orthologous to the yeast Hrd1p (Nadav et al., 2003), RNF5/RMA1 (Morito et al., 2008), TEB4 (ortholog of yeast Doa10p) (Hassink et al., 2005), TRC8 (Stagg et al., 2009), RFP2 (Lerner et al., 2007) and CHIP (non-ER protein) (Meacham et al., 2001). These E3s are known to interact with the mammalian homolog of yeast E2 enzymes Ubc6p (Ube2j1p and Ube2j2p) and Ubc7p (Ube2g1p and Ube2g2p) (Lenk et al., 2002). Ube2g2p is strongly implicated in ERAD, functioning with multiple ERAD E3s (Chen et al., 2006; Hassink et al., 2005; Kikkert et al., 2004) while there is also evidence supporting roles for ER localized Ube2j1p and Ube2j2p in the degradation of ERAD substrates (Elangovan et al., 2017).

Figure 2: Schematic overview of the different steps and branches in ER membrane protein degradation by the ERAD pathway in *S. cerevisiae*. (Left) Chaperones (Ssa1p and Ydj1p) recognize ER membrane proteins (Pma1p and Ste6*) with misfolded cytosolic domains (ERAD-C substrates) and mediate the transfer of substrates to the E3 ligase Doa10p (1). Doa10p together with E2 enzymes (Ubc6p and Ubc7p) promotes ubiquitination of ERAD-C substrates, which are then extracted from the ER membrane by the Cdc48p/Ufd1p/Npi4p complex (2) and targeted for degradation by the proteasome (3). (Right) ER membrane proteins (Hmg2p, Sec61-2 and Pdr5*) with a misfolded transmembrane domain (ERAD-M) are recognized by the Hrd1p/Hrd3 complex (1). ERAD-M

substrates are then ubiquitinated by Ubc7p and Hrd1p/Hrd3p, retro translocated from the ER to the cytosol (2) and finally degraded by the proteasome (3). Misfolded domains on the proteins are indicated with a red star.

Much of our knowledge on the ERAD pathway comes from experiments in yeast. Membrane proteins with misfolded TMDs (referred to as ERAD-M substrates) are targeted by the HRD complex (Sato et al., 2009) while the DOA complex preferentially removes membrane proteins with misfolded cytosolic domains (ERAD-C substrates) (Carvalho et al., 2006; Vashist and Ng, 2004). ERAD-C substrates are initially recognized by cytosolic chaperones, which promote interactions with (and subsequent ubiquitination by) Doa10p (Buck et al., 2007; Nakatsukasa et al., 2008). For instance, the degradation of a mutant form of the ATP binding cassette transporter Ste6p (Ste6*), the mutated plasma membrane ATPase Pma1p, mammalian CFTR expressed in yeast and mammalian apolipoprotein B (apoB) relies on the Hsp70 (Ssa1p), Hsp40 (Hlj1p and Ydj1p) and Bip (Kar2p) chaperones. Mutations in these chaperones prevent Doa10p dependent ubiquitination and degradation of ERAD substrates (Han et al., 2007; Zhang et al., 2001). The simplest view could be that chaperones act as an initial recognition factor by binding hydrophobic amino acid clusters in membrane proteins to prevent aggregation (Nishikawa et al., 2005) and augment membrane protein access to Doa10p. Contrary to the well-explored role of chaperones in substrate recognition, the mechanism by which Doa10p recognizes the substrate or chaperone-substrate complex remains unclear. A conserved C-terminal element (CTE) in Doa10p is involved in the recognition of certain ERAD substrates (Zattas et al., 2016) however, no direct interaction between Doa10 CTE and substrate proteins was observed so far. Furthermore, the degradation of Ste6* occurred independently of the Doa10 CTE (Zattas et al., 2016), suggesting that there might be other sequences in Doa10p that are required for the recognition of individual membrane protein substrates.

Unlike substrates of the DOA pathway, membrane proteins with defective transmembrane segments (ERAD-M) appear to be recognized directly by the E3 ligase Hrd1p (Gardner et al., 2000). Early insight into this mechanism was revealed by studies using Hmg2p, a yeast isozyme of the mammalian HMG-CoA reductase (HMGR) and a key enzyme in sterol biosynthesis (Hampton and Rine, 1994). Both in yeast and mammals, HMGR degradation by ERAD follows a feedback mechanism (Hampton and Garza, 2009; Ravid et al., 2000). For instance, high production of sterol pathway products causes reversible misfolding of the Hmg2p transmembrane domain (TMD), rendering it more susceptible to degradation by the HRD pathway (Gardner and Hampton, 1999; Gardner et al., 2001). The misfolded TMD of Hmg2p would expose buried residues which could then be detected by the E3 ligase Hrd1p. Interestingly, mutation of the hydrophilic intramembrane residues in Hmg2p or the E3 ligase Hrd1p prevents Hmg2p degradation (Sato et al., 2009). These data suggest that the detection of misfolded Hmg2p by Hrd1p could occur through inter-TMD interactions. Like Hrd1p, the transmembrane segment of other E3 ligases, including yeast Doa10p and mammalian Hrd1p

and gp78p (required for mammalian HMGR degradation) possess hydrophilic amino acids (Habeck et al., 2015), indicating that the recognition of hydrophilic TMD residues in membrane proteins by similar residues within a ligase may be a broadly employed strategy.

However, a number of Hrd1p substrates, including the thermolabile Sec61-2 protein translocon and a mutant form of the ABC transporter Pdr5p appear to be recognized by non-hydrophilic residues in the TMD of Hrd1p (Sato et al., 2009). Intriguingly, these membrane proteins, despite carrying mutations outside the transmembrane segments, such as in the cytosolic domain (Sec61-2) or luminal loop (Pdr5*), behave as ERAD-M substrates due to the mutations that either precede an unusually short TM segment or remain close to the TM segment (Carvalho et al., 2006; Plemper et al., 1998). These mutations could result in the destabilization of the TM segment, causing a structural change recognized by Hrd1p (Sato et al., 2009). Together, these examples indicate that the Hrd1p TMD specifically recognizes and mediates the degradation of ERAD-M substrates.

Followed by Hrd1p- or Doa10p-mediated substrate recognition and ubiquitination, the final steps in the degradation of ERAD substrates includes the extraction and proteasomal degradation of ubiquitinated membrane proteins. Generally the extraction of ERAD substrates are mediated by the highly conserved and widely studied AAA ATPase known as Cdc48p in yeast and p97 in mammals (Ye et al., 2001). Cdc48p/p97 forms heterotrimeric complex with cytosolic proteins Ufd1p and Npi4p to extract proteins from the ER membrane for cytosolic degradation (Figure 2, step 2) (Meyer et al., 2000; Park et al., 2005; Stolz et al., 2011). Poly-Ub chains in membrane protein substrates act as a recognition signal for the Cdc48p/p97 complex. Due to the mechanical force provided by the Cdc48p/p97 complex, certain membrane bound proteins can be dislodged entirely from the membrane and released to the cytosol for degradation by the proteasome (Figure 2, step 4). This has been observed for substrates containing one or more TM segments such as MHC class I heavy chains and unpaired T- Cell receptor subunits. Whether the dislocation of these membrane proteins require a protein conducting channel formed by Sec61p, Derlin1 or the Hrd1p ligase itself is not known (Plemper et al., 1999; Schoebel et al., 2017; Wiertz et al., 1996). Intriguingly, certain membrane proteins were observed to be degraded directly at the ER membrane instead of being released in the cytosol (Smith et al., 2016). In this report, the authors demonstrated that under conditions of proteasome inhibition, the poly-ubiquitinated membrane proteins Hgm2p, Pca1p Ste6* remained stable on the ER membrane instead of being released to the cytosol (Smith et al., 2016). Here, Cdc48p serves to, on the one hand bind poly ubiquitinated proteins on the ER membrane and, on the other hand recruit the proteasome to the ER membrane, to mediate the extraction and immediate degradation of substrates. Such a mechanism would not only increase the efficiency of membrane protein extraction due to the presence of multiple AAA ATPase proteasome subunits but would also prevent the aggregation of substrates through the exposure of hydrophobic residues to the cytosol.

6. Membrane protein degradation from mitochondria

Similar to ERAD, a pathway exists that targets mitochondrial membrane proteins present at both the mitochondrial outer membrane (MOM) and inner membrane (IOM), termed mitochondrial associated degradation (MAD). The mechanism of substrate recognition by MAD is not well-known. The repertoire of MAD substrates that have been identified to date is still limited and currently includes Mfn1p, Mfn2p and Mcl1p in humans and Fzo1p, Mdm34p, Msp1p and Tom70p in yeast (Heo et al., 2010; Tanaka et al., 2010). Mitochondria form a highly dynamic network in the cell and this network is regulated by opposing fusion and fission events (Friedman and Nunnari, 2014; Pernas and Scorrano, 2016). Several key effector proteins of mitochondrial fusion (mitofusins; Fzo1p in yeast and Mfn1p and Mfn2p in humans) and fission (Fis1p, Mffp and Mdv1p in humans) are located at the OM (Song et al., 2015). With their domains exposed at the cytosolic side of the membrane, these proteins are directly accessible by the MAD pathway. By selectively removing fusion or fission components, MAD provides a highly effective level of regulation (Escobar-Henriques and Langer, 2014). Furthermore, MAD likely provides quality control for OM proteins in general as demonstrated by a recent study on the MAD-mediated degradation of nitrosylated OM proteins (Benischke et al., 2014). Future studies would be needed to provide additional insights into the relationship between different mechanisms of OM protein degradation through the UPS.

Like ERAD, the degradation of membrane proteins by MAD requires component of the ubiquitination machinery (E1, E2 and E3 enzymes) for their proteasomal degradation. Several components of the ubiquitination machinery have been implicated in MAD, including the E3 ligases Dma1p, Rsp5p and Mdm30p in yeast and MARCH5/MITOL, MULAN/MAPL and Parkin in mammalian cells (Heo et al., 2010; Kim et al., 2013; Wu et al., 2016; Yoo et al., 2016). Significantly Parkin, a cytosolic E3 ligase, is recruited to the MOM upon mitochondrial stress (Lee et al., 2019). In addition to these E3 ligases, the MAD pathway is potentially modulated by de-ubiquitinating enzymes (DUBs) such as Usp30p in mammals and Ubp16p, Ubp2p and Ubp6p in yeast (Anton et al., 2013; Cunningham et al., 2015; Kinner and Kolling, 2003).

The machinery of the MAD pathway shares some key components with ERAD. For instance, substrate-recruiting factors, namely Vms1p (VMS1 in mammals) and Ufd1p/Doa1p (UFD1L in mammals) are common to both pathways (Heo et al., 2010; Wu et al., 2016). Vms1p and Ufd1p/Doa1p binds Npl4p (NPL4 in mammals) in a mutually exclusive manner. Then, either the Vms1p–Npl4p complex or the Ufd1p/Doa1p–Npl4p complex binds to the N terminus of Cdc48p (Heo et al., 2010; Tran and Brodsky, 2012). Vms1p is recruited to mitochondria mainly under stress conditions, whereas the Doa1p–Cdc48p–Ufd1p–Npl4p complex contributes to basal MAD activity. This indicates that different Cdc48-dependent MAD pathways exist, with potentially distinct substrate specificities and biological functions.

Interestingly, proteasomes were observed to recruit to the surface of stressed mitochondria (Nakagawa et al., 2007; Yoshii et al., 2011) and the localization of Pre6p, a component of the 20S core particle, at the surface of mitochondria further raises the possibility of spatial regulation of proteasome assembly (Heinemeyer et al., 1994).

7. Peroxisomes

Our understanding of ER and mitochondrial membrane protein degradation is in sharp contrast to what is known on peroxisomal membrane protein (PMP) degradation. Peroxisomes are cell organelles present in almost all eukaryotic cells and encompass a multitude of matrix and membrane proteins that participate in various metabolic process/activities, depending on the species and/or cell type (Gabaldon, 2010). The degradation of fatty acids through β -oxidation and detoxification of hydrogen peroxide (H_2O_2) are two widely distributed and well-conserved functions but many more exist. These include plasmalogen and bile acid synthesis in mammals (Ferdinandusse et al., 2009), methanol degradation in yeast (van der Klei et al., 2006), penicillin biosynthesis in fungi (Muller et al., 1991) and the glyoxylate cycle, biosynthesis of isoprenoids and plant hormones and photorespiration in plants (Hayashi and Nishimura, 2003; Li et al., 2005; Mano and Nishimura, 2005). In humans, a defect in peroxisome biogenesis or deficiency in the activity of a single peroxisomal enzyme or transporter protein ultimately leads to serious, often lethal diseases (e.g., Zellweger syndrome and ALD) (Braverman et al., 2016; Waterham et al., 2016). The existence of many such inherited peroxisomal disorders highlights the significance of peroxisomes in human health.

Apart from the diversity in metabolic functions, peroxisomes show remarkable dynamics in morphology, abundance and protein content that vary based on the external cues such as a response to a specific nutrient source (Lingard et al., 2009; Ribeiro et al., 2012; Schrader et al., 2012; Wang et al., 2015). For instance in *S. cerevisiae*, it is well established that the expression of many genes coding for peroxisomal proteins such as metabolic enzymes but also proteins required for peroxisome biogenesis are repressed in the presence of glucose and specifically induced on fatty acids such as oleic acid, ultimately leading to an increase in peroxisome number and size (Gurvitz and Rottensteiner, 2006; Karpichev and Small, 1998; Smith et al., 2002). Proteins that control peroxisome biogenesis are collectively termed as peroxins and encoded by *PEX* genes (Distel et al., 1996; Smith and Aitchison, 2009). More than 35 peroxins have been identified so far and majority of them are peroxisomal membrane proteins (PMPs) which fulfil a variety of functions such as matrix and membrane protein import, metabolite and ion transport, peroxisome inheritance and organelle membrane tethering (Baerends et al., 2000; Schrader and Fahimi, 2008; Smith and Aitchison, 2013). This clearly indicates that PMPs play a crucial role in regulating the processes of peroxisome formation and maintenance. It goes without saying that PMP homeostasis plays a crucial role in peroxisome function. Below we

will briefly discuss how PMPs are imported into peroxisomes, their function in matrix protein import and the underlying effects of cells lacking PMPs.

7.1 PMP import into peroxisomes

Since peroxisomes are devoid of a protein synthesis machinery, all peroxisomal proteins are nuclear encoded, synthesized by poly-free ribosomes in the cytosol (Lazarow and Fujiki, 1985) and post-translationally imported into peroxisomes with the help of a peroxisomal targeting signal (PTS) sequence (Pieuchot and Jedd, 2012). Upon the induction of peroxisome biogenesis, the synthesis and targeting of PMPs was shown to precede that of matrix components (Luers et al., 1990; Veenhuis and Goodman, 1990). The trafficking of PMPs to peroxisomes depend on mPTS, an arbitrary membrane targeting signal present in the N-terminal (Pex3p), C-terminal (Pex15p) or in an internal (PMP47) position within the PMP sequence (Baerends et al., 1996; Dyer et al., 1996; Elgersma et al., 1997). Although the mechanisms of PMP import are not well understood, three peroxins - Pex3p, Pex19p and Pex16p - have been implicated in the targeting and insertion of PMPs into the peroxisomal membrane (Liu et al., 2016). While Pex3p and Pex19p are present in all organisms, Pex16p has been identified only in mammalian cells, *Yarrowia lipolytica*, *Arabidopsis thaliana* and *Pencillium chrysogenum* (Kiel et al., 2006; Kim and Mullen, 2013; Opalinski et al., 2012). Generally, PMPs synthesized in the cytosol are recognized by the cytosolic chaperone Pex19p, the Pex19p-PMP complex then docks at the peroxisomal membrane through the interaction of Pex19p with Pex3p which then facilitates the insertion of PMPs in the peroxisome by a yet unknown mechanism. PMPs targeted by this way are referred to as Class I PMPs (Imanaka et al., 1996). Alternatively, Class II PMPs may traffic via the ER and subsequently insert into the peroxisomes independently of Pex19p (Thoms et al., 2012; Yonekawa et al., 2011). Several PMPs including mammalian Pex3p (Mayerhofer et al., 2016), Pex2p (RING E3 ligase) and Pex16p in *Y. lipolytica*, have been suggested to target to peroxisomes via the ER (van der Zand et al., 2010), although there exists much debate on this topic (Knoops et al., 2014; Otzen et al., 2004; Wroblewska et al., 2017). The detection of PMPs at the ER was performed in *pex3* cells which were assumed to completely lack peroxisomes (Baerends et al., 1997). However, recent observations in *H. polymorpha pex3* and *pex19* cells as well as in *S. cerevisiae pex3* cells indicates the presence of pre-peroxisomal vesicles (PPVs) harbouring a subset of PMPs (including Pex8p, Pex13p, Pex14p, Pex15p, Pex17p, Pex25p and Pex22p (Knoops et al., 2014; Otzen et al., 2004; Wroblewska et al., 2017). Thus, it is not clear whether these proteins target to peroxisomes via the ER or via a different mechanism.

7.2 Peroxisomal matrix protein import

Like membrane proteins, the transport of peroxisomal matrix proteins are mediated by specific import sequences (Brocard and Hartig, 2006; Lazarow, 2006). However, matrix proteins utilize a sorting machinery distinct from that used for PMP import. Proteins destined for the

peroxisomal matrix contain a C-terminal (tripeptide) PTS1 sequence (Swinkels et al., 1992) or an N-terminal (nonapeptide) PTS2 sequence (Lazarow, 2006; Petriv et al., 2004). After synthesis, most matrix proteins containing a PTS1 are recognized by Pex5p (Gatto et al., 2000), while PTS2 proteins are recognized by Pex7p (Lazarow, 2006), which functions together with the co-receptor proteins Pex18p/Pex21p in *S. cerevisiae*, Pex20p in *H. polymorpha* and fungi species and the long form of Pex5p (Pex5L) in mammals/higher eukaryotes (Schliebs and Kunau, 2006). Recently Pex9p, a new PTS receptor was identified and was shown to import a subset of PTS1-containing proteins such as malate synthase 1 and 2 under certain conditions (Effelsberg et al., 2016). In addition to the cytosolic PTS receptors mentioned above, matrix protein import is mediated by several PMPs (Pex13p, Pex14p and in yeast Pex17p) that collectively form the matrix protein import machinery, which is responsible for docking of the cargo-bound import receptor at the peroxisomal membrane (Eckert and Erdmann, 2003) and translocation of cargo into the peroxisomal lumen by a dynamic translocation pore (Meinecke et al., 2010). Generally, receptors are recycled back to the cytosol after cargo release through the action of several membrane bound E3 ligases (Pex2p, Pex10p and Pex12p), together with an E2 enzyme Pex4p that is bound to the peroxisomal membrane via its interaction with the PMP Pex22p (Ali et al., 2018; El Magraoui et al., 2014). Recycling of the receptor involves ubiquitylation (Platta et al., 2007) and extraction from the membrane by an AAA-type ATPase complex consisting of the proteins Pex1p and Pex6p (Platta et al., 2005). This method of recycling resembles, to a certain extent, the degradation of misfolded proteins by the ERAD quality control machinery (Schliebs et al., 2010).

Mutations or absence of any of the PMPs mentioned above can have a significant impact on protein import to peroxisomes and the underlying metabolic functions. For instance, loss of either one of the docking complex PMPs (Pex13p, Pex14p or Pex17p) abolishes PTS1 and PTS2 protein import and renders peroxisomal metabolic pathways inactive (Azevedo and Schliebs, 2006; Williams and Distel, 2006). Cells lacking Pex3p, which is involved in early peroxisome biogenesis and PMP import, are devoid of functional peroxisomes and characterized by the presence of small vesicle like structures (Knoops et al., 2014; Wroblewska et al., 2017). Since peroxisome proliferation is controlled by the Pex11p family of proteins, the loss of these PMPs results in cells with a reduced number of peroxisomes (Schrader and Fahimi, 2006a). Furthermore, mutations in the gene encoding for peroxisomal ABC transporter (ABCD1) results in protein instability leading to loss of fatty acyl-coA transport to peroxisomes and thereby reduced β -oxidation activity (Cartier et al., 1995; Engelen et al., 2014). This clearly demonstrates that PMPs play a crucial role in regulating peroxisomal functions but also in controlling the processes of peroxisome formation and maintenance. Due to this vital role, there must be a tight control between PMP synthesis, import and degradation. While the first two have been the subject of many studies, little is currently known on PMP degradation.

8. Membrane protein degradation from peroxisomes

Since peroxisomes generate large amount of ROS (Schrader and Fahimi, 2006b), a known cause of damage to proteins, it is highly likely that PMPs residing in this ROS rich environment are potentially vulnerable to oxidative damage (Figure 3, left). Furthermore, because PMPs play vital roles in regulating the wide range of peroxisomal functions, it can be imagined that targeted down-regulation of certain PMPs may exert control over such functions (Figure 3, right). Therefore, investigating the degradation of individual PMPs would allow us to understand how PMP homeostasis is regulated, to what extent PMP degradation is involved in regulating peroxisome function and what would be the impact of inhibiting PMP degradation on peroxisome function. For this reason, a pathway to selectively degrade damaged or redundant PMPs, would be an invaluable asset, particularly when the alternative, wholesome destruction of peroxisomes by pexophagy (Eberhart and Kovacs, 2018), may not be desirable. With this in mind, it is surprising that until relatively recently, PMP degradation has received very little attention. In this following section, we shall first sum up the current knowledge on the peroxisomal ubiquitination machinery and its role in ubiquitinating peroxisomal proteins. Then, we will provide an overview on PMPs which are reported to be degraded and further elaborate on the PMPs that are suggested to undergo degradation.

Figure 3: Model depicting possible functions of PMP turnover in quality control and targeted degradation. (Left) Reactive oxygen species (ROS) such as hydrogen peroxide produced in the peroxisome lumen could cause damage to PMPs resulting in protein misfolding and possibly loss in functionality.

Misfolded/unfolded PMPs can then be selectively recognized and ubiquitinated by the ubiquitination machinery (E2 and E3), extracted from the peroxisomal membrane and targeted for proteasomal degradation. (Right) Upon a signal from an event occurring at the peroxisome, functional PMPs could also undergo Ub-proteasome dependent degradation to regulate various peroxisomal process.

8.1 Insights from PTS receptor recycling and degradation

The PTS receptors Pex5p and Pex20p family co-receptor proteins (Pex18p and Pex20p) were shown to undergo ubiquitination after delivering matrix proteins to peroxisomes (Liu and Subramani, 2013; Platta et al., 2008; Purdue and Lazarow, 2001). Depending on the nature of ubiquitination, these receptor proteins promote two different outcomes- receptor recycling or degradation. For instance, Pex5p and co-receptors Pex18p and Pex20p harbouring single Ub on their conserved cysteine residues near the N-termini of these proteins are recycled back to the cytosol, allowing the receptors to take part in further rounds of import (Hensel et al., 2011; Kiel et al., 2005; Leon and Subramani, 2007; Platta et al., 2007; Williams et al., 2007). Alternatively, when receptor proteins are unable to recycle properly either due to a mutation in their conserved cysteine residues or lack of recycling machinery components, these peroxisomal proteins were shown to undergo poly-ubiquitination on one or more conserved lysine's (Hensel et al., 2011; Kiel et al., 2005; Platta et al., 2007). The available evidence suggests that poly-ubiquitination serves a quality control function, priming receptors that are unable to recycle for proteasomal degradation (Kiel et al., 2005; Leon et al., 2006; Williams and Distel, 2010).

Figure 4: Model depicting the steps of PTS receptor recycling and degradation in *S. cerevisiae*. Matrix proteins harbouring a PTS1 or PTS2 are recognized by the cytosolic receptors Pex5p and Pex7p/Pex18p/Pex21p

respectively. Receptor bound matrix proteins first associate with the docking complex (Pex13p/Pex14p/Pex17p) at the peroxisomal membrane (PM), followed by the translocation of the matrix proteins into peroxisome lumen (PL). After cargo release, (co) receptor proteins are ubiquitinated by the peroxisomal ubiquitination machinery (Pex12p and Pex4p) and recycled by the AAA complex (Pex1p/Pex6p) back to the cytosol. Alternatively, PTS receptors are poly-ubiquitinated by E3s (Pex2p/Pex10p) and E2s (Ubc4p/Ubc5p) and targeted for proteasomal degradation.

Peroxisomal receptor ubiquitination requires the typical ubiquitination enzymes- E1, E2 and E3. Pex4p, the E2 enzyme together with its peroxisomal anchor protein Pex22p promotes mono-ubiquitination of Pex5p (in *S. cerevisiae* and *Arabidopsis*) (Figure 4) and Pex20p (in *Pichia pastoris*) (El Magraoui et al., 2014; Liu and Subramani, 2013; Williams et al., 2012; Williams et al., 2007) whereas the cytosolic protein Ubc4p (but also Ubc5p) in *S. cerevisiae* functions as the E2 enzyme in the poly-ubiquitination of Pex5p and Pex18p (Figure 4) (El Magraoui et al., 2013; Kiel et al., 2005). In mammals, members of the E2D (UbCH5/Ube2D) family of E2s were shown to regulate both the recycling (mono-ubiquitination) and probably degradation (poly-ubiquitination) of Pex5p (Grou et al., 2008; Okumoto et al., 2014). The three PMPs Pex2p, Pex10p and Pex12p also referred as RING peroxins that exhibit E3 ligase activity (El Magraoui et al., 2012) facilitate Ub attachment to the (co-) receptor proteins. In *S. cerevisiae*, each E3 ligase is assigned a distinct function for either mono-ubiquitination or poly-ubiquitination of Pex5p and Pex18p (Figure 4), whereas in *P. pastoris*, all three E3 ligases are essential for both mono-ubiquitination and poly-ubiquitination of Pex5p and Pex20p (El Magraoui et al., 2013; Liu and Subramani, 2013). The roles of the RING peroxins in ubiquitination of Pex5p were first determined in *S. cerevisiae* *in vitro* where the authors showed that Pex12p is required for Pex5p mono-ubiquitination (Figure 4), whereas Pex2p and/or Pex10p is required for Pex5p poly-ubiquitination (Figure 4) (Platta et al., 2009; Williams et al., 2008b). In *Arabidopsis*, the Ub domain-containing proteins DSK2a and DSK2b interact with PEX2 and PEX12 and may help shuttle poly-ubiquitinated proteins to the proteasome for degradation (Kaur et al., 2013).

Once the receptor proteins are ubiquitinated by the peroxisomal ubiquitination machinery, they are extracted from the peroxisomal membrane in an ATP dependent manner through the action of two AAA proteins, Pex1p and Pex6p (Schwerter et al., 2017) which are anchored to the peroxisomes by Pex15p in yeast (Figure 4) (Birschmann et al., 2003) or Pex26p in mammals (Matsumoto et al., 2003). In *S. cerevisiae* it is reported that the Pex1p/Pex6p complex facilitates the extraction of both mono and poly-ubiquitinated Pex5p (Figure 4) (Platta et al., 2008). During the membrane extraction process, the deubiquitinating enzyme Ubp15p in *S. cerevisiae* (Debelyy et al., 2011) and USP9X in mammals (Grou et al., 2012) is required to remove ubiquitin from mono-ubiquitinated Pex5p (and likely Pex20p family members), which then allows the receptor to take part in another round of import (El Magraoui et al., 2019). Unlike the yeast Pex5p, whose levels were stabilized in *pex6* mutants, Pex5p levels in human fibroblast

and *P. pastoris pex6* mutants were observed to be reduced (Chang et al., 1999; Collins et al., 2000). This would suggest that alternative mechanisms exist to remove poly-ubiquitinated proteins from the peroxisomal membrane in humans and *P. pastoris* and target them for degradation. To this end, Law et al., showed that loss of AAA-complex in humans result in the accumulation of ubiquitinated Pex5p on the peroxisomal membrane that signals pexophagy, thereby suggesting an additional role for AAA proteins in preventing pexophagy, additional to its role in receptor recycling (Law et al., 2017).

Like the PTS1 receptor Pex5p, the PTS2 co-receptor Pex7p was shown to undergo UPS-mediated degradation in the yeast *P. pastoris* (Hagstrom et al., 2014) and in humans (Miyauchi-Nanri et al., 2014). Interestingly, the peroxisomal ubiquitination machinery had no role in Pex7p degradation instead, the cytosolic E3 ligase complex CRL4A (Cullin4A-RING Ub E3 ligase) was required for Pex7p ubiquitination/degradation in humans. In both cases, the authors reported that non-functional Pex7p was targeted for degradation, defining these degradation events as quality control related. These examples demonstrate that the UPS, particularly the peroxisomal ubiquitination machinery, but also non-peroxisomal proteins, function in the quality control of receptor proteins by ubiquitinating them and targeting them for proteasomal degradation. Furthermore, the PMPs needed for (co) receptor ubiquitination and retro translocation resemble ERAD components (Schliebs et al., 2010), suggesting that damaged peroxisomal proteins may be retro translocated out of the peroxisome and degraded in the cytosol by the 26S proteasome.

8.2 Lessons from matrix protein degradation

Three mechanisms of peroxisomal matrix protein quality control has been proposed so far (i) degradation of the proteins in the peroxisome lumen by proteases (Aksam et al., 2007; Bartoszewska et al., 2012) (ii) degradation of the whole organelles by pexophagy (Eberhart and Kovacs, 2018) and (iii) retro-translocation of matrix proteins from peroxisomes followed by cytosolic degradation, a process similar to the ERAD pathway and called peroxisome-associated matrix protein degradation (PexAD) (Lingard et al., 2009). Several studies performed in *Arabidopsis* demonstrate a role for PexAD pathway in the degradation of Isocitrate lyase (ICL) and malate synthase (MLS). ICL and MLS are peroxisomal glyoxylate cycle enzymes that enable carbon from acetyl-CoA to be utilized in gluconeogenesis, thus providing energy for germinating seedlings (reviewed in (Graham, 2008)). A few days after germination, when seedlings are able to grow photo-autotrophically, ICL and MLS are degraded as they are no longer required (Lingard et al., 2009; Zolman et al., 2005) and peroxisomes instead function in the photo respiratory pathway. Here, the removal of obsolete matrix proteins such as ICL and MLS by the PexAD pathway (Figure 5, adapted from (Williams, 2014)) acts as a mechanism to adapt their protein content to coincide with the development progression. The E2 Pex4p and its peroxisomal anchor Pex22p were shown to be

required for ICL degradation (Zolman et al., 2005) (Figure 5). In addition, the degradation of ICL and MLS was inhibited in cells disrupted for the import receptor Pex5p or the ATPase Pex6p (Lingard et al., 2009). Significantly, these enzymes were stabilized in the cytosol in the *pex5* mutant but localized to the peroxisomes in the *pex6*. These data indicate that import is a pre-requisite for degradation and also that the degradation event does not occur inside peroxisomes. Interestingly a study by Farmer et al., showed that the deletion of LON2 in autophagy mutants increased the stability of ICL and MLS, suggesting a role for pexophagy as well as LON2 in matrix protein turnover (Farmer et al., 2013). The authors suggested that LON2 could partially disaggregate matrix proteins, to allow their ubiquitin-dependent retro translocation out of the organelle. Thus, in the absence of LON2, ubiquitinated matrix proteins that are not able to retro translocate efficiently might accumulate, which in turn may trigger pexophagy. Alternatively, LON2 could function in recognizing the substrates and targeting them to the peroxisomal ubiquitination machinery (Williams, 2014). Either way, future studies will be needed to resolve how the degradation of MLS and ICS is achieved.

Figure 5: Model of isocitrate lyase (ICL) and malate synthetase (MLS) degradation by the Peroxisome-associated matrix protein degradation (PexAD) pathway in Arabidopsis. Influenced by peroxisomal metabolism and/or possibly hydrogen peroxide damage (1), Lon protease could facilitate ICL/MLS targeting to

the export machinery (2). Next, ICL and MLS ubiquitination is mediated by the peroxisomal E2 Pex4p, probably with the aid of the peroxisomal E3 ligases Pex2p, Pex10p and Pex12p (3). Ubiquitinated ICL and MLS is then recognized by Pex6p and exported to the cytosol (4), where degradation occurs, most likely through the action of the proteasome (5). Defects in the proteins shown in reddish yellow are known to inhibit ICL/MLS degradation while the role for other proteins (depicted in grey) on ICL/MLS turnover is currently unknown.

8.3 What we know about PMP degradation

As mentioned already, PMP degradation has not been well explored. In the next sections, we will present what is currently known on this topic and we will elaborate on both the (suspected) mechanisms as well as the functions of these degradation events.

8.3.1 Pex3p

One of the first reports on peroxisomal membrane protein (PMP) degradation concerns Pex3p in the yeast *H. polymorpha* (Bellu et al., 2002). Pex3p plays a critical role in peroxisome formation by functioning as a docking factor for Pex19p (Fang et al., 2004), the cycling receptor that binds and transports newly synthesized PMPs to peroxisomes (Sacksteder et al., 2000). In addition to its role in peroxisome biogenesis, Pex3p functions in peroxisome inheritance (Munck et al., 2009) and the selective degradation of peroxisomes, a process referred to as pexophagy (Eberhart and Kovacs, 2018). Incubating the methylotrophic yeasts *H. polymorpha* and *P. pastoris* in methanol or the budding yeast *S. cerevisiae* in oleic acid induces peroxisome proliferation, whereas pexophagy is triggered when methanol or oleic acid grown cells are shifted to glucose, ethanol or nitrogen-depleted starvation medium (Bellu et al., 2002; Farre et al., 2008; Motley et al., 2012; Oku and Sakai, 2016). Under these conditions, peroxisomal metabolism is no longer critical for viability and cell growth, resulting in the degradation of peroxisomes by the vacuole. This process in *H. polymorpha* is initiated by the removal of Pex3p from peroxisomes by the proteasome system (Bellu et al., 2002; Williams and van der Klei, 2013). Interestingly, Pex3p removal was shown to also induce peroxisome degradation under peroxisome proliferating conditions (van Zutphen et al., 2011). Since peroxisomes are involved in various oxidative reactions such as methanol catabolism which produce ROS, they are prone to oxidative damage (Walker et al., 2018). As dysfunction of yeast peroxisomes may result in necrotic cell death, timely recognition and constitute turnover of damaged peroxisomes is of crucial importance in cell vitality. This demonstrates that Pex3p dependent pexophagy is essential for cellular housekeeping as it triggers the removal of damaged or redundant peroxisomes.

The role for the proteasome in Pex3p turnover came from the observation that Pex3p degradation in cells shifted from methanol to glucose was inhibited by the treatment of the proteasome inhibitor MG132 (Bellu et al., 2002). The involvement of the proteasome further suggests a role for ubiquitination in Pex3p turnover. To this end, Pex3p was shown to be

ubiquitinated and mutating lysine residues in Pex3p to arginines or deleting the genes encoding for peroxisomal RING E3 ligases Pex2p and Pex10p blocked Pex3p degradation and hence glucose induced pexophagy (Williams and van der Klei, 2013), indicating a role for these proteins in degrading PMPs. However, unlike Pex5p, the AAA ATPase Pex1p was not required for Pex3p degradation (Williams and van der Klei, 2013) suggesting that there could be other AAA proteins that act to remove Pex3p from peroxisomes. Collectively, these results demonstrate that *H. polymorpha* Pex3p is degraded by a process that is dependent on the peroxisomal ubiquitin machinery and the proteasome. Currently, it is unclear if a similar requirement of Pex3p removal from peroxisome membrane to initiate pexophagy exists in *P. pastoris* or in *S. cerevisiae*, where Pex3p is shown to recruit the pexophagy receptor Atg30 and Atg36 to the peroxisome respectively (Farre et al., 2008; Motley et al., 2012). Interestingly, Pex3p ubiquitination and degradation is also observed in mammals and pexophagy is initiated only upon Pex3p overexpression (Yamashita et al., 2014). However, blocking Pex3p ubiquitination through introduction of mutations in lysine residues of Pex3p had no effect on peroxisome degradation (Yamashita et al., 2014) suggesting that unlike yeasts, ubiquitination of Pex3p is dispensable for pexophagy in mammals.

8.3.2 Pex13p

The PMPs Pex13p, Pex14p (and Pex17p in yeast) form a docking complex that functions in importing proteins destined for the peroxisomal matrix (Williams and Distel, 2006). Loss of either of the PMPs results in the absence of a functional docking complex, leading to a defect in matrix protein import. Recently, Pex13p from *H. polymorpha* was shown to be ubiquitinated and degraded by a process that is dependent on the peroxisomal E2 Pex4p and the peroxisomal RING E3 ligase Pex2p (Figure 6A) (Chen et al., 2018). Intriguingly, Pex13p degradation was only partially inhibited in cells lacking Pex4p, suggesting a role for another E2 enzyme in Pex13p turnover. While these proteins affected the degradation of Pex13p, how *H. polymorpha* Pex13p is recognized, extracted from the peroxisomal membrane and targeted for degradation is not clear at present (Figure 6A, steps 1 & 3). Furthermore, the reason why Pex13p is degraded remains unknown. However, considering its function it is likely that Pex13p turnover might play a role in matrix protein import. In this light, an interesting comparison can be drawn from the recent studies on *Arabidopsis* Pex13p, which was shown to be degraded by SP1 (Figure 6B, left) (Pan et al., 2016). SP1, a RING-type E3 ligase regulates chloroplast biogenesis by binding the components of the TOC protein import complex and targeting them for degradation (Ling et al., 2012). In addition to chloroplasts, SP1 associates with the peroxisomal membrane and interacts with Pex13p (Pan and Hu, 2018). The authors showed that SP1 affected the stability of Pex13p in a manner that is dependent on its catalytic RING domain thus suggesting a role for SP1 in Pex13p ubiquitination and degradation (Pan et al., 2016). Furthermore, the deletion of SP1 in *pex14* cells, which are defective in matrix protein targeting, not only inhibited Pex13p

degradation, but also resulted in an increase in peroxisomal β -oxidation activity due to the increased import of matrix proteins (Pan et al., 2016). These data strongly suggest that SP1 negatively regulates peroxisomal matrix protein import by down-regulating import complex proteins Pex13p (Figure 6B, right) and possibly Pex14p on the peroxisomal membrane. Like *Arabidopsis*, Pex13p in *H. polymorpha* could undergo targeted degradation to regulate matrix protein import. Alternatively, Pex13p can also be degraded by the peroxisomal ubiquitination machinery for quality control purposes, possibly to remove damaged or incorrectly folded Pex13p. Since Pex13p degradation has already been reported in plants and the yeast *H. polymorpha*, it would be interesting to investigate if Pex13p turnover is conserved across various yeast species as well as humans.

A

B

Figure 6: A) A working model for Pex13p degradation in *H. polymorpha*. Pex13p is recognized for degradation by an unknown mechanism (1) and undergoes ubiquitination mediated by the E3 ligase Pex2p and the E2 Pex4p (2). Ubiquitinated Pex13p is extracted from the peroxisome in an unknown manner (3) and targeted for proteasomal degradation (4). Inhibiting the function of proteins shown in red/yellow blocks Pex13p ubiquitination and degradation while proteins denoted in grey are thought to function in Pex13p turnover but their role is currently unknown.

B) Model of Pex13p degradation in Arabidopsis. (Right) Pex13p acts as a docking complex protein that facilitates PTS receptor-cargo complex to dock at the peroxisomal membrane followed by the translocation of cargo into peroxisome lumen. PTS receptors are then recycled back to the cytosol by a process dependent on the peroxisomal ubiquitination machinery (E2 and E3) and Pex1p/Pex6p. (Left) The E3 ligase SP1 present at the peroxisome promotes ubiquitination and proteasomal degradation of Pex13p which negatively regulates protein import possibly by acting at the docking step and inhibiting the docking of receptor-cargo proteins (indicated by red arrow) and the underlying steps in matrix protein import.

8.3.3 Pex15p

While the degradation of the PMPs Pex3p and Pex13p likely play a role in regulating peroxisomal activities, the degradation of the tail anchored (TA) peroxisomal membrane protein Pex15p by the AAA-ATPase Msp1 serves as a first example of PMP quality control in this yeast. The PMP Pex15p, which anchors the AAA proteins (Pex1p and Pex6p) to the peroxisomes, is involved in the recycling of matrix proteins receptor back to the cytosol (Birschmann et al., 2003). Msp1p on the other hand, consists of an N-terminal transmembrane (TM) anchor followed by a C-terminal cytoplasmic ATPase domain and is localized mainly in the mitochondrial outer membrane (OMM) but also partially in the peroxisomal membrane (Nakai et al., 1993; Okreglak and Walter, 2014). Several studies have shown that Msp1p is involved in removing incorrectly targeted TA proteins such as the PMP Pex15p lacking the peroxisomal targeting signal of the C-terminal 30 residues (Pex15 Δ 30) that is mislocalized to mitochondria (Figure 7A) (Okreglak and Walter, 2014; Weir et al., 2017; Wohlever et al., 2017). Like other AAA-ATPases, Msp1p was proposed to be involved in the extraction of mistargeted Pex15 Δ 30 from the OMM and target them for degradation in the cytosol. However a recent study showed that Pex15 Δ 30 is first recognized by Msp1p in the outer mitochondrial membrane (Figure 7A, steps 1 & 2), then targeted to the ER (Figure 7A, step 3) and ubiquitinated by Doa10p (ER embedded E3 ligase) and its co-factors (Ubc6p and Ubc7p) (Matsumoto et al., 2019) (Figure 7A, step 4). Subsequently, the Cdc48-Ufd1-Npl4 complex mediates the extraction of ubiquitinated Pex15 Δ 30 present at the ER membrane for proteasomal degradation in the cytosol (Figure 7A, steps 5 & 6). It is not clear why only Cdc48p and not Msp1p can extract Pex15 Δ 30 and target it for degradation. One possible explanation could be that substrate extraction and unfolding by Cdc48p may be closely coupled with proteasomal processing (Bodnar and Rapoport, 2017). In contrast to Msp1p-mediated clearance of Pex15p that relies on the ER-associated degradation (ERAD) system, a study by Dederer et al., showed

that Msp1p and Doa10p acts in parallel to degrade Pex15 Δ 30 (Dederer et al., 2019). Here, Doa10p was suggested to play a major role in Pex15 Δ 30 degradation while Msp1p deals only with a fraction of Pex15 Δ 30 that is mistargeted to mitochondria. Because the authors followed the steady protein levels and not the rate of Pex15 Δ 30 turnover, it is plausible that in cells lacking Msp1p or Doa10p, Pex15 Δ 30 degradation is inhibited to a similar extent, suggesting that Msp1p and Doa10p act in the same pathway as proposed by (Matsumoto et al., 2019). Nevertheless, it is clear that Pex15 Δ 30 degradation is mediated by the ubiquitin proteasome system (UPS) which involves Msp1p, Doa10p and Cdc48p complex. Most importantly, these examples demonstrate that PMP quality control pathways exists in yeast and acts to remove mislocalized or redundant PMPs. Similar to the yeast Msp1p, its human homolog ATAD1 also localizes to both mitochondria and peroxisomes and has been shown to remove mislocalized TA proteins such as Pex26p (Chen et al., 2014).

Intriguingly Msp1p localized to the peroxisomal membrane functions in degrading peroxisomal Pex15p when present in excess of its interacting partner Pex3p (Weir et al., 2017) (Figure 7B). Although the exact mechanism and additional factors involved in Pex15p degradation remain unknown, the authors suggested that Pex3p binding to Pex15p rapidly converts Pex15p from a Msp1p-susceptible to a Msp1p-resistant form and protects it from degradation by Msp1p (Figure 7B). Together, a role for Msp1p/ATAD1 in TA membrane protein (Pex15p or Pex26p) quality control is clearly known. Considering that Msp1p recognizes the substrate TA membrane proteins such as Pex15p via their exposed hydrophobic core residues that are normally buried inside (Li et al., 2019), it is possible that Msp1p might also be involved in the general quality control of other peroxisomal membrane proteins.

Figure 7: A) Model of Msp1p-mediated clearance of the mitochondrially mistargeted Pex15p mutant (Pex15 Δ 30). The Pex15p mutant is first recognized by Msp1p, which mediates the extraction from the mitochondrial membrane and targets the mistargeted Pex15p to the ER. Within the ER membrane, Pex15p is ubiquitinated by a process dependent on Doa10p and Ubc6p/Ubc7p, extracted by the Cdc48p complex and released into cytosol for proteasomal degradation.

B) Model of Msp1p-mediated degradation of peroxisomal Pex15p. Following insertion into peroxisomes, nascent Pex15p can be recognized by Msp1p however, this requires either Pex15p and/or Msp1p to be present above their usual levels. The interaction of Pex3p with Pex15p promotes maturation of nascent Pex15p and blocks recognition and degradation by Msp1p (indicated by red line).

8.3.4 ALDP

Like yeast, the role for quality control in humans has been suggested for the PMP Adrenoleukodystrophy (ALD) protein (ALDP). The involvement of peroxisomes in metabolic pathways such as fatty acid β -oxidation (Wanders and Waterham, 2006) necessitate the transport of metabolites in and out of peroxisomes. Peroxisomes possess specialized transport systems such as ATP-binding cassette (ABC) transporters (Theodoulou et al., 2006), which are mainly involved in trafficking various compounds across membranes, ranging from ions to peptides. ABC transporters are found either as complete or half transporters which can homo- or hetero dimerize to form an active transporter (Liu et al., 1999). In mammals, four half-ABC transporter superfamily members belonging to the subclass D have been identified in the peroxisomal membrane and they include ALDP, an ALD-related protein (ALDRP), a 70-kDa PMP (PMP70) and the PMP70-related protein (PMP70R) (Baker et al., 2015; Theodoulou et al., 2006; Wanders et al., 2007). Of these transporters, ALDP has been studied extensively due to its role in ALD, a human disorder caused by the mutations in the ALDP encoding gene ABCD1 (Kemp et al., 2011; Mosser et al., 1993). ALDP is suggested to be involved in the import of CoA esters of very-long-chain fatty acyl-coA (VLCFA) into the peroxisome (*van Roermund et al., 2008*), in a similar fashion as performed by their yeast homologs Pxa1p/Pxa2p in *S. cerevisiae* (Hettema et al., 1996; Shani et al., 1996). To date, more than 340 non-recurrent missense mutations of the ABCD1 gene have been identified (<https://adrenoleukodystrophy.info/>). In these missense mutations, approximately 70% of the ABCD1/ALDP proteins are either not detected or reduced in amount in ALD fibroblasts (Kemp et al., 2001; Takahashi et al., 2007). As a result of this lack of ALDP, patients suffering from ALD fail to oxidize VLCFAs, leading to the accumulation of fatty acids in plasma and tissues (Engelen et al., 2014). Until now neither the mechanism of instability nor the proteases responsible for degradation of the mutant proteins have not been fully characterized. Recently, Takahashi et al., reported that the degradation of mutant ALDP protein was partially inhibited by treatment with MG132, suggesting that these ALDP mutants are degraded via the proteasome (Takahashi et al., 2007). Interestingly, a subset of unstable ALDP mutant proteins in patient cells became stable under low-temperature culture conditions and exhibited proper peroxisomal localization (Morita et al., 2019) while under these conditions patient cells displayed increased residual β -oxidation activity (Zhang et al., 2011). Some of the ALDP mutants which were rapidly degraded *in vivo* still possessed ATP hydrolysis activity *in vitro* (Roerig et al., 2001), indicating that mutant forms of ALDP retain a certain degree of functionality.

8.4 Potential PMP substrates of the UPS/PMP degradation pathway

Several reports in the literature strongly suggest that in addition to the PMPs discussed above, there are other PMPs that may undergo degradation via the UPS.

8.4.1 Inp1p/Inp2p

The budding yeast *S. cerevisiae* undergoes asymmetrical division, where a portion of its organelles, including peroxisomes, are delivered to the bud while the remaining organelles are retained in the mother cell (Fagarasanu and Rachubinski, 2007). Inp1p and Inp2p have important but opposite roles during peroxisome partition (Fagarasanu et al., 2010). While Inp1p associates with Pex3p and functions in tethering peroxisomes to the cortical ER in the mother cell (Knoblauch et al., 2013), Inp2p acts as an adaptor that mediates peroxisome recruitment by the myosin Myo2p for transport to the daughter cells. A fine balance between Inp1p and Inp2p is therefore required to ensure the correct partitioning of peroxisomes between mother and daughter cells. Fagarasanu et al., showed that the levels of Inp2p fluctuate during the cell cycle (Fagarasanu et al., 2006). Similar to Inp2p, Inp1p levels also varied during the cell cycle (Fagarasanu et al., 2005), which could suggest that both Inp1p and Inp2p undergo degradation. Nevertheless, little is known about the cellular abundance of Inp1p or Inp2p and their role in peroxisome inheritance (Fagarasanu et al., 2005).

Inp2p levels were observed to be maximal during peroxisome inheritance and decreased later in the cell cycle when around half of the peroxisomes have been delivered to the bud (Fagarasanu et al., 2006). This could indicate that Inp2p undergoes degradation at later stages in peroxisome inheritance and could possibly function in terminating peroxisome motility. Evidence for Inp2p degradation comes from data on *myo2* mutants that are defective in binding and transporting peroxisomes, producing buds devoid of peroxisomes. Unlike wild-type cells which exhibit fluctuation in the levels of Inp2, an overall increase in Inp2p levels was observed at all stages of cell cycle in *myo2* mutants (Fagarasanu et al., 2009). The higher levels of Inp2p was not due to increased synthesis but rather reflected a result of reduced degradation. Since Inp2p can only be detected on bud-localized peroxisomes in wild-type cells (Fagarasanu et al., 2006) it is possible that Inp2p undergoes degradation in the bud. However based on this data, together with the data on *myo2* mutants where Inp2p is predominantly localized to mother cells (Fagarasanu et al., 2009), Knoblauch et al., proposed that the Inp2p degradation machinery could be present in both the bud and the mother cell, the activation of which would cause the release of transferred peroxisomes in bud and prevent additional recruitment of peroxisomes from the mother cell.

The degradation of Inp2p to terminate peroxisome movement to daughter cells agrees with current models for the inheritance of other organelles, such as yeast vacuoles. Vac17p, the vacuole-specific receptor for Myo2p, undergoes degradation following ubiquitination mediated by a novel E3-ubiquitin ligase Dma1p and its paralogue Dma2p (Tang et al., 2003; Yau et al., 2014), to release the vacuole in the bud. Interestingly, Dma1p and Dma2p are also required for peroxisome movements in yeast (Yau et al., 2014). Furthermore a PEST motif, a protein domain consisting mainly of the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T)

that mediates Vac17p degradation (Tang et al., 2003) is also present in Inp2. Whether PEST sequences, the proteasome and ubiquitination machinery proteins such as E3- ligase Dma1p and Dma2p play a role in Inp2p turnover would be interesting to investigate.

8.4.2 Pex14p

In addition to the docking protein Pex13p, which was shown to be ubiquitinated and degraded by the UPS, ubiquitinated peptides of Pex14p have been detected in *S. cerevisiae* and human cells (Kim et al., 2011; Mayor et al., 2007; Pan et al., 2016). Pex14p, a Pex13p interacting partner and docking complex protein (Azevedo and Schliebs, 2006; Urquhart et al., 2000), serves as a first site of contact for peroxisomal receptor proteins at the peroxisomal. In *H. polymorpha*, the levels of non-phosphorylated form of Pex14p were reduced in the cells deleted for *PMP20* (Bener Aksam et al., 2008) while the phosphorylated form of Pex14p remained unaffected in this mutant. PMP20 is a peroxiredoxin involved in the degradation of H₂O₂ and the absence of Pmp20p results in enhanced oxidative stress in cells (Aksam et al., 2008). Such an increase in oxidative stress could result in damage to PMPs such as Pex14p, possibly by post-translation modifications, that could lead to protein misfolding and target them for degradation. Interestingly, Pex14p levels appear increased in cells lacking one of the peroxisomal E3 ligases Pex2p, Pex10p or Pex12p or the E2 enzyme Pex4p (Chen et al., 2018; Koek et al., 2007; van der Klei et al., 1998). The increased stability of Pex14p in these mutants may indicate that Pex14p degradation is inhibited. This also suggests that like *pmp20* mutants, Pex14p in wild-type cells might be damaged or incorrectly folded and thereby be targeted for quality control purposes. It is highly likely that the presence of damaged or redundant PMPs, when not removed from peroxisomes, could result in aggregation of misfolded PMPs. Whether UPS play a role in the reduced levels of non-phosphorylated Pex14p needs to be determined.

9. Perspectives

The metabolic functions of peroxisomes are largely determined by protein content – which matrix and peroxisomal membrane proteins (PMPs) reside in peroxisomes. PMPs regulate most aspects of peroxisome biology and for the peroxisomes to function effectively, PMPs homeostasis is essential - this includes the regulation of PMP targeting as well as PMP quality control processes and degradation. We currently have a good idea which proteins are involved in PMP targeting. However, we do not understand how PMPs are inserted into the membrane. Likewise, our knowledge on PMP degradation is very limited.

To date only four PMPs- Pex3p (yeast), Pex13p (yeast and plants), Pex15p (yeast) and ALDP (humans) have been observed to undergo degradation by the proteasome (Chen et al., 2018; Morita et al., 2019; Pan et al., 2016; Williams and van der Klei, 2013). Significantly, the role for ubiquitin in Pex3p, Pex13p and Pex15p turnover has been established however, the ubiquitination machinery (i.e) E2s and E3s involved in ALDP degradation have not been

identified yet. The abundance of Inp2p in cells has been suggested to regulate peroxisome inheritance (Fagarasanu et al., 2009) and the data on Pex14p suggests that they are prone to oxidative damage (Aksam et al., 2008). The question remains whether these PMPs undergo UPS dependent degradation and if there are other potential PMP substrates that might be targeted for degradation (Figure 8). Furthermore, it is essential to investigate the function behind the turnover of individual PMPs, including whether the PMP degradation event serves a particular purpose, such as with Pex3p or whether the PMP degradation event is for quality control purposes, as observed for Pex15p.

Figure 8: Schematic overview of future research areas that could provide detailed insights into PMP degradation. (1) Which of the PMPs are targeted for degradation? and (2) how PMP substrates are specifically recognized from other PMPs present at the peroxisomes, (3) which mechanisms are involved in PMP ubiquitination and (4) extraction of ubiquitinated PMPs from the peroxisomes are currently not well-known and requires further investigation.

While our knowledge on the involvement of peroxisomal ubiquitin machinery in PMP degradation is increasing, how PMPs are recognized and extracted from peroxisomal membrane to facilitate degradation by the proteasome is unknown (Figure 8). To this end, the role for Pex1p/Pex6p in PMP degradation is unclear at the moment and these proteins were observed to be dispensable for Pex3p turnover (Williams and van der Klei, 2013), suggesting a role for

other AAA proteins in PMP extraction. Whether peroxisomes, like ER and Mitochondria, require the activity of Cdc48p would be interesting to investigate. Alternatively Msp1p, which functions in degrading Pex15p (Matsumoto et al., 2019), may also function in the degradation of other PMPs. Currently, we do not have direct evidence to show PMPs are first released in the cytosol in an ATP dependent manner before being degraded by the proteasome. This raises the possibility that proteasomes could transiently localize to the peroxisomes and degrade PMPs, a characteristic similar to what had been observed for ER and Mitochondria, particularly for degrading membrane proteins with multiple TMDs.

The involvement of the E2 Pex4p and RING E3 peroxins (Pex2p and Pex10p) indicates a role for peroxisomal ubiquitination machinery in PMP degradation (Chen et al., 2018; Williams and van der Klei, 2013) but there is also evidence that support a role for non-peroxisomal proteins in peroxisomal protein degradation, such as the E2 enzyme Ubc4p, involved in Pex5p degradation (Kiel et al., 2005) and the cytosolic E3 ligase complex CRL4A, that participates in Pex7p degradation (Miyauchi-Nanri et al., 2014). Furthermore, the observation that Pex13p is only partially inhibited in *pex4* cells could suggest that there are other E2 enzymes involved in Pex13p turnover. One obvious candidate would be Ubc4p as its role in Pex5p degradation has already been demonstrated. Interestingly the E3 ligase Dma1p (and its paralogue Dma2p), involved in Vac17p degradation, regulates peroxisome movements (Yau et al., 2014). Furthermore, the cytosolic E3 ligase Ubr2p has been implicated in peroxisome biogenesis (Lockshon et al., 2007; Saleem et al., 2010). It would be interesting to investigate whether these E3 ligases are involved in PMP degradation while future work that focusses on identifying non-peroxisomal factors that might regulate the degradation of PMPs will surely provide new insights into how PMP degradation is connected to general cellular degradation pathways, including which UPS components are shared amongst the different degradation pathways. Therefore, studying PMP degradation would not only help us to understand whether PMPs are degraded through conserved degradation pathways but it will also help us understand how PMPs are regulated and to what extent PMP turnover controls peroxisomal functions. Furthermore, it would also provide valuable insights into role of PMP quality control in human health and diseases.

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References

- Adams, J. 2003. The proteasome: structure, function, and role in the cell. *Cancer Treat Rev.* 29 Suppl 1:3-9.
- Adle, D.J., D. Sinani, H. Kim, and J. Lee. 2007. A cadmium-transporting P-1B-type ATPase in yeast *Saccharomyces cerevisiae*. *Journal of Biological Chemistry.* 282:947-955.

- Adle, D.J., W.Z. Wei, N. Smith, J.J. Bies, and J. Lee. 2009. Cadmium-mediated rescue from ER-associated degradation induces expression of its exporter. *Proceedings of the National Academy of Sciences of the United States of America*. 106:10189-10194.
- Aksam, E.B., H. Jungwirth, S.D. Kohlwein, J. Ring, F. Madeo, M. Veenhuis, and I.J. van der Klei. 2008. Absence of the peroxiredoxin Pmp20 causes peroxisomal protein leakage and necrotic cell death. *Free Radical Bio Med*. 45:1115-1124.
- Aksam, E.B., A. Koek, J.A. Kiel, S. Jourdan, M. Veenhuis, and I.J. van der Klei. 2007. A peroxisomal lon protease and peroxisome degradation by autophagy play key roles in vitality of *Hansenula polymorpha* cells. *Autophagy*. 3:96-105.
- Ali, A.M., J. Atmaj, A. Adawy, S. Lunev, N. Van Oosterwijk, S.R. Yan, C. Williams, and M.R. Groves. 2018. The Pex4p-Pex22p complex from *Hansenula polymorpha*: biophysical analysis, crystallization and X-ray diffraction characterization. *Acta crystallographica. Section F, Structural biology communications*. 74:76-81.
- Anton, F., G. Dittmar, T. Langer, and M. Escobar-Henriques. 2013. Two Deubiquitylases Act on Mitofusin and Regulate Mitochondrial Fusion along Independent Pathways. *Molecular Cell*. 49:487-498.
- Azevedo, J.E., and W. Schliebs. 2006. Pex14p, more than just a docking protein. *Bba-Mol Cell Res*. 1763:1574-1584.
- Baerends, R.J., K.N. Faber, J.A. Kiel, I.J. van der Klei, W. Harder, and M. Veenhuis. 2000. Sorting and function of peroxisomal membrane proteins. *FEMS Microbiol Rev*. 24:291-301.
- Baerends, R.J., F.A. Salomons, K.N. Faber, J.A. Kiel, I.J. Van der Klei, and M. Veenhuis. 1997. Deviant Pex3p levels affect normal peroxisome formation in *Hansenula polymorpha*: high steady-state levels of the protein fully abolish matrix protein import. *Yeast*. 13:1437-1448.
- Baerends, R.J.S., S.W. Rasmussen, R.E. Hilbrands, M. vanderHeide, K.N. Faber, P.T.W. Reuvekamp, J.A.K.W. Kiel, J.M. Cregg, I.J. vanderKlei, and M. Veenhuis. 1996. The *Hansenula polymorpha* PER9 gene encodes a peroxisomal membrane protein essential for peroxisome assembly and integrity. *Journal of Biological Chemistry*. 271:8887-8894.
- Bagola, K., M. Mehnert, E. Jarosch, and T. Sommer. 2011. Protein dislocation from the ER. *Biochim Biophys Acta*. 1808:925-936.
- Baker, A., D.J. Carrier, T. Schaedler, H.R. Waterham, C.W. van Roermund, and F.L. Theodoulou. 2015. Peroxisomal ABC transporters: functions and mechanism. *Biochemical Society Transactions*. 43:959-965.
- Barford, D. 2011. Structural insights into anaphase-promoting complex function and mechanism. *Philos T R Soc B*. 366:3605-3624.
- Bartoszewska, M., C. Williams, A. Kikhney, L. Opalinski, C.W.T. van Roermund, R. de Boer, M. Veenhuis, and I.J. van der Klei. 2012. Peroxisomal Proteostasis Involves a Lon Family Protein That Functions as Protease and Chaperone. *Journal of Biological Chemistry*. 287:27380-27395.
- Bassermann, F., R. Eichner, and M. Pagano. 2014. The ubiquitin proteasome system - implications for cell cycle control and the targeted treatment of cancer. *Biochim Biophys Acta*. 1843:150-162.
- Baugh, J.M., E.G. Viktorova, and E.V. Pilipenko. 2009. Proteasomes Can Degrade a Significant Proportion of Cellular Proteins Independent of Ubiquitination. *Journal of Molecular Biology*. 386:814-827.
- Bays, N.W., R.G. Gardner, L.P. Seelig, C.A. Joazeiro, and R.Y. Hampton. 2001. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat Cell Biol*. 3:24-29.
- Bellu, A.R., F.A. Salomons, J.A.K.W. Kiel, M. Veenhuis, and I.J. van der Klei. 2002. Removal of Pex3p is an important initial stage in selective peroxisome degradation in *Hansenula polymorpha*. *Journal of Biological Chemistry*. 277:42875-42880.

- Ben-Nissan, G., and M. Sharon. 2014. Regulating the 20S proteasome ubiquitin-independent degradation pathway. *Biomolecules*. 4:862-884.
- Bener Aksam, E., H. Jungwirth, S.D. Kohlwein, J. Ring, F. Madeo, M. Veenhuis, and I.J. van der Klei. 2008. Absence of the peroxiredoxin Pmp20 causes peroxisomal protein leakage and necrotic cell death. *Free Radic Biol Med*. 45:1115-1124.
- Benischke, A.S., C. Hemion, J. Flammer, and A. Neutznern. 2014. Proteasome-mediated quality control of S-nitrosylated mitochondrial proteins. *Mitochondrion*. 17:182-186.
- Bercovich, Z., Y. Rosenberghasson, A. Ciechanover, and C. Kahana. 1989. Degradation of Ornithine Decarboxylase in Reticulocyte Lysate Is Atp-Dependent but Ubiquitin-Independent. *Journal of Biological Chemistry*. 264:15949-15952.
- Birschmann, I., A.K. Stroobants, M. van den Berg, A. Schafer, K. Rosenkranz, W.H. Kunau, and H.F. Tabak. 2003. Pex15p of *Saccharomyces cerevisiae* provides a molecular basis for recruitment of the AAA peroxin Pex6p to peroxisomal membranes. *Molecular Biology of the Cell*. 14:2226-2236.
- Bodnar, N.O., and T.A. Rapoport. 2017. Molecular Mechanism of Substrate Processing by the Cdc48 ATPase Complex. *Cell*. 169:722-735.
- Bowie, J.U. 2011. Membrane protein folding: how important are hydrogen bonds? *Curr Opin Struct Biol*. 21:42-49.
- Braten, O., I. Livneh, T. Ziv, A. Admon, I. Kehat, L.H. Caspi, H. Gonen, B. Bercovich, A. Godzik, S. Jahandideh, L. Jaroszewski, T. Sommer, Y.T. Kwon, M. Guharoy, P. Tompa, and A. Ciechanover. 2016. Numerous proteins with unique characteristics are degraded by the 26S proteasome following monoubiquitination. *Proceedings of the National Academy of Sciences of the United States of America*. 113:E4639-E4647.
- Braverman, N.E., G.V. Raymond, W.B. Rizzo, A.B. Moser, M.E. Wilkinson, E.M. Stone, S.J. Steinberg, M.F. Wangler, E.T. Rush, J.G. Hacia, and M. Bose. 2016. Peroxisome biogenesis disorders in the Zellweger spectrum: An overview of current diagnosis, clinical manifestations, and treatment guidelines. *Mol Genet Metab*. 117:313-321.
- Brocard, C., and A. Hartig. 2006. Peroxisome targeting signal 1: Is it really a simple tripeptide? *Biochim Biophys Acta*. 1763:1565-1573.
- Buck, T.M., C.M. Wright, and J.L. Brodsky. 2007. The activities and function of molecular chaperones in the endoplasmic reticulum. *Semin Cell Dev Biol*. 18:751-761.
- Cartier, N., J. Lopez, P. Moullier, F. Rocchiccioli, M.O. Rolland, P. Jorge, J. Mosser, J.L. Mandel, P.F. Bougneres, O. Danos, and et al. 1995. Retroviral-mediated gene transfer corrects very-long-chain fatty acid metabolism in adrenoleukodystrophy fibroblasts. *Proc Natl Acad Sci U S A*. 92:1674-1678.
- Carvalho, P., V. Goder, and T.A. Rapoport. 2006. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell*. 126:361-373.
- Chang, C.C., S. South, D. Warren, J. Jones, A.B. Moser, H.W. Moser, and S.J. Gould. 1999. Metabolic control of peroxisome abundance. *Journal of Cell Science*. 112:1579-1590.
- Chen, B., J. Mariano, Y.C. Tsai, A.H. Chan, M. Cohen, and A.M. Weissman. 2006. The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. *Proceedings of the National Academy of Sciences of the United States of America*. 103:341-346.
- Chen, X., S. Devarajan, N. Danda, and C. Williams. 2018. Insights into the Role of the Peroxisomal Ubiquitination Machinery in Pex13p Degradation in the Yeast *Hansenula polymorpha*. *Journal of Molecular Biology*. 430:1545-1558.

- Chen, Y.C., G.K.E. Umanah, N. Dephoure, S.A. Andrabi, S.P. Gygi, T.M. Dawson, V.L. Dawson, and J. Rutter. 2014. Msp1/ATAD1 maintains mitochondrial function by facilitating the degradation of mislocalized tail-anchored proteins. *Embo Journal*. 33:1548-1564.
- Christianson, J.C., and Y. Ye. 2014. Cleaning up in the endoplasmic reticulum: ubiquitin in charge. *Nat Struct Mol Biol*. 21:325-335.
- Christis, C., N.H. Lubsen, and I. Braakman. 2008. Protein folding includes oligomerization - examples from the endoplasmic reticulum and cytosol. *Febs J*. 275:4700-4727.
- Ciechanover, A., Y. Hod, and A. Hershko. 1978. Heat-Stable Polypeptide Component of an Atp-Dependent Proteolytic System from Reticulocytes. *Biochemical and Biophysical Research Communications*. 81:1100-1105.
- Ciechanover, A., A. Orian, and A.L. Schwartz. 2000. Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays*. 22:442-451.
- Ciechanover, A., and A.L. Schwartz. 2004. The ubiquitin system: pathogenesis of human diseases and drug targeting. *Biochim Biophys Acta*. 1695:3-17.
- Collins, C.S., J.E. Kalish, J.C. Morrell, J.M. McCaffery, and S.J. Gould. 2000. The peroxisome biogenesis factors Pex4p, Pex22p, Pex1p, and Pex6p act in the terminal steps of peroxisomal matrix protein import. *Molecular and Cellular Biology*. 20:7516-7526.
- Conaway, R.C., C.S. Brower, and J.W. Conaway. 2002. Emerging roles of ubiquitin in transcription regulation. *Science*. 296:1254-1258.
- Coux, O., K. Tanaka, and A.L. Goldberg. 1996. Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem*. 65:801-847.
- Cunningham, C.N., J.M. Baughman, L. Phu, J.S. Tea, C. Yu, M. Coons, D.S. Kirkpatrick, B. Bingol, and J.E. Corn. 2015. USP30 and parkin homeostatically regulate atypical ubiquitin chains on mitochondria. *Nature Cell Biology*. 17:160-+.
- Cymer, F., G. von Heijne, and S.H. White. 2015. Mechanisms of Integral Membrane Protein Insertion and Folding. *Journal of Molecular Biology*. 427:999-1022.
- Dahlmann, B., F. Kopp, L. Kuehn, B. Nidel, G. Pfeifer, R. Hegerl, and W. Baumeister. 1989. The Multicatalytic Proteinase (Prosome) Is Ubiquitous from Eukaryotes to Archaeobacteria. *Febs Letters*. 251:125-131.
- Deak, P.M., and D.H. Wolf. 2001. Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. *J Biol Chem*. 276:10663-10669.
- Debelyy, M.O., H.W. Platta, D. Saffian, A. Hensel, S. Thoms, H.E. Meyer, B. Warscheid, W. Girzalsky, and R. Erdmann. 2011. Ubp15p, a Ubiquitin Hydrolase Associated with the Peroxisomal Export Machinery. *Journal of Biological Chemistry*. 286:28223-28234.
- Dederer, V., A. Khmelinskii, A.G. Huhn, V. Okreglak, M. Knop, and M.K. Lemberg. 2019. Cooperation of mitochondrial and ER factors in quality control of tail-anchored proteins. *eLife*. 8.
- DeWire, S.M., S. Ahn, R.J. Lefkowitz, and S.K. Shenoy. 2007. Beta-arrestins and cell signaling. *Annu Rev Physiol*. 69:483-510.
- Distel, B., R. Erdmann, S.J. Gould, G. Blobel, D.I. Crane, J.M. Cregg, G. Dodt, Y. Fujiki, J.M. Goodman, W.W. Just, J.A. Kiel, W.H. Kunau, P.B. Lazarow, G.P. Mannaerts, H.W. Moser, T. Osumi, R.A. Rachubinski, A. Roscher, S. Subramani, H.F. Tabak, T. Tsukamoto, D. Valle, I. van der Klei, P.P. van Veldhoven, and M. Veenhuis. 1996. A unified nomenclature for peroxisome biogenesis factors. *J Cell Biol*. 135:1-3.
- Dobson, C.M. 2004. Principles of protein folding, misfolding and aggregation. *Semin Cell Dev Biol*. 15:3-16.

- Drummond, D.A., and C.O. Wilke. 2008. Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. *Cell*. 134:341-352.
- Du, K., M. Sharma, and G.L. Lukacs. 2005. The DeltaF508 cystic fibrosis mutation impairs domain-domain interactions and arrests post-translational folding of CFTR. *Nat Struct Mol Biol*. 12:17-25.
- Dyer, J.M., J.A. McNew, and J.M. Goodman. 1996. The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. *Journal of Cell Biology*. 133:269-280.
- Eagle, H., K.A. Piez, R. Fleischman, and V.I. Oyama. 1959. Protein Turnover in Mammalian Cell Cultures. *Journal of Biological Chemistry*. 234:592-597.
- Eberhart, T., and W.J. Kovacs. 2018. Pexophagy in yeast and mammals: an update on mysteries. *Histochemistry and Cell Biology*. 150:473-488.
- Eckert, J.H., and R. Erdmann. 2003. Peroxisome biogenesis. *Rev Physiol Bioch P*. 147:75-121.
- Ee, G., and N. Lehming. 2012. How the ubiquitin proteasome system regulates the regulators of transcription. *Transcription*. 3:235-239.
- Effelsberg, D., L.D. Cruz-Zaragoza, W. Schliebs, and R. Erdmann. 2016. Pex9p is a new yeast peroxisomal import receptor for PTS1-containing proteins. *Journal of Cell Science*. 129:4057-4066.
- El Magraoui, F., B.E. Baumer, H.W. Platta, J.S. Baumann, W. Girzalsky, and R. Erdmann. 2012. The RING-type ubiquitin ligases Pex2p, Pex10p and Pex12p form a heteromeric complex that displays enhanced activity in an ubiquitin conjugating enzyme-selective manner. *FEBS J*. 279:2060-2070.
- El Magraoui, F., R. Brinkmeier, T. Mastalski, A. Hupperich, C. Strehl, D. Schwerter, W. Girzalsky, H.E. Meyer, B. Warscheid, R. Erdmann, and H.W. Platta. 2019. The deubiquitination of the PTS1-import receptor Pex5p is required for peroxisomal matrix protein import. *Bba-Mol Cell Res*. 1866:199-213.
- El Magraoui, F., R. Brinkmeier, A. Schrotter, W. Girzalsky, T. Muller, K. Marcus, H.E. Meyer, R. Erdmann, and H.W. Platta. 2013. Distinct Ubiquitination Cascades Act on the Peroxisomal Targeting Signal Type 2 Co-receptor Pex18p. *Traffic*. 14:1290-1301.
- El Magraoui, F., A. Schrotter, R. Brinkmeier, L. Kunst, T. Mastalski, T. Muller, K. Marcus, H.E. Meyer, W. Girzalsky, R. Erdmann, and H.W. Platta. 2014. The Cytosolic Domain of Pex22p Stimulates the Pex4p-Dependent Ubiquitination of the PTS1-Receptor. *Plos One*. 9.
- Elangovan, M., H.K. Chong, J.H. Park, E.J. Yeo, and Y.J. Yoo. 2017. The role of ubiquitin-conjugating enzyme Ube2j1 phosphorylation and its degradation by proteasome during endoplasmic stress recovery. *J Cell Commun Signal*. 11:265-273.
- Elgersma, Y., L. Kwast, M. van den Berg, W.B. Snyder, B. Distel, S. Subramani, and H.F. Tabak. 1997. Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in *S-cerevisiae*, causes proliferation of the endoplasmic reticulum membrane. *Embo Journal*. 16:7326-7341.
- Ella, H., Y. Reiss, and T. Ravid. 2019. The Hunt for Degrons of the 26S Proteasome. *Biomolecules*. 9.
- Elsasser, S., D. Chandler-Militello, B. Muller, J. Hanna, and D. Finley. 2004. Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. *Journal of Biological Chemistry*. 279:26817-26822.
- Enenkel, C., A. Lehmann, and P.M. Kloetzel. 1998. Subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope-ER network in yeast. *EMBO J*. 17:6144-6154.
- Engelen, M., S. Kemp, and B.T. Poll-The. 2014. X-linked adrenoleukodystrophy: pathogenesis and treatment. *Current neurology and neuroscience reports*. 14:486.
- Escobar-Henriques, M., and T. Langer. 2014. Dynamic survey of mitochondria by ubiquitin. *Embo Reports*. 15:231-243.

- Fagarasanu, A., M. Fagarasanu, G.A. Eitzen, J.D. Aitchison, and R.A. Rachubinski. 2006. The peroxisomal membrane protein Inp2p is the peroxisome-specific receptor for the myosin V motor Myo2p of *Saccharomyces cerevisiae*. *Developmental Cell*. 10:587-600.
- Fagarasanu, A., F.D. Mast, B. Knoblach, Y. Jin, M.J. Brunner, M.R. Logan, J.N.M. Glover, G.A. Eitzen, J.D. Aitchison, L.S. Weisman, and R.A. Rachubinski. 2009. Myosin-driven peroxisome partitioning in *S. cerevisiae*. *Journal of Cell Biology*. 186:541-554.
- Fagarasanu, A., F.D. Mast, B. Knoblach, and R.A. Rachubinski. 2010. Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast. *Nat Rev Mol Cell Bio*. 11:644-654.
- Fagarasanu, A., and R.A. Rachubinski. 2007. Orchestrating organelle inheritance in *Saccharomyces cerevisiae*. *Current Opinion in Microbiology*. 10:528-538.
- Fagarasanu, M., A. Fagarasanu, Y.Y.C. Tam, J.D. Aitchison, and R.A. Rachubinski. 2005. Inp1p is a peroxisomal membrane protein required for peroxisome inheritance in *Saccharomyces cerevisiae*. *Journal of Cell Biology*. 169:765-775.
- Fang, S.Y., M. Ferrone, C.H. Yang, J.P. Jensen, S. Tiwari, and A.M. Weissman. 2001. The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America*. 98:14422-14427.
- Fang, Y., J.C. Morrell, J.M. Jones, and S.J. Gould. 2004. PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. *Journal of Cell Biology*. 164:863-875.
- Farmer, L.M., M.A. Rinaldi, P.G. Young, C.H. Danan, S.E. Burkhart, and B. Bartel. 2013. Disrupting Autophagy Restores Peroxisome Function to an Arabidopsis lon2 Mutant and Reveals a Role for the LON2 Protease in Peroxisomal Matrix Protein Degradation. *Plant Cell*. 25:4085-4100.
- Farre, J.C., R. Manjithaya, R.D. Mathewson, and S. Subramani. 2008. PpAtg30 tags peroxisomes for turnover by selective autophagy. *Developmental Cell*. 14:365-376.
- Ferdinandusse, S., S. Denis, P.L. Faust, and R.J. Wanders. 2009. Bile acids: the role of peroxisomes. *J Lipid Res*. 50:2139-2147.
- Fiedler, S., J. Broecker, and S. Keller. 2010. Protein folding in membranes. *Cell Mol Life Sci*. 67:1779-1798.
- Finley, D. 2009. Recognition and Processing of Ubiquitin-Protein Conjugates by the Proteasome. *Annual Review of Biochemistry*. 78:477-513.
- Finley, D., H.D. Ulrich, T. Sommer, and P. Kaiser. 2012. The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. *Genetics*. 192:319-360.
- Fonseca, R., R.M. Vabulas, F.U. Hartl, T. Bonhoeffer, and U.V. Nagerl. 2006. A balance of protein synthesis and proteasome-dependent degradation determines the maintenance of LTP. *Neuron*. 52:239-245.
- Fredrickson, E.K., and R.G. Gardner. 2012. Selective destruction of abnormal proteins by ubiquitin-mediated protein quality control degradation. *Semin Cell Dev Biol*. 23:530-537.
- Friedman, J.R., and J. Nunnari. 2014. Mitochondrial form and function. *Nature*. 505:335-343.
- Funakoshi, M., T. Sasaki, T. Nishimoto, and H. Kobayashi. 2002. Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. *Proceedings of the National Academy of Sciences of the United States of America*. 99:745-750.
- Furth, N., O. Gertman, A. Shiber, O.S. Alfassy, I. Cohen, M.M. Rosenberg, N.K. Doron, A. Friedler, and T. Ravid. 2011. Exposure of bipartite hydrophobic signal triggers nuclear quality control of Ndc10 at the endoplasmic reticulum/nuclear envelope. *Molecular Biology of the Cell*. 22:4726-4739.
- Gabaldon, T. 2010. Peroxisome diversity and evolution. *Philos Trans R Soc Lond B Biol Sci*. 365:765-773.

- Gardner, R.G., and R.Y. Hampton. 1999. A highly conserved signal controls degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes. *Journal of Biological Chemistry*. 274:31671-31678.
- Gardner, R.G., A.G. Shearer, and R.Y. Hampton. 2001. In vivo action of the HRD ubiquitin ligase complex: Mechanisms of endoplasmic reticulum quality control and sterol regulation. *Molecular and Cellular Biology*. 21:4276-4291.
- Gardner, R.G., G.M. Swarbrick, N.W. Bays, S.R. Cronin, S. Wilhovsky, L. Seelig, C. Kim, and R. Hampton. 2000. Endoplasmic reticulum degradation requires lumen to cytosol signaling: Transmembrane control of Hrd1p by Hrd3p. *Journal of Cell Biology*. 151:69-82.
- Gatto, G.J., Jr., B.V. Geisbrecht, S.J. Gould, and J.M. Berg. 2000. A proposed model for the PEX5-peroxisomal targeting signal-1 recognition complex. *Proteins*. 38:241-246.
- Gibson, J.S., and J.C. Ellory. 2002. Membrane transport in sickle cell disease. *Blood cells, molecules & diseases*. 28:303-314.
- Gille, C., A. Goede, C. Schloetelburg, R. Preissner, P.M. Kloetzel, U.B. Gobel, and C. Frommel. 2003. A comprehensive view on proteasomal sequences: Implications for the evolution of the proteasome. *Journal of Molecular Biology*. 326:1437-1448.
- Glickman, M.H., and D. Raveh. 2005. Proteasome plasticity. *FEBS Lett*. 579:3214-3223.
- Goldberg, A.L. 1972. Degradation of Abnormal Proteins in Escherichia-Coli. *Proceedings of the National Academy of Sciences of the United States of America*. 69:422-+.
- Goldberg, A.L. 2003. Protein degradation and protection against misfolded or damaged proteins. *Nature*. 426:895-899.
- Graham, I.A. 2008. Seed storage oil mobilization. *Annual Review of Plant Biology*. 59:115-142.
- Groll, M., M. Bajorek, A. Kohler, L. Moroder, D.M. Rubin, R. Huber, M.H. Glickman, and D. Finley. 2000. A gated channel into the proteasome core particle. *Nature Structural Biology*. 7:1062-1067.
- Groll, M., L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H.D. Bartunik, and R. Huber. 1997. Structure of 20S proteasome from yeast at 2.4 angstrom resolution. *Nature*. 386:463-471.
- Grou, C.P., A.F. Carvalho, M.P. Pinto, S. Wiese, H. Piechura, H.E. Meyer, B. Warscheid, C. Sa-Miranda, and J.E. Azevedo. 2008. Members of the E2D (UbcH5) family mediate the ubiquitination of the conserved cysteine of Pex5p, the peroxisomal import receptor. *Journal of Biological Chemistry*. 283:14190-14197.
- Grou, C.P., T. Francisco, T.A. Rodrigues, M.O. Freitas, M.P. Pinto, A.F. Carvalho, P. Domingues, S.A. Wood, J.E. Rodriguez-Borges, C. Sa-Miranda, M. Fransen, and J.E. Azevedo. 2012. Identification of Ubiquitin-specific Protease 9X (USP9X) as a Deubiquitinase Acting on Ubiquitin-Peroxin 5 (PEX5) Thioester Conjugate. *Journal of Biological Chemistry*. 287:12815-12827.
- Gurvitz, A., and H. Rottensteiner. 2006. The biochemistry of oleate induction: transcriptional upregulation and peroxisome proliferation. *Biochim Biophys Acta*. 1763:1392-1402.
- Guterman, A., and M.H. Glickman. 2004. Deubiquitinating enzymes are IN/(trinsic to proteasome function). *Current protein & peptide science*. 5:201-211.
- Habeck, G., F.A. Ebner, H. Shimada-Kreft, and S.G. Kreft. 2015. The yeast ERAD-C ubiquitin ligase Doa10 recognizes an intramembrane degron (vol 209, pg 261, 2015). *Journal of Cell Biology*. 209:165-165.
- Hagstrom, D., C. Ma, S. Guha-Polley, and S. Subramani. 2014. The unique degradation pathway of the PTS2 receptor, Pex7, is dependent on the PTS receptor/coreceptor, Pex5 and Pex20. *Mol Biol Cell*. 25:2634-2643.
- Hampton, R.Y., R.G. Gardner, and J. Rine. 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Molecular Biology of the Cell*. 7:2029-2044.

- Hampton, R.Y., and R.M. Garza. 2009. Protein Quality Control as a Strategy for Cellular Regulation: Lessons from Ubiquitin-Mediated Regulation of the Sterol Pathway. *Chem Rev.* 109:1561-1574.
- Hampton, R.Y., and J. Rine. 1994. Regulated Degradation of Hmg-CoA Reductase, an Integral Membrane-Protein of the Endoplasmic-Reticulum, in Yeast. *Journal of Cell Biology.* 125:299-312.
- Han, S., Y. Liu, and A. Chang. 2007. Cytoplasmic hsp70 promotes ubiquitination for endoplasmic reticulum-associated degradation of a misfolded mutant of the yeast plasma membrane ATPase, PMA1. *Journal of Biological Chemistry.* 282:26140-26149.
- Hardy, J., and D.J. Selkoe. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science.* 297:353-356.
- Hartl, F.U., A. Bracher, and M. Hayer-Hartl. 2011. Molecular chaperones in protein folding and proteostasis. *Nature.* 475:324-332.
- Hartmann-Petersen, R., and C. Gordon. 2004. Integral UBL domain proteins: a family of proteasome interacting proteins. *Semin Cell Dev Biol.* 15:247-259.
- Hassink, G., M. Kikkert, S. van Voorden, S.J. Lee, R. Spaapen, T. van Laar, C.S. Coleman, E. Bartee, K. Fruh, V. Chau, and E. Wiertz. 2005. TEB4 is a C4HC3 RING finger-containing ubiquitin ligase of the endoplasmic reticulum. *Biochemical Journal.* 388:647-655.
- Hayashi, M., and M. Nishimura. 2003. Entering a new era of research on plant peroxisomes. *Current opinion in plant biology.* 6:577-582.
- He, C., and D.J. Klionsky. 2009. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet.* 43:67-93.
- Heinemeyer, W., N. Trondle, G. Albrecht, and D.H. Wolf. 1994. Pre5 and Pre6, the Last Missing Genes Encoding 20s Proteasome Subunits from Yeast - Indication for a Set of 14 Different Subunits in the Eukaryotic Proteasome Core. *Biochemistry.* 33:12229-12237.
- Hensel, A., S. Beck, F. El Magraoui, H.W. Platta, W. Girzalsky, and R. Erdmann. 2011. Cysteine-dependent Ubiquitination of Pex18p Is Linked to Cargo Translocation across the Peroxisomal Membrane. *Journal of Biological Chemistry.* 286:43495-43505.
- Heo, J.M., N. Livnat-Levanon, E.B. Taylor, K.T. Jones, N. Dephoure, J. Ring, J. Xie, J.L. Brodsky, F. Madeo, S.P. Gygi, K. Ashrafi, M.H. Glickman, and J. Rutter. 2010. A stress-responsive system for mitochondrial protein degradation. *Mol Cell.* 40:465-480.
- Hershko, A., and A. Ciechanover. 1998. The ubiquitin system. *Annu Rev Biochem.* 67:425-479.
- Hettema, E.H., C.W.T. vanRoermund, B. Distel, M. vandenBerg, C. Vilela, C. RodriguesPousada, R.J.A. Wanders, and H.F. Tabak. 1996. The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. *Embo Journal.* 15:3813-3822.
- Hicke, L. 2001. Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol.* 2:195-201.
- Hoppe, T. 2005. Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. *Trends in Biochemical Sciences.* 30:183-187.
- Husnjak, K., and I. Dikic. 2012. Ubiquitin-Binding Proteins: Decoders of Ubiquitin-Mediated Cellular Functions. *Annual Review of Biochemistry, Vol 81.* 81:291-322.
- Husnjak, K., S. Elsassner, N.X. Zhang, X. Chen, L. Randles, Y. Shi, K. Hofmann, K.J. Walters, D. Finley, and I. Dikic. 2008. Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature.* 453:481-488.
- Ikeda, F., and I. Dikic. 2008. Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep.* 9:536-542.
- Imanaka, T., Y. Shiina, T. Takano, T. Hashimoto, and T. Osumi. 1996. Insertion of the 70-kDa peroxisomal membrane protein into peroxisomal membranes in vivo and in vitro. *J Biol Chem.* 271:3706-3713.

- Jarosch, E., C. Taxis, C. Volkwein, J. Bordallo, D. Finley, D.H. Wolf, and T. Sommer. 2002. Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol.* 4:134-139.
- Karpichev, I.V., and G.M. Small. 1998. Global regulatory functions of Oaf1p and Pip2p (Oaf2p), transcription factors that regulate genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 18:6560-6570.
- Kaur, N., Q.Z. Zhao, Q. Xie, and J.P. Hu. 2013. Arabidopsis RING Peroxins are E3 Ubiquitin Ligases that Interact with Two Homologous Ubiquitin Receptor Proteins. *Journal of integrative plant biology.* 55:108-+.
- Kemp, S., A. Pujol, H.R. Waterham, B.M. van Geel, C.D. Boehm, G.V. Raymond, G.R. Cutting, R.J. Wanders, and H.W. Moser. 2001. ABCD1 mutations and the X-linked adrenoleukodystrophy mutation database: role in diagnosis and clinical correlations. *Hum Mutat.* 18:499-515.
- Kemp, S., F.L. Theodoulou, and R.J.A. Wanders. 2011. Mammalian peroxisomal ABC transporters: from endogenous substrates to pathology and clinical significance. *British journal of pharmacology.* 164:1753-1766.
- Kiel, J.A.K.W., K. Emmrich, H.E. Meyer, and W.H. Kunau. 2005. Ubiquitination of the peroxisomal targeting signal type 1 receptor, Pex5p, suggests the presence of a quality control mechanism during peroxisomal matrix protein import. *Journal of Biological Chemistry.* 280:1921-1930.
- Kiel, J.A.K.W., M. Veenhuis, and I.J. van der Klei. 2006. PEX genes in fungal genomes: Common, rare or redundant. *Traffic.* 7:1291-1303.
- Kikkert, M., R. Doolman, M. Dai, R. Avner, G. Hassink, S. van Voorden, S. Thanedar, J. Roitelman, V. Chau, and E. Wiertz. 2004. Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *Journal of Biological Chemistry.* 279:3525-3534.
- Kim, N.C., E. Tresse, R.M. Kolaitis, A. Molliex, R.E. Thomas, N.H. Alami, B. Wang, A. Joshi, R.B. Smith, G.P. Ritson, B.J. Winborn, J. Moore, J.Y. Lee, T.P. Yao, L. Pallanck, M. Kundu, and J.P. Taylor. 2013. VCP Is Essential for Mitochondrial Quality Control by PINK1/Parkin and this Function Is Impaired by VCP Mutations (vol 78, pg 65, 2013). *Neuron.* 78:403-403.
- Kim, P.K., and R.T. Mullen. 2013. PEX16: a multifaceted regulator of peroxisome biogenesis. *Frontiers in physiology.* 4.
- Kim, W., E.J. Bennett, E.L. Huttlin, A. Guo, J. Li, A. Possemato, M.E. Sowa, R. Rad, J. Rush, M.J. Comb, J.W. Harper, and S.P. Gygi. 2011. Systematic and Quantitative Assessment of the Ubiquitin-Modified Proteome. *Molecular Cell.* 44:325-340.
- Kinner, A., and R. Kolling. 2003. The yeast deubiquitinating enzyme Ubp16 is anchored to the outer mitochondrial membrane. *Febs Letters.* 549:135-140.
- Kirisako, T., K. Kamei, S. Murata, M. Kato, H. Fukumoto, M. Kanie, S. Sano, F. Tokunaga, K. Tanaka, and K. Iwai. 2006. A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.* 25:4877-4887.
- Kitagawa, K., Y. Kotake, and M. Kitagawa. 2009. Ubiquitin-mediated control of oncogene and tumor suppressor gene products. *Cancer science.* 100:1374-1381.
- Kleijnen, M.F., R.M. Alarcon, and P.M. Howley. 2003. The ubiquitin-associated domain of hPLIC-2 interacts with the proteasome. *Molecular Biology of the Cell.* 14:3868-3875.
- Kleijnen, M.F., A.H. Shih, P.B. Zhou, S. Kumar, R.E. Soccio, N.L. Kedersha, G. Gill, and P.M. Howley. 2000. The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. *Molecular Cell.* 6:409-419.
- Knoblauch, B., X.J. Sun, N. Coquelle, A. Fagarasanu, R.L. Poirier, and R.A. Rachubinski. 2013. An ER-peroxisome tether exerts peroxisome population control in yeast. *Embo Journal.* 32:2439-2453.
- Knoops, K., S. Manivannan, M.N. Cepinska, A.M. Krikken, A.M. Kram, M. Veenhuis, and I.J. van der Klei. 2014. Preperoxisomal vesicles can form in the absence of Pex3. *Journal of Cell Biology.* 204:659-668.

- Koek, A., M. Komori, M. Veenhuis, and I.J. van der Klei. 2007. A comparative study of peroxisomal structures in *Hansenula polymorpha* pex mutants. *Fems Yeast Research*. 7:1126-1133.
- Komander, D., and M. Rape. 2012. The Ubiquitin Code. *Annual Review of Biochemistry*, Vol 81. 81:203-229.
- Kravtsova-Ivantsiv, Y., S. Cohen, and A. Ciechanover. 2011. Modification by Single Ubiquitin Moieties Rather Than Polyubiquitination Is Sufficient for Proteasomal Processing of the p105 NF-kappa B Precursor. *Advances in Tnf Family Research*. 691:95-106.
- Krogh, A., B. Larsson, G. von Heijne, and E.L.L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *Journal of Molecular Biology*. 305:567-580.
- Langosch, D., C. Scharnagl, H. Steiner, and M.K. Lemberg. 2015. Understanding intramembrane proteolysis: from protein dynamics to reaction kinetics. *Trends in Biochemical Sciences*. 40:318-327.
- Law, K.B., D. Bronte-Tinkew, E. Di Pietro, A. Snowden, R.O. Jones, A. Moser, J.H. Brumell, N. Braverman, and P.K. Kim. 2017. The peroxisomal AAA ATPase complex prevents pexophagy and development of peroxisome biogenesis disorders. *Autophagy*. 13:868-884.
- Lazarow, P.B. 2006. The import receptor Pex7p and the PTS2 targeting sequence. *Biochim Biophys Acta*. 1763:1599-1604.
- Lazarow, P.B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu Rev Cell Biol*. 1:489-530.
- Lecker, S.H., A.L. Goldberg, and W.E. Mitch. 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *Journal of the American Society of Nephrology : JASN*. 17:1807-1819.
- Lee, B.H., Y. Lu, M.A. Prado, Y. Shi, G. Tian, S.W. Sun, S. Elsasser, S.P. Gygi, R.W. King, and D. Finley. 2016. USP14 deubiquitinates proteasome-bound substrates that are ubiquitinated at multiple sites. *Nature*. 532:398-+.
- Lee, L., R. Seager, Y. Nakamura, K.A. Wilkinson, and J.M. Henley. 2019. Parkin-mediated ubiquitination contributes to the constitutive turnover of mitochondrial fission factor (Mff). *PLoS One*. 14:e0213116.
- Lee, M.J., and M.B. Yaffe. 2016. Protein Regulation in Signal Transduction. *Cold Spring Harbor perspectives in biology*. 8.
- Lemberg, M.K. 2011. Intramembrane Proteolysis in Regulated Protein Trafficking. *Traffic*. 12:1109-1118.
- Lenk, U., H. Yu, J. Walter, M.S. Gelman, E. Hartmann, R.R. Kopito, and T. Sommer. 2002. A role for mammalian Ubc6 homologues in ER-associated protein degradation. *Journal of Cell Science*. 115:3007-3014.
- Leon, S., and S. Subramani. 2007. A conserved cysteine residue of *Pichia pastoris* Pex20p is essential for its recycling from the peroxisome to the cytosol. *Journal of Biological Chemistry*. 282:7424-7430.
- Leon, S., L. Zhang, W.H. McDonald, J. Yates, J.M. Cregg, and S. Subramani. 2006. Dynamics of the peroxisomal import cycle of PpPex20p: ubiquitin-dependent localization and regulation. *Journal of Cell Biology*. 172:67-78.
- Lerner, M., M. Corcoran, D. Cepeda, M.L. Nielsen, R. Zubarev, F. Ponten, M. Uhlen, S. Hober, D. Grander, and O. Sangfelt. 2007. The RBCC gene RFP2 (leu5) encodes a novel transmembrane E3 ubiquitin ligase involved in ERAD. *Molecular Biology of the Cell*. 18:1670-1682.
- Li, C., A.L. Schillmiller, G. Liu, G.I. Lee, S. Jayanty, C. Sageman, J. Vrebalov, J.J. Giovannoni, K. Yagi, Y. Kobayashi, and G.A. Howe. 2005. Role of beta-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. *Plant Cell*. 17:971-986.
- Li, L.L., J. Zheng, X. Wu, and H. Jiang. 2019. Mitochondrial AAA-ATPase Msp1 detects mislocalized tail-anchored proteins through a dual-recognition mechanism. *Embo Reports*. 20.

- Li, W., M.H. Bengtson, A. Ulbrich, A. Matsuda, V.A. Reddy, A. Orth, S.K. Chanda, S. Batalov, and C.A.P. Joazeiro. 2008. Genome-Wide and Functional Annotation of Human E3 Ubiquitin Ligases Identifies MULAN, a Mitochondrial E3 that Regulates the Organelle's Dynamics and Signaling. *Plos One*. 3.
- Ling, Q.H., W.H. Huang, A. Baldwin, and P. Jarvis. 2012. Chloroplast Biogenesis Is Regulated by Direct Action of the Ubiquitin-Proteasome System. *Science*. 338:655-659.
- Lingard, M.J., M. Monroe-Augustus, and B. Bartel. 2009a. Peroxisome-associated matrix protein degradation in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*. 106:4561-4566.
- Liu, L.X., K. Janvier, V. Berteaux-Lecellier, N. Cartier, R. Benarous, and P. Aubourg. 1999. Homo- and heterodimerization of peroxisomal ATP-binding cassette half-transporters. *J Biol Chem*. 274:32738-32743.
- Liu, X.Q., and S. Subramani. 2013. Unique Requirements for Mono- and Polyubiquitination of the Peroxisomal Targeting Signal Co-receptor, Pex20. *Journal of Biological Chemistry*. 288:7230-7240.
- Liu, Y.Q., Y. Yagita, and Y. Fujiki. 2016. Assembly of Peroxisomal Membrane Proteins via the Direct Pex19p-Pex3p Pathway. *Traffic*. 17:433-455.
- Lockshon, D., L.E. Surface, E.O. Kerr, M. Kaeberlein, and B.K. Kennedy. 2007. The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. *Genetics*. 175:77-91.
- Luers, G., K. Beier, T. Hashimoto, H.D. Fahimi, and A. Volkl. 1990. Biogenesis of Peroxisomes - Sequential Biosynthesis of the Membrane and Matrix Proteins in the Course of Hepatic Regeneration. *European Journal of Cell Biology*. 52:175-184.
- Madura, K. 2004. Rad23 and Rpn10: perennial wallflowers join the melee. *Trends in Biochemical Sciences*. 29:637-640.
- Mandelkow, E.M., and E. Mandelkow. 2012. Biochemistry and cell biology of tau protein in neurofibrillary degeneration. *Cold Spring Harbor perspectives in medicine*. 2:a006247.
- Mano, S., and M. Nishimura. 2005. Plant peroxisomes. *Vitamins and hormones*. 72:111-154.
- Martin, I., V.L. Dawson, and T.M. Dawson. 2011. Recent advances in the genetics of Parkinson's disease. *Annu Rev Genomics Hum Genet*. 12:301-325.
- Matsumoto, N., S. Tamura, and Y. Fujiki. 2003. The pathogenic peroxin Pex26p recruits the Pex1p-Pex6p AAA ATPase complexes to peroxisomes. *Nature Cell Biology*. 5:454-460.
- Matsumoto, S., K. Nakatsukasa, C. Kakuta, Y. Tamura, M. Esaki, and T. Endo. 2019. Msp1 Clears Mistargeted Proteins by Facilitating Their Transfer from Mitochondria to the ER. *Molecular Cell*. 76:191-+.
- Mayerhofer, P.U., M. Bano-Polo, I. Mingarro, and A.E. Johnson. 2016. Human Peroxin PEX3 Is Co-translationally Integrated into the ER and Exits the ER in Budding Vesicles. *Traffic*. 17:117-130.
- Mayor, T., J. Graumann, J. Bryan, M.J. MacCoss, and R.J. Deshaies. 2007. Quantitative profiling of ubiquitylated proteins reveals proteasome substrates and the substrate repertoire influenced by the Rpn10 receptor pathway. *Molecular & Cellular Proteomics*. 6:1885-1895.
- Meacham, G.C., C. Patterson, W.Y. Zhang, J.M. Younger, and D.M. Cyr. 2001. The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nature Cell Biology*. 3:100-105.
- Meinecke, M., C. Cizmowski, W. Schliebs, V. Kruger, S. Beck, R. Wagner, and R. Erdmann. 2010. The peroxisomal importomer constitutes a large and highly dynamic pore. *Nature Cell Biology*. 12:273-U268.
- Metzger, M.B., V.A. Hristova, and A.M. Weissman. 2012. HECT and RING finger families of E3 ubiquitin ligases at a glance. *Journal of Cell Science*. 125:531-537.
- Meyer, H.H., J.G. Shorter, J. Seemann, D. Pappin, and G. Warren. 2000. A complex of mammalian Ufd1 and Npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *Embo Journal*. 19:2181-2192.

- Miyauchi-Nanri, Y., S. Mukai, K. Kuroda, and Y. Fujiki. 2014. CUL4A-DDB1-Rbx1 E3 ligase controls the quality of the PTS2 receptor Pex7p. *Biochemical Journal*. 463:65-74.
- Morita, M., S. Matsumoto, A. Sato, K. Inoue, D.G. Kostsin, K. Yamazaki, K. Kawaguchi, N. Shimozawa, S. Kemp, R.J. Wanders, H. Kojima, T. Okabe, and T. Imanaka. 2019. Stability of the ABCD1 Protein with a Missense Mutation: A Novel Approach to Finding Therapeutic Compounds for X-Linked Adrenoleukodystrophy. *JIMD reports*. 44:23-31.
- Morito, D., K. Hirao, Y. Oda, N. Hosokawa, F. Tokunaga, D.M. Cyr, K. Tanaka, K. Iwai, and K. Nagata. 2008. Gp78 cooperates with RMA1 in endoplasmic reticulum-associated degradation of CFTRDeltaF508. *Molecular Biology of the Cell*. 19:1328-1336.
- Mosser, J., A.M. Douar, C.O. Sarde, P. Kioschis, R. Feil, H. Moser, A.M. Poustka, J.L. Mandel, and P. Aubourg. 1993. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature*. 361:726-730.
- Motley, A.M., J.M. Nuttall, and E.H. Hettema. 2012. Pex3-anchored Atg36 tags peroxisomes for degradation in *Saccharomyces cerevisiae*. *Embo Journal*. 31:2852-2868.
- Muchowski, P.J., and J.L. Wacker. 2005. Modulation of neurodegeneration by molecular chaperones. *Nature Reviews Neuroscience*. 6:11-22.
- Muller, W.H., T.P. van der Krift, A.J. Krouwer, H.A. Wosten, L.H. van der Voort, E.B. Smaal, and A.J. Verkleij. 1991. Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. *EMBO J*. 10:489-495.
- Munck, J.M., A.M. Motley, J.M. Nuttall, and E.H. Hettema. 2009. A dual function for Pex3p in peroxisome formation and inheritance. *Journal of Cell Biology*. 187:463-471.
- Nadav, E., A. Shmueli, H. Barr, H. Gonen, A. Ciechanover, and Y. Reiss. 2003. A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. *Biochemical and Biophysical Research Communications*. 303:91-97.
- Nakagawa, T., M. Shirane, S.I. Iemura, T. Natsume, and K.I. Nakayama. 2007. Anchoring of the 26S proteasome to the organellar membrane by FKBP38. *Genes to Cells*. 12:709-719.
- Nakai, M., T. Endo, T. Hase, and H. Matsubara. 1993. Intramitochondrial Protein Sorting - Isolation and Characterization of the Yeast Msp1 Gene Which Belongs to a Novel Family of Putative Atpases. *Journal of Biological Chemistry*. 268:24262-24269.
- Nakatsukasa, K., G. Huyer, S. Michaelis, and J.L. Brodsky. 2008. Dissecting the ER-Associated degradation of a misfolded polytopic membrane protein. *Cell*. 132:101-112.
- Navon, A., and A.L. Goldberg. 2001. Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. *Molecular Cell*. 8:1339-1349.
- Ng, D.P., B.E. Poulsen, and C.M. Deber. 2012. Membrane protein misassembly in disease. *Biochim Biophys Acta*. 1818:1115-1122.
- Nishikawa, S., J.L. Brodsky, and K. Nakatsukasa. 2005. Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). *Journal of Biochemistry*. 137:551-555.
- Ogura, T., and A.J. Wilkinson. 2001. AAA+ superfamily ATPases: common structure--diverse function. *Genes to cells : devoted to molecular & cellular mechanisms*. 6:575-597.
- Okreglak, V., and P. Walter. 2014. The conserved AAA-ATPase Msp1 confers organelle specificity to tail-anchored proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 111:8019-8024.
- Oku, M., and Y. Sakai. 2016. Pexophagy in yeasts. *Bba-Mol Cell Res*. 1863:992-998.

- Okumoto, K., H. Noda, and Y. Fujiki. 2014. Distinct Modes of Ubiquitination of Peroxisome-targeting Signal Type 1 (PTS1) Receptor Pex5p Regulate PTS1 Protein Import. *Journal of Biological Chemistry*. 289:14089-14108.
- Opalinski, L., M. Bartoszewska, S. Fekken, H.Y. Liu, R. de Boer, I. van der Klei, M. Veenhuis, and J.A.K.W. Kiel. 2012. De Novo Peroxisome Biogenesis in *Penicillium Chrysogenum* Is Not Dependent on the Pex11 Family Members or Pex16. *Plos One*. 7.
- Otzen, M., U. Perband, D. Wang, R.J. Baerends, W.H. Kunau, M. Veenhuis, and I.J. Van der Klei. 2004. *Hansenula polymorpha* Pex19p is essential for the formation of functional peroxisomal membranes. *J Biol Chem*. 279:19181-19190.
- Ozkan, E., H.T. Yu, and J. Deisenhofer. 2005. Mechanistic insight into the allosteric activation of a ubiquitin-conjugating enzyme by RING-type ubiquitin ligases. *Proceedings of the National Academy of Sciences of the United States of America*. 102:18890-18895.
- Pan, R.H., and J.P. Hu. 2018. The Arabidopsis E3 Ubiquitin Ligase SP1 Targets to Chloroplasts, Peroxisomes, and Mitochondria. *Plant Physiology*. 176:480-482.
- Pan, R.H., J. Satkovich, and J.P. Hu. 2016. E3 ubiquitin ligase SP1 regulates peroxisome biogenesis in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*. 113:E7307-E7316.
- Park, S., R. Isaacson, H.T. Kim, P.A. Silver, and G. Wagner. 2005. Ufd1 exhibits the AAA-ATPase fold with two distinct ubiquitin interaction sites. *Structure*. 13:995-1005.
- Pernas, L., and L. Scorrano. 2016. Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. *Annu Rev Physiol*. 78:505-531.
- Peth, A., N. Kukushkin, M. Bosse, and A.L. Goldberg. 2013. Ubiquitinated Proteins Activate the Proteasomal ATPases by Binding to Usp14 or Uch37 Homologs. *Journal of Biological Chemistry*. 288:7781-7790.
- Petriv, O.I., L. Tang, V.I. Titorenko, and R.A. Rachubinski. 2004. A new definition for the consensus sequence of the peroxisome targeting signal type 2. *J Mol Biol*. 341:119-134.
- Petroski, M.D., G. Kleiger, and R.J. Deshaies. 2006. Evaluation of a diffusion-driven mechanism for substrate ubiquitination by the SCF-Cdc34 ubiquitin ligase complex. *Molecular Cell*. 24:523-534.
- Pickart, C.M. 2000. Ubiquitin in chains. *Trends Biochem Sci*. 25:544-548.
- Pickart, C.M. 2001. Mechanisms underlying ubiquitination. *Annu Rev Biochem*. 70:503-533.
- Pickart, C.M., and M.J. Eddins. 2004. Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta*. 1695:55-72.
- Pickering, A.M., and K.J.A. Davies. 2012. Degradation of Damaged Proteins: The Main Function of the 20S Proteasome. *Prog Mol Biol Transl*. 109:227-248.
- Pieuchot, L., and G. Jedd. 2012. Peroxisome assembly and functional diversity in eukaryotic microorganisms. *Annu Rev Microbiol*. 66:237-263.
- Platta, H.W., F. El Magraoui, B.E. Baumer, D. Schlee, W. Girzalsky, and R. Erdmann. 2009. Pex2 and Pex12 Function as Protein-Ubiquitin Ligases in Peroxisomal Protein Import. *Molecular and Cellular Biology*. 29:5505-5516.
- Platta, H.W., F. El Magraoui, S. Grunau, D. Schlee, W. Girzalsky, and R. Erdmann. 2008. Ubiquitination of the peroxisomal import receptor Pex5p is required for its receptor cycle. *European Journal of Cell Biology*. 87:56-56.
- Platta, H.W., F. El Magraoui, D. Schlee, S. Grunau, W. Girzalsky, and R. Erdmann. 2007. Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling. *Journal of Cell Biology*. 177:197-204.
- Platta, H.W., S. Grunau, K. Rosenkranz, W. Girzalsky, and R. Erdmann. 2005. Functional role of the AAA peroxins in dislocation of the cycling PTS1 receptor back to the cytosol. *Nature Cell Biology*. 7:817-U883.

- Plempner, R.K., J. Bordallo, P.M. Deak, C. Taxis, R. Hitt, and D.H. Wolf. 1999. Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. *Journal of Cell Science*. 112:4123-4134.
- Plempner, R.K., R. Egner, K. Kuchler, and D.H. Wolf. 1998. Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *Journal of Biological Chemistry*. 273:32848-32856.
- Poirier, Y., V.D. Antonenkov, T. Glumoff, and J.K. Hiltunen. 2006. Peroxisomal beta-oxidation--a metabolic pathway with multiple functions. *Biochim Biophys Acta*. 1763:1413-1426.
- Purdue, P.E., and P.B. Lazarow. 2001. Pex18p is constitutively degraded during peroxisome biogenesis. *Journal of Biological Chemistry*. 276:47684-47689.
- Radi, R. 2013. Protein Tyrosine Nitration: Biochemical Mechanisms and Structural Basis of Functional Effects. *Accounts Chem Res*. 46:550-559.
- Rao, H., and A. Sastry. 2002. Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *Journal of Biological Chemistry*. 277:11691-11695.
- Ravid, T., R. Doolman, R. Avner, D. Harats, and J. Roitelman. 2000. The ubiquitin-proteasome pathway mediates the regulated degradation of mammalian 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Journal of Biological Chemistry*. 275:35840-35847.
- Ravid, T., and M. Hochstrasser. 2008. Diversity of degradation signals in the ubiquitin-proteasome system. *Nat Rev Mol Cell Bio*. 9:679-U625.
- Ravid, T., S.G. Kreft, and M. Hochstrasser. 2006. Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. *Embo J*. 25:533-543.
- Raynes, R., L.C.D. Pomatto, and K.J.A. Davies. 2016. Degradation of oxidized proteins by the proteasome: Distinguishing between the 20S, 26S, and immunoproteasome proteolytic pathways. *Molecular Aspects of Medicine*. 50:41-55.
- Reggiori, F., and H.R. Pelham. 2002. A transmembrane ubiquitin ligase required to sort membrane proteins into multivesicular bodies. *Nat Cell Biol*. 4:117-123.
- Ribeiro, D., I. Castro, H.D. Fahimi, and M. Schrader. 2012. Peroxisome morphology in pathology. *Histol Histopathol*. 27:661-676.
- Roerig, P., P. Mayerhofer, A. Holzinger, and J. Gartner. 2001. Characterization and functional analysis of the nucleotide binding fold in human peroxisomal ATP binding cassette transporters. *FEBS Lett*. 492:66-72.
- Rosenbaum, J.C., E.K. Fredrickson, M.L. Oeser, C.M. Garrett-Engele, M.N. Locke, L.A. Richardson, Z.W. Nelson, E.D. Hetrick, T.I. Milac, D.E. Gottschling, and R.G. Gardner. 2011. Disorder Targets Misorder in Nuclear Quality Control Degradation: A Disordered Ubiquitin Ligase Directly Recognizes Its Misfolded Substrates. *Molecular Cell*. 41:93-106.
- Ross, C.A., and M.A. Poirier. 2004. Protein aggregation and neurodegenerative disease. *Nature medicine*. 10 Supl:S10-17.
- Ruggiano, A., O. Foresti, and P. Carvalho. 2014. Quality control: ER-associated degradation: protein quality control and beyond. *J Cell Biol*. 204:869-879.
- Sacksteder, K.A., J.M. Jones, S.T. South, X.L. Li, Y.F. Liu, and S.J. Gould. 2000. PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *Journal of Cell Biology*. 148:931-944.
- Saleem, R.A., R. Long-O'Donnell, D.J. Dilworth, A.M. Armstrong, A.P. Jamakhandi, Y. Wan, T.A. Knijnenburg, A. Niemisto, J. Boyle, R.A. Rachubinski, I. Shmulevich, and J.D. Aitchison. 2010. Genome-Wide Analysis of Effectors of Peroxisome Biogenesis. *Plos One*. 5.

- Sato, B.K., D. Schulz, P.H. Do, and R.Y. Hampton. 2009. Misfolded Membrane Proteins Are Specifically Recognized by the Transmembrane Domain of the Hrd1p Ubiquitin Ligase. *Molecular Cell*. 34:212-222.
- Scazzari, M., I. Amm, and D.H. Wolf. 2015. Quality Control of a Cytoplasmic Protein Complex CHAPERONE MOTORS AND THE UBIQUITIN-PROTEASOME SYSTEM GOVERN THE FATE OF ORPHAN FATTY ACID SYNTHASE SUBUNIT Fas2 OF YEAST. *Journal of Biological Chemistry*. 290:4677-4687.
- Scheffner, M., U. Nuber, and J.M. Huibregtse. 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature*. 373:81-83.
- Schliebs, W., W. Girzalsky, and R. Erdmann. 2010. Peroxisomal protein import and ERAD: variations on a common theme. *Nat Rev Mol Cell Bio*. 11:885-890.
- Schliebs, W., and W.H. Kunau. 2006. PTS2 co-receptors: diverse proteins with common features. *Biochim Biophys Acta*. 1763:1605-1612.
- Schoebel, S., W. Mi, A. Stein, S. Ovchinnikov, R. Pavlovicz, F. DiMaio, D. Baker, M.G. Chambers, H.Y. Su, D.S. Li, T.A. Rapoport, and M.F. Liao. 2017. Cryo-EM structure of the protein-conducting ERAD channel Hrd1 in complex with Hrd3. *Nature*. 548:352-+.
- Schrader, M., N.A. Bonekamp, and M. Islinger. 2012. Fission and proliferation of peroxisomes. *Biochim Biophys Acta*. 1822:1343-1357.
- Schrader, M., and H.D. Fahimi. 2006a. Growth and division of peroxisomes. *International Review of Cytology - a Survey of Cell Biology, Vol 255*. 255:237-+.
- Schrader, M., and H.D. Fahimi. 2006b. Peroxisomes and oxidative stress. *Biochim Biophys Acta*. 1763:1755-1766.
- Schrader, M., and H.D. Fahimi. 2008. The peroxisome: still a mysterious organelle. *Histochem Cell Biol*. 129:421-440.
- Schulman, B.A., and J.W. Harper. 2009. Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nat Rev Mol Cell Biol*. 10:319-331.
- Schwerter, D.P., I. Grimm, H.W. Platta, and R. Erdmann. 2017. ATP-driven processes of peroxisomal matrix protein import. *Biological Chemistry*. 398:607-624.
- Seglen, P.O., and P. Bohley. 1992. Autophagy and other vacuolar protein degradation mechanisms. *Experientia*. 48:158-172.
- Shani, N., A. Sapag, P.A. Watkins, and D. Valle. 1996. An S-cerevisiae peroxisomal transporter, orthologous to the human adrenoleukodystrophy protein, appears to be a heterodimer of two half ABC transporters: Pxa1p and Pxa2p. *Peroxisomes: Biology and Role in Toxicology and Disease*. 804:770-772.
- Sheaff, R.J., J.D. Singer, J. Swanger, M. Smitherman, J.M. Roberts, and B.E. Clurman. 2000. Proteasomal turnover of p21(Cip1) does not require p21(Cip1) ubiquitination. *Molecular Cell*. 5:403-410.
- Sherman, M.Y., and A.L. Goldberg. 2001. Cellular defenses against unfolded proteins: A cell biologist thinks about neurodegenerative diseases. *Neuron*. 29:15-32.
- Sitte, N., K. Merker, T. Von Zglinicki, K.J.A. Davies, and T. Grune. 2000. Protein oxidation and degradation during cellular senescence of human BJ fibroblasts: part II - aging of nondividing cells. *Faseb Journal*. 14:2503-2510.
- Skowrya, D., K.L. Craig, M. Tyers, S.J. Elledge, and J.W. Harper. 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*. 91:209-219.
- Smith, J.J., and J.D. Aitchison. 2009. Regulation of peroxisome dynamics. *Curr Opin Cell Biol*. 21:119-126.
- Smith, J.J., and J.D. Aitchison. 2013. Peroxisomes take shape. *Nat Rev Mol Cell Biol*. 14:803-817.

- Smith, J.J., M. Marelli, R.H. Christmas, F.J. Vizeacoumar, D.J. Dilworth, T. Ideker, T. Galitski, K. Dimitrov, R.A. Rachubinski, and J.D. Aitchison. 2002. Transcriptome profiling to identify genes involved in peroxisome assembly and function. *J Cell Biol.* 158:259-271.
- Smith, N., D.J. Adle, M. Zhao, X. Qin, H. Kim, and J. Lee. 2016. Endoplasmic Reticulum-associated Degradation of Pcalp, a Polytopic Protein, via Interaction with the Proteasome at the Membrane. *J Biol Chem.* 291:15082-15092.
- Song, M.S., K. Mihara, Y. Chen, L. Scorrano, and G.W. Dorn. 2015. Mitochondrial Fission and Fusion Factors Reciprocally Orchestrate Mitophagic Culling in Mouse Hearts and Cultured Fibroblasts. *Cell metabolism.* 21:273-285.
- Soto, C. 2003. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nature reviews. Neuroscience.* 4:49-60.
- Sriramachandran, A.M., and R.J. Dohmen. 2014. SUMO-targeted ubiquitin ligases. *Bba-Mol Cell Res.* 1843:75-85.
- Stagg, H.R., M. Thomas, D. van den Boomen, E.J.H.J. Wiertz, H.A. Drabkin, R.M. Gemmill, and P.J. Lehner. 2009. The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER. *Journal of Cell Biology.* 186:685-692.
- Stirling, P.C., V.F. Lundin, and M.R. Leroux. 2003. Getting a grip on non-native proteins. *EMBO Rep.* 4:565-570.
- Stolz, A., W. Hilt, A. Buchberger, and D.H. Wolf. 2011. Cdc48: a power machine in protein degradation. *Trends Biochem Sci.* 36:515-523.
- Sung, M.K., J.M. Reitsma, M.J. Sweredoski, S. Hess, and R.J. Deshaies. 2016. Ribosomal proteins produced in excess are degraded by the ubiquitin-proteasome system. *Molecular Biology of the Cell.* 27:2642-2652.
- Swanson, R., M. Locher, and M. Hochstrasser. 2001. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes Dev.* 15:2660-2674.
- Swatek, K.N., and D. Komander. 2016. Ubiquitin modifications. *Cell research.* 26:399-422.
- Sweeney, H.L., and A. Houdusse. 2010. Structural and Functional Insights into the Myosin Motor Mechanism. *Annual Review of Biophysics, Vol 39.* 39:539-557.
- Swinkels, B.W., S.J. Gould, and S. Subramani. 1992. Targeting efficiencies of various permutations of the consensus C-terminal tripeptide peroxisomal targeting signal. *FEBS Lett.* 305:133-136.
- Takahashi, N., M. Morita, T. Maeda, Y. Harayama, N. Shimozaawa, Y. Suzuki, H. Furuya, R. Sato, Y. Kashiwayama, and T. Imanaka. 2007. Adrenoleukodystrophy: subcellular localization and degradation of adrenoleukodystrophy protein (ALDP/ABCD1) with naturally occurring missense mutations. *Journal of neurochemistry.* 101:1632-1643.
- Tanaka, A., M.M. Cleland, S. Xu, D.P. Narendra, D.F. Suen, M. Karbowski, and R.J. Youle. 2010. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J Cell Biol.* 191:1367-1380.
- Tanaka, K. 2009. The proteasome: Overview of structure and functions. *P Jpn Acad B-Phys.* 85:12-36.
- Tang, F.S., E.J. Kauffman, J.L. Novak, J.J. Nau, N.L. Catlett, and L.S. Weisman. 2003. Regulated degradation of a class V myosin receptor directs movement of the yeast vacuole. *Nature.* 422:87-92.
- Theodoulou, F.L., M. Holdsworth, and A. Baker. 2006. Peroxisomal ABC transporters. *Febs Letters.* 580:1139-1155.
- Thoms, S., I. Harms, K.U. Kalies, and J. Gartner. 2012. Peroxisome formation requires the endoplasmic reticulum channel protein Sec61. *Traffic.* 13:599-609.

- Thrower, J.S., L. Hoffman, M. Rechsteiner, and C.M. Pickart. 2000. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19:94-102.
- Tran, J.R., and J.L. Brodsky. 2012. A Cdc48p-associated factor modulates endoplasmic reticulum-associated degradation, cell stress, and ubiquitinated protein homeostasis. *Faseb Journal.* 26.
- Tsoi, H., T.C. Lau, S.Y. Tsang, K.F. Lau, and H.Y. Chan. 2012. CAG expansion induces nucleolar stress in polyglutamine diseases. *Proc Natl Acad Sci U S A.* 109:13428-13433.
- Urquhart, A.J., D. Kennedy, S.J. Gould, and D.I. Crane. 2000. Interaction of Pex5p, the type 1 peroxisome targeting signal receptor, with the peroxisomal membrane proteins Pex14p and Pex13p. *Journal of Biological Chemistry.* 275:4127-4136.
- Uversky, V.N. 2007. Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation. *Journal of neurochemistry.* 103:17-37.
- van der Klei, I.J., R.E. Hilbrands, J.A.K.W. Kiel, S.W. Rasmussen, J.M. Cregg, and M. Veenhuis. 1998. The ubiquitin-conjugating enzyme Pex4p of *Hansenula polymorpha* is required for efficient functioning of the PTS1 import machinery. *Embo Journal.* 17:3608-3618.
- van der Klei, I.J., H. Yurimoto, Y. Sakai, and M. Veenhuis. 2006. The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Bba-Mol Cell Res.* 1763:1453-1462.
- van der Zand, A., I. Braakman, and H.F. Tabak. 2010. Peroxisomal Membrane Proteins Insert into the Endoplasmic Reticulum. *Molecular Biology of the Cell.* 21:2057-2065.
- van Roermund, C.W.T., W.F. Visser, L. Ijlst, A. van Cruchten, M. Boek, W. Kulik, H.R. Waterham, and R.J.A. Wanders. 2008. The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. *Faseb Journal.* 22:4201-4208.
- van Zutphen, T., M. Veenhuis, and I.J. van der Klei. 2011. Damaged peroxisomes are subject to rapid autophagic degradation in the yeast *Hansenula polymorpha*. *Autophagy.* 7:863-872.
- Varshavsky, A. 1991. Naming a Targeting Signal. *Cell.* 64:13-15.
- Varshavsky, A. 2005. Regulated protein degradation. *Trends Biochem Sci.* 30:283-286.
- Varshavsky, A. 2019. N-degron and C-degron pathways of protein degradation. *Proceedings of the National Academy of Sciences of the United States of America.* 116:358-366.
- Vashist, S., and D.T. Ng. 2004. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J Cell Biol.* 165:41-52.
- Veenhuis, M., and J.M. Goodman. 1990. Peroxisomal Assembly - Membrane Proliferation Precedes the Induction of the Abundant Matrix Proteins in the Methylotrophic Yeast *Candida Boidinii*. *Journal of Cell Science.* 96:583-590.
- Walker, C.L., L.C.D. Pomatto, D.N. Tripathi, and K.J.A. Davies. 2018. Redox Regulation of Homeostasis and Proteostasis in Peroxisomes. *Physiological Reviews.* 98:89-115.
- Walker, L.C., and H. LeVine. 2012. Corruption and Spread of Pathogenic Proteins in Neurodegenerative Diseases. *Journal of Biological Chemistry.* 287:33109-33115.
- Wanders, R.J.A., W.F. Visser, C.W.T. van Roermund, S. Kemp, and H.R. Waterham. 2007. The peroxisomal ABC transporter family. *Pflug Arch Eur J Phy.* 453:719-734.
- Wanders, R.J.A., and H.R. Waterham. 2006. Biochemistry of mammalian peroxisomes revisited. *Annual Review of Biochemistry.* 75:295-332.
- Wang, X., S. Li, Y. Liu, and C. Ma. 2015. Redox regulated peroxisome homeostasis. *Redox biology.* 4:104-108.
- Ward, S.M., D.S. Himmelstein, J.K. Lancia, and L.I. Binder. 2012. Tau oligomers and tau toxicity in neurodegenerative disease. *Biochem Soc Trans.* 40:667-671.

- Waterham, H.R., S. Ferdinandusse, and R.J. Wanders. 2016. Human disorders of peroxisome metabolism and biogenesis. *Biochim Biophys Acta*. 1863:922-933.
- Weir, N.R., R.A. Kamber, J.S. Martenson, and V. Denic. 2017. The AAA protein Msp1 mediates clearance of excess tail-anchored proteins from the peroxisomal membrane. *eLife*. 6.
- Wenzel, D.M., A. Lissounov, P.S. Brzovic, and R.E. Klevit. 2011. UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature*. 474:105-U136.
- Wiertz, E.J., D. Tortorella, M. Bogoy, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*. 384:432-438.
- Wilkens, S. 2015. Structure and mechanism of ABC transporters. *F1000Prime Rep*. 7:14.
- Wilkinson, C.R.M., M. Seeger, R. Hartmann-Petersen, M. Stone, M. Wallace, C. Semple, and C. Gordon. 2001. Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nature Cell Biology*. 3:939-943.
- Willems, A.R., S. Lanker, E.E. Patton, K.L. Craig, T.F. Nason, N. Mathias, R. Kobayashi, C. Wittenberg, and M. Tyers. 1996. Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. *Cell*. 86:453-463.
- Williams, A., S. Sarkar, P. Cuddon, E.K. Ttofi, S. Saiki, F.H. Siddiqi, L. Jahreiss, A. Fleming, D. Pask, P. Goldsmith, C.J. O'Kane, R.A. Floto, and D.C. Rubinshtein. 2008a. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nature chemical biology*. 4:295-305.
- Williams, C. 2014. Going against the flow: a case for peroxisomal protein export. *Biochim Biophys Acta*. 1843:1386-1392.
- Williams, C., and B. Distel. 2006. Pex13p: Docking or cargo handling protein? *Biochim Biophys Acta*. 1763:1585-1591.
- Williams, C., and B. Distel. 2010. Protein Quality Control in Peroxisomes: Ubiquitination of the Peroxisomal Targeting Signal Receptors. In *Handbook of Cell Signaling*. Vol. 3. R.A. Bradshaw and E.A. Dennis, editors. Academic press. 2489-2497.
- Williams, C., M. van den Berg, E. Geers, and B. Distel. 2008b. Pex10p functions as an E3 ligase for the Ubc4p-dependent ubiquitination of Pex5p. *Biochem Biophys Res Commun*. 374:620-624.
- Williams, C., M. van den Berg, S. Panjikar, W.A. Stanley, B. Distel, and M. Wilmanns. 2012. Insights into ubiquitin-conjugating enzyme/ co-activator interactions from the structure of the Pex4p:Pex22p complex. *EMBO J*. 31:391-402.
- Williams, C., M. van den Berg, R.R. Sprenger, and B. Distel. 2007. A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p. *J Biol Chem*. 282:22534-22543.
- Williams, C., and I.J. van der Klei. 2013. Pexophagy-linked degradation of the peroxisomal membrane protein Pex3p involves the ubiquitin-proteasome system. *Biochem Biophys Res Commun*. 438:395-401.
- Winklhofer, K.F., J. Tatzelt, and C. Haass. 2008. The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. *EMBO Journal*. 27:336-349.
- Wohlever, M.L., A. Mateja, P.T. McGilvray, K.J. Day, and R.J. Keenan. 2017. Msp1 Is a Membrane Protein Dislocase for Tail-Anchored Proteins. *Molecular Cell*. 67:194-+.
- Wroblewska, J.P., L.D. Cruz-Zaragoza, W. Yuan, A. Schummer, S.G. Chuartzman, R. de Boer, S. Oeljeklaus, M. Schuldiner, E. Zalckvar, B. Warscheid, R. Erdmann, and I.J. van der Klei. 2017. *Saccharomyces cerevisiae* cells lacking Pex3 contain membrane vesicles that harbor a subset of peroxisomal membrane proteins. *Bba-Mol Cell Res*. 1864:1656-1667.
- Wu, X., L.L. Li, and H. Jiang. 2016. Doa1 targets ubiquitinated substrates for mitochondria-associated degradation. *Journal of Cell Biology*. 213:49-63.

- 1
- Yamashita, S.I., K. Abe, Y. Tatemichi, and Y. Fujiki. 2014. The membrane peroxin PEX3 induces peroxisome-ubiquitination-linked pexophagy. *Autophagy*. 10:1549-1564.
- Yau, R., and M. Rape. 2016. The increasing complexity of the ubiquitin code. *Nature Cell Biology*. 18:579-586.
- Yau, R.G., Y.T. Peng, R.R. Valiathan, S.R. Birkeland, T.E. Wilson, and L.S. Weisman. 2014. Release from Myosin V via Regulated Recruitment of an E3 Ubiquitin Ligase Controls Organelle Localization. *Developmental Cell*. 28:520-533.
- Ye, Y., H.H. Meyer, and T.A. Rapoport. 2001. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature*. 414:652-656.
- Yonekawa, S., A. Furuno, T. Baba, Y. Fujiki, Y. Ogasawara, A. Yamamoto, M. Tagaya, and K. Tani. 2011. Sec16B is involved in the endoplasmic reticulum export of the peroxisomal membrane biogenesis factor peroxin 16 (Pex16) in mammalian cells. *Proc Natl Acad Sci U S A*. 108:12746-12751.
- Yoo, Y.S., Y.Y. Park, J.S. Lee, and H. Cho. 2016. The mitochondrial E3 ubiquitin ligase MARCH5 resolves MAVS aggregates during antiviral signaling. *Journal of Immunology*. 196.
- Yoshii, S.R., C. Kishi, N. Ishihara, and N. Mizushima. 2011. Parkin Mediates Proteasome-dependent Protein Degradation and Rupture of the Outer Mitochondrial Membrane. *Journal of Biological Chemistry*. 286:19630-19640.
- Zattas, D., J.M. Berk, S.G. Kreft, and M. Hochstrasser. 2016. A Conserved C-terminal Element in the Yeast Doa10 and Human MARCH6 Ubiquitin Ligases Required for Selective Substrate Degradation. *J Biol Chem*. 291:12105-12118.
- Zhang, X., C. De Marcos Lousa, N. Schutte-Lensink, R. Ofman, R.J. Wanders, S.A. Baldwin, A. Baker, S. Kemp, and F.L. Theodoulou. 2011. Conservation of targeting but divergence in function and quality control of peroxisomal ABC transporters: an analysis using cross-kingdom expression. *Biochem J*. 436:547-557.
- Zhang, Y., G. Nijbroek, M.L. Sullivan, A.A. McCracken, S.C. Watkins, S. Michaelis, and J.L. Brodsky. 2001. Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. *Mol Biol Cell*. 12:1303-1314.
- Zolman, B.K., M. Monroe-Augustus, I.D. Silva, and B. Bartel. 2005. Identification and functional characterization of Arabidopsis PEROXIN4 and the interacting protein PEROXIN22. *Plant Cell*. 17:3422-3435.

A large, stylized white number '2' is centered on a black, ink-splattered background. The background has a rough, textured appearance with white speckles and splatters, giving it a high-contrast, graphic look. The number '2' is a simple, bold, sans-serif font.

Chapter 2

Investigating peroxisomal membrane protein turnover with a tandem fluorescent timer

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Author contributions

CW conceived and supervised the project, with input from MK. CW and SD designed the experiments. SD and CW analysed the data with the help from other authors.

SD performed biochemical and fluorescence microscopy experiments. SD and MM performed and analysed tF²T stability assay. SD and CW wrote the manuscript with the contribution from all the authors.

Abstract

Protein homeostasis is the balance between protein synthesis and degradation. Changes in protein homeostasis have a severe impact on cell health and can lead to severe human diseases such as Parkinson's disease. Despite the significance of peroxisomes in human health, very little is known about the degradation of peroxisomal membrane proteins (PMPs). Therefore, we were interested in developing a technique that can be used to investigate the stability of PMPs in *S. cerevisiae* cells *in vivo*. A tandem fluorescent protein timer (tFT) is a powerful tool for assessing protein degradation in living cells. A tFT is a fusion of two different fluorescent proteins with distinct fluorophore maturation kinetics. When coupled to a protein of interest, the stability of the protein of interest can be determined from the ratio of fluorescent intensities of the two fluorescent domains. In this chapter, we have validated the tFT approach for investigating the degradation of PMPs in *S. cerevisiae*. Our data demonstrates that tFT tagging does not appear to affect the induction or peroxisomal localization of the tFT-tagged PMPs we tested, two major characteristics of PMPs that are crucial for peroxisome biogenesis. Furthermore, we show that the tFT approach has the potential to report on the stability of PMPs as well as to identify unstable PMPs that undergo rapid degradation. In addition, we present evidence, which demonstrates that our tFT approach can provide insights into the reasons why PMP degradation occurs.

Keywords: tFT, protein stability, peroxisome, PMP turnover

Introduction

Cellular proteins are being continually synthesized and degraded. Alterations to this equilibrium, for instance due to reduced or increased degradation, often results in changes to cellular protein abundance, which can dramatically impact on cell health. As an example, disturbances in the degradation of cell cycle control proteins such as the p53 tumour suppressor plays a vital role in many forms of human cancers (Nakayama and Nakayama, 2006). Also, accumulation of damaged or misfolded mitochondrial and Endoplasmic reticulum (ER) proteins in the cell are linked to neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases (Heo and Rutter, 2011; Matus et al., 2011; Morimoto, 2008). The impaired degradation of ER or mitochondrial proteins, resulting in protein aggregation, often disturbs the normal physiology of the corresponding organelles, leading to dysfunctional mitochondria or ER, which can dramatically affect cell health. These examples demonstrate that correct regulation of protein degradation plays a vital role not only in regulating protein homeostasis and the underlying protein function but also in cell health.

Peroxisomes are organelles found in virtually all eukaryotic cells, encompassing a multitude of proteins that are involved in various metabolic processes, depending on the species and/or cell type (Gabaldon, 2010). Common functions of peroxisomes include fatty acid β -oxidation, lipid biosynthesis and de-toxification of hydrogen peroxide but many more exist (Hiltunen et al., 2003; Islinger et al., 2012; Smith and Aitchison, 2013). The significance of peroxisomes in humans is demonstrated by the fact that deficiencies in peroxisomes often lead to lethal human diseases such as Adrenoleukodystrophy or Zellweger syndrome (Waterham et al., 2016). Despite the importance of peroxisomes in human health, little is known about peroxisomal protein homeostasis.

Depending on conditions, peroxisomes can undergo dynamic changes not only in size, shape and number but also in protein composition (Lingard et al., 2009; Ribeiro et al., 2012; Schrader et al., 2012; Wang et al., 2015). More than 30 proteins, collectively termed as peroxins and encoded by *PEX* genes, are involved in the biogenesis of peroxisomes (Distel et al., 1996; Smith and Aitchison, 2009). The majority of these peroxins are peroxisomal membrane proteins (PMPs), which are involved in various aspects of peroxisome biogenesis, including peroxisome formation, division of peroxisomes and peroxisomal matrix and membrane protein import (Baerends et al., 2000a; Schrader and Fahimi, 2008; Smith and Aitchison, 2013). Because PMPs play a vital role in regulating peroxisome biogenesis and function, there must be a tight control of PMP levels, through the translation and degradation of PMPs. The expression of *PEX* genes encoding for PMPs is regulated and depends, for example, on the nature of nutrients available to the cell. For the yeast *Saccharomyces cerevisiae*, it is well known that the expression of genes encoding for PMPs involved in fatty acid β -oxidation is low when cells grow on glucose but is

highly induced when fatty acids such as oleate are provided as sole carbon source (Gurvitz and Rottensteiner, 2006; Karpichev and Small, 1998; Smith et al., 2002). However, a subset of genes coding for PMPs that are essential for peroxisome formation and maintenance are expressed under peroxisome repressing conditions and their expression is only moderately enhanced on oleate (Rottensteiner et al., 2003a). These examples demonstrate that PMP regulation occurs at the level of transcription and that it can be specific for individual PMPs.

While the transcription dependent regulation of PMPs has been the subject of many studies, the stability or the degradation of PMPs is less well studied. Recently, the PMPs Pex3p and Pex13p from the yeast *Hansenula polymorpha* were shown to be removed from peroxisomes and subsequently degraded by the Ubiquitin Proteasome System (UPS). While Pex3p removal was shown to initiate pexophagy (Bellu et al., 2002; Williams and van der Klei, 2013), the degradation of Pex13p was suggested to have a role in matrix protein import (Chen et al., 2018). Furthermore, the levels of Inp1p and Inp2p, proteins involved in peroxisome inheritance in yeast, were shown to fluctuate during the cell cycle in a pattern that parallels the dynamics of peroxisomes (Fagarasanu et al., 2006; Fagarasanu et al., 2005), suggesting that the turnover of these proteins could act as a signalling mechanism during peroxisome inheritance. However, the degradation of Inp1p and Inp2p has not been investigated further. Peroxisomes also generate large amounts of reactive oxygen species (ROS) such as hydrogen peroxide (Schrader and Fahimi, 2006). Therefore, it is highly likely that PMPs residing in this ROS rich environment are vulnerable to oxidative damage yet little is known about the degradation of damaged PMPs. Hence, our knowledge on PMP degradation is far from complete but it is highly likely that, in addition to Pex3p and Pex13p, other PMPs are targeted for degradation because they are non-functional, damaged or because their degradation regulates a peroxisomal process (Williams, 2014). Therefore, investigating the degradation of individual PMPs would allow us to understand how PMPs are regulated, to what extent PMP degradation is involved in regulating peroxisome function and what would be the impact of an imbalance in PMP homeostasis on peroxisome function.

As a first step towards investigating PMP degradation, it is mandatory to know globally which PMPs undergo degradation. An array of methodologies exist that would allow PMP degradation to be followed in cell cultures. Pulse-chase analysis, a classical method involving the radioactive labelling of a protein for a short period of time (pulse) and the decay in radioactivity measured over time (chase) can be used to infer protein half-life (Schimke and Doyle, 1970). Radiolabelling, combined with mass spectrometry, allows the turnover of a large number of proteins to be analysed in parallel (Pratt et al., 2002). Protein stability can also be assayed by blocking protein synthesis using cycloheximide, after which the levels of the protein of interest present are determined by immunoblotting (Belle et al., 2006). However, both these methods are less suitable for monitoring protein turnover in living cells, a feature important for

a systems level understanding of protein function. To measure the rate of protein turnover in living cells, several different techniques exist, two of which are discussed here.

The first method employs a photo-switchable fluorescent protein. Here, the protein of interest is tagged with a fluorescent protein, which can either acquire (photo-activation), change (photo-conversion) or lose (photo-bleaching) fluorescence by illuminating the protein population with a high-intensity laser light (Geva-Zatorsky et al., 2012; Lippincott-Schwartz et al., 2003; Zhang et al., 2007). Followed by irradiation, the decrease in fluorescence signal is monitored over time and the measured signal intensities are fitted to an appropriate model, thus yielding turnover or half-life estimates of protein fusions.

The second approach employs a single fluorescent protein that acts as a timer (Tersikh et al., 2000). In this method, a fluorescent protein (which is fused to a protein of interest) changes colour over time as it undergoes maturation. The process by which a fluorescent protein acquires its chromophore is termed as maturation and the time needed to form the chromophore is called maturation time (Iizuka et al., 2011). The ratio of fluorescence intensities of the second and first fluorescent state (i.e. the second and first colour that the fluorescent protein adopts) provides a measure of protein age and thereby reports on the stability of the tagged protein. However, photo-switchable fluorescent proteins and single fluorescent timer have several shortcomings, including the photo toxic effect caused by high laser light, the low brightness of certain fluorophores or their tendency to form oligomers, which can influence the behaviour of tagged proteins (Dobrucki et al., 2007; Subach et al., 2009; Tersikh et al., 2000; Tsuboi et al., 2010).

A recently described approach, involving the use of a tandem fluorescence timer (tFT), can overcome some of these limitations (Khmelinskii et al., 2012). The tFT technique employs two monomeric bright fluorescent proteins with different maturation kinetics: a fast maturing green (e.g. GFP) and a slow maturing red (e.g. mCherry) fluorescent protein. The intensity ratio between the slow and fast maturing fluorescent domains (mCherry/GFP) reports on the stability of a protein fused to tFT (Khmelinskii et al., 2012). Because a tFT consists of a fast maturing fluorescent domain it enables us to identify unstable fusion proteins with a rapid turnover while at the same time display the potential to track most stable or long-lived proteins by the use of red fluorescence.

In this chapter, we have validated the suitability of the tFT approach to investigate PMP turnover. Our data demonstrates that tFT analysis is a valid and robust approach to examine PMP stability and identify unstable PMPs with a fast turnover.

Results

Tagging peroxisomal membrane proteins (PMPs) with a tandem fluorescent timer (tFT)

The tandem fluorescent timer (tFT) approach has been previously used to investigate protein turnover in living cells of different organisms, including *S. cerevisiae* and Zebrafish (Barry et al., 2016; Dona et al., 2013; Khmelinskii et al., 2012). We wanted to determine the suitability of the tFT approach in investigating the turnover of PMPs *in vivo* in *S. cerevisiae*. The tFT tag is composed of super folded green fluorescent protein (sfGFP), which is a rapidly folding and fast maturing fluorescent protein with a half-life of around 6 mins (Khmelinskii et al., 2012; Khmelinskii et al., 2016; Pedelacq et al., 2006) and mCherry, which matures with a half-time of around 40 mins (Khmelinskii et al., 2012; Merzlyak et al., 2007). The ratio of mCherry to sfGFP intensities (mCherry/sfGFP) provides a measure of the age of the tFT fusion protein and thereby its stability (Khmelinskii et al., 2012). We fused a tFT tag to different PMPs at their C-terminus and expressed PMP-tFT fusions under the control of their endogenous promoter (Figure 1A and 1C). Shortly after the synthesis of the PMP-tFT fusion, tFT tagged PMP molecules will mostly display green fluorescence (Figure 1A, step 1) and gradually display red fluorescence over time (Figure 1A, step 2). PMP-tFT fusions undergoing fast turnover will mostly be degraded prior to mCherry maturation, resulting in a pool of newly synthesized PMP-tFT molecules exhibiting low red fluorescence (Figure 1B, left). The mCherry/sfGFP ratio would therefore be lower and fusion proteins are said to be unstable. By contrast, the intensity of mCherry and the ratio would be higher for stable PMP-tFT fusions with a slow turnover (Figure 1B, right).

By using the tFT approach, we assessed the stability of PMPs and identified unstable PMPs in *S. cerevisiae*. For this, we fused a tFT tag to 15 PMPs, in order to examine the stability of individual PMP-tFT protein fusions. PMPs selected for tFT tagging, which are involved in various peroxisomal functions, include Pex3p (involved in PMP import) and Pex13p, Pex14p and Pex17p (all are members of the docking complex required for peroxisomal matrix protein import) (Brown and Baker, 2003; Subramani et al., 2000). In addition, we also selected Pex11p, Pex25p and Pex27p (three PMPs involved in peroxisome fission) and Inp1p and Inp2p (both required for peroxisome inheritance) (Fagarasanu et al., 2010; Schrader et al., 2012). The peroxisomal E3 ligase complex consisting of Pex2p, Pex10p and Pex12p (required for recycling and degradation of peroxisomal matrix protein receptor Pex5p) and peroxisomal E2 anchor-Pex22p (involved in Pex5p recycling) were also selected for tFT tagging (El Magraoui et al., 2014; Platta et al., 2009; Williams et al., 2008). Finally, we fused a tFT tag to the fatty acid transporters Pxa1p and Ant1p, which are involved in transporting long and medium chain fatty acids to peroxisomes, respectively (Baker et al., 2015; van Roermund et al., 2012).

Compared to conventional gene tagging, which involves the introduction of a tag and marker sequence together into the desired chromosomal loci (Wach et al., 1997), we used an

efficient endonuclease-driven approach for tFT tagging of PMPs (Khmelniskii et al., 2011). This method involves the use of I-SceI meganuclease to remove all the auxiliary sequences including the marker coding sequences, that are reported to affect endogenous gene expression by changing mRNA abundance, stability or localization (Lind and Norbeck, 2009). By using this tagging approach, we seamlessly fused a tFT tag to the PMPs of interest by homologous recombination (Figure 1C). I-SceI targets a rare 18-base pair sequence absent from the genome of *S. cerevisiae* (Colleaux et al., 1986). In this tagging approach, the tFT module consists of full length mCherry coding sequence followed by a I-SceI site, selection marker (*URA3* gene), second I-SceI site and a portion of mCherry (*mCherry^{ΔN}*) and full length sfGFP coding sequence (Figure 1C). Here, *mCherry^{ΔN}* (mCherry sequence lacking last 91 amino acids) is used for homologous recombination (see below- Figure 1C step 3). First, the tagging module is amplified in a PCR reaction with short overhangs homologous to C-terminus of the PMP locus and integrated in to the genome of *S. cerevisiae* which expresses I-SceI under a galactose inducible promoter (*GALI*) (Figure 1C, step 1). The positive clones are selected using URA marker and correct integration of tFT module is checked by colony PCR. Followed by correct module integration, cells expressing PMP-tFT fusions are grown on galactose containing medium to induce the expression of I- SceI, resulting in the excision of heterologous terminator sequences and URA marker (Figure 1C step 2). The double stranded break can then be repaired by homologous recombination between the mCherry and *mCherry^{ΔN}* sequences (Figure 1C step 3). This effectively removes all the auxiliary sequences from the integrated module, leaving a single copy of the tFT tagged PMP in the genome. Cell expressing tFT fusions will exhibit sfGFP fluorescence only after URA marker excision and homologous recombination between mCherry sequences.

Figure 1: Tagging peroxisomal membrane proteins (PMPs) with a tandem fluorescent timer (tFT)

A) A PMP is fused to a tandem fluorescent timer (tFT) tag, which consists of two fluorescent domains with distinct fluorophore maturation rates. Super folded GFP (sfGFP) matures approximately with a half-time of ~6 mins

(indicated by 1) whereas, mCherry matures with a half-time of ~40 mins (indicated by 2). The ratio of the mCherry/sfGFP fluorescent signals reports on the stability of a tFT tagged PMP (PMP-tFT) fusion protein.

B) For PMP-tFT fusions with fast turnover, peroxisomes contain large fraction of PMPs displaying higher green fluorescence compared to mCherry, resulting in low mCherry/sfGFP ratio. For PMP-tFT fusions with slow turnover, tFT tagged PMPs in peroxisomes exhibit high green and red fluorescence, leading to higher mCherry/sfGFP ratio. tFT tagged PMPs with low and high mCherry/sfGFP ratio are therefore categorized as unstable and stable proteins respectively.

C) The seamless gene tagging approach was used to construct PMP-tFT strains. Strains also expressed the I-SceI meganuclease under a galactose inducible promoter. First, a module for the C-terminal tagging of a PMP with a mCherry-sfGFP tFT is integrated into the genome locus of the PMP of interest. Subsequent I-SceI expression leads to the excision of heterologous terminator (term- indicated in red) and the *URA3* selection marker. Followed by this, the repair of the double strand break occurs by homologous recombination between the *mCherry* and *mCherry*^{ΔN} sequences. In the final strains, the tFT fusions (PMP-tFT) are expressed under the control of their endogenous promoter and terminator sequences.

D) Table indicating which PMPs were tagged with a tFT in our study.

The stability of tFT tagged proteins vary, ranging from unstable to highly stable

Following seamless tFT tagging of 15 individual PMPs, we assessed the stability of each PMP-tFT fusion *in vivo* under two different conditions: peroxisome repressing (glucose – Figure 2A) and peroxisome inducing conditions (oleate medium containing 0.1% glucose – Figure 2B). Since most PMPs tested here play a crucial role in peroxisome biogenesis, any effect due to tFT tagging of PMPs could affect peroxisome function resulting in reduced growth on oleate. Therefore, 0.1% glucose was added to our oleate medium (hereafter referred to as oleate-plus medium) to facilitate proper growth (see materials and methods for details). In *S cerevisiae*, peroxisomes are the sole compartment for β -oxidation of fatty acids such as oleate. Growth on oleate induces the expression of several genes encoding for peroxisomal proteins (including PMPs) involved in fatty acid oxidation and it also stimulates the import of matrix enzymes into peroxisomes required for fatty acid oxidation (Gurvitz and Rottensteiner, 2006). This oleate dependent induction is accompanied by a proliferation of peroxisomes (Veenhuis et al., 1987). By contrast, growth on fermentable carbon sources such as glucose represses the expression of genes encoding for proteins (including PMPs) involved in β -oxidation and therefore peroxisomes are few in numbers. Since a subset of PMPs are present only in low levels on glucose conditions (Karpichev and Small, 1998), it would be interesting to know whether PMP levels under these conditions are regulated by degradation. Therefore, we examined the stability of PMPs involved in different peroxisomal functions (Figure 1D) under peroxisome repressing and peroxisome inducing conditions. We used yMaM344, a strain expressing truncated non-fluorescent mCherry^{ΔN} protein (Khmelinskii et al., 2014) as negative control. The fluorescence intensities of mCherry and sfGFP were measured every day for four days, from whole colonies of cells expressing PMP-tFT fusions and were corrected for auto fluorescence using intensity

values of the negative control strain (see materials and methods). The normalized mCherry and sfGFP values were then used to calculate mCherry/sfGFP ratios in cells expressing individual PMP-tFT fusions (see materials and methods for further details). The ratios obtained depict the relative stability of PMP-tFT fusions and are displayed in a heat map (Figure 2), colour coded from dark green (mCherry/sfGFP of 0) to red (mCherry/sfGFP of 2). We categorized PMP-tFT fusions with mCherry/sfGFP ratios lower than 1.0 as unstable proteins.

We observed that individual PMP-tFT fusion proteins display different stability profiles (Figure 2A and 2B). Under peroxisome repressing conditions Pex2-tFT, Pex13-tFT, Pxa1-tFT, Ant1-tFT and Inp2-tFT displayed low mCherry/sfGFP ratios, ranging from 0.5 to 0.9 on indicated days (Figure 2A). On the other hand, Pex11-tFT appeared to be the most stable tFT fusion, with the highest ratio ranging between 1.5 and 1.8. The remaining PMP-tFT fusions were categorized as stable proteins as they exhibited ratios ranging from 1.0 to 1.5 (Figure 2A). Similarly, under peroxisome inducing conditions (Figure 2B), Pex2-tFT, Pxa1-tFT, Pex13-tFT and Inp1-tFT were observed to have low mCherry/sfGFP ratios (<1.0), indicating that these four proteins are unstable fusion proteins and could undergo degradation in cells grown on oleate containing medium. Again, Pex11-tFT appeared to be the most stable tFT fusion with ratios ranging between 1.7 and 2.0 on indicated days. The remaining ten of the fifteen PMP-tFT fusions are categorized as stable proteins as they displayed ratios between 1.0 and 1.7.

Furthermore, we observed that most PMP-tFT fusions in cells grown on oleate-plus plates displayed lower ratios on day three and four compared to days one and two (Figure 2B). *S. cerevisiae* cells grown on oleate-plus liquid medium reach stationary phase after approximately 24 hrs (Kawalek et al., 2016) compared to growth on oleate liquid medium, where the cells require around 64 hrs (~2.5 days) to reach stationary phase (Al-Saryi et al., 2017). While it is challenging to determine the growth phase of cells growing on plates, we suspect that cells expressing PMP-tFT fusions would be in the later stages of growth after three or four days on oleate-plus plates. At this stage, most of the oleate will have been utilized and there will be little oleate available to induce the oleate response element (ORE) involved in the upregulation of a subset of PMPs (Gurvitz and Rottensteiner, 2006). This may result in reduced tFT fusion protein expression and thereby a reduced ratio at later time points (Figure 2B- day 3 and day 4). Furthermore, the mCherry/sfGFP ratio in Inp2-tFT cells grown on oleate-plus medium was very low (<0.3) and the ratio on days one and four was observed to be negative (Figure 2B, indicated in grey). The mCherry intensity values of Inp2-tFT were similar or slightly lower compared to background (WT) fluorescence, resulting in negative mCherry/sfGFP ratio. This suggests that Inp2-tFT levels in oleate-plus medium are below the detection limit of the fluorescence-based plate reader.

Based on our tFT approach, we concluded that Pxa1-tFT and Pex13-tFT are unstable proteins, suggesting that they could possibly undergo degradation. The peroxisomal fatty acid transporter Pxa1p is a homologue of the human protein ALDP (Adrenoleukodystrophy protein)

(Shani et al., 1995) and mutations in ALDP leads to severe disease called ALD (Adrenoleukodystrophy) (Engelen et al., 2014; Mosser et al., 1993). Many ALD causing ALDP mutants are targeted for degradation yet the mechanisms underlying ALDP instability are unknown (Morita et al., 2018). Hence, studying how Pxa1p degradation is facilitated could provide new insights on ALDP mutant degradation in ALD. Furthermore, the degradation of Pex13p and UPS proteins involved in Pex13 degradation has already been reported in *H. polymorpha* (Chen et al., 2018), making it interesting to investigate the instability of tFT tagged *S. cerevisiae* Pex13p further.

Figure 2: The stability of tFT tagged PMPs under peroxisome inducing and repressing conditions.

Heat map representing the stability of PMP-tFT fusions in A) Glucose (peroxisome repressing medium) and B) Oleate-plus medium (peroxisome inducing medium). mCherry and sfGFP fluorescence was measured from whole colonies of cells expressing PMP-tFT fusions at the indicated time points (days 1-4). The fluorescent intensities obtained on days 1, 2, 3 and 4 from each strain were used to calculate mCherry/sfGFP ratios. mCherry/sfGFP ratios representing the stability of PMP-tFT fusions are colour coded from green (unstable) to red (highly-stable). Grey indicates a negative mCherry/sfGFP ratio (see text for details).

Pex13-tFT is functional while tFT tagged Pxa1p exhibits reduced functionality

Next, we wanted to validate our tFT setup and for this, we selected two unstable PMP-tFT fusions- Pex13-tFT and Pxa1-tFT fusion proteins identified from our stability analysis (Figure 2). We examined whether tFT tagging to Pxa1p and Pex13p affects the functionality of these proteins. Because PMP-tFT fusions were constructed with a seamless gene tagging approach, PMP-tFT fusions are the only copy of the PMP present in the cell. Therefore, the functionality of PMP-tFT fusions can be easily assessed. Since Pex13p is involved in the import of peroxisomal matrix enzymes required for the β -oxidation of fatty acids (Hiltunen et al., 2003),

the loss of Pex13p leads to a block in matrix protein import and thereby contributes to loss of functional peroxisomes (Elgersma et al., 1996; Erdmann and Blobel, 1996). We reasoned that if tFT tagging to Pex13p affects its functionality, cells would be unable to utilize oleate for growth. Unlike *pex13* cells, which display a growth defect on media containing oleate as sole carbon source (unlike oleate-plus media, which contains glucose), the growth of cells expressing Pex13-tFT was similar to WT (Figure 3A), establishing that Pex13-tFT is functional.

Compared to Pex13p, loss of Pxa1p function does not lead to a growth defect in cells because its function (oleate transport to peroxisomes for β -oxidation) can be taken over by the fatty acyl-CoA synthetase Faa2p (Hettema et al., 1996). Therefore, in order to assess the functionality of Pxa1p alone, we performed growth experiments with cells lacking Faa2p. As expected *pxa1faa2* cells were unable to grow on oleate (Figure 3B). Interestingly, *faa2* cells expressing Pxa1-tFT (*faa2.Pxa1-tFT*) displayed poorer growth on oleate compared to WT cells (Figure 3B). In order to examine the extent to which growth of *faa2.Pxa1-tFT* cells are affected, we also assessed the growth of this strain on oleate liquid medium (Figure 3C). *faa2.Pxa1-tFT* cells grew better compared to *pxa1faa2* or *pex4* cells. However, the growth of *faa2.Pxa1-tFT* was slower compared to WT cells, suggesting that Pxa1-tFT retains only a certain degree of functionality (Figure 3C). Overall, our growth assays suggest that Pex13-tFT is functional while also indicating that Pxa1-tFT is reduced in functionality.

Figure 3: Growth of cells expressing Pex13-tFT and Pxa1-tFT on oleate medium. A) Ten-fold serial dilutions of WT and Pex13-tFT cells, together with *pex13* and *pex4* cells were spotted on oleate plates and grown at 30° C for 7 days. *pex13* and *pex4* cells act as negative control. B) Spot assay to test the growth of tFT tagged Pxa1p in *faa2* cells. Ten-fold serial dilutions of WT and *faa2* cells producing Pxa1-tFT (*faa2.Pxa1-tFT*) together with *pxa1faa2* and *pex4* cells were spotted on oleate plates and grown at 30° C for 9 days. *pxa1faa2* and *pex4* cells acts as negative control. C) The functionality of Pxa1-tFT was also tested by checking the growth of WT and *faa2.Pxa1-tFT* cells on oleate medium (0, 6, 14, 18, 24, 30, 38, 48, 60 and 74 hours). The growth of *pxa1 faa2* and *pex4* cells acts as control. Values represent the mean $\pm$ SD of two independent experiments.

PMP-tFT fusions are induced under peroxisome inducing conditions

Following on from our functionality analysis, we next assessed whether the tFT tagged PMPs are induced under peroxisome inducing conditions. For this, we checked Pxa1-tFT and Pex13-tFT levels after shifting the cells from peroxisome repressing (glucose) to peroxisome inducing conditions (oleate-plus medium). Pxa1-tFT levels were observed to be very low in cells grown

on glucose (Figure 4A- 0h) while the levels were upregulated in oleate-plus medium with only a minor decrease in protein levels after 24 hours of growth (Figure 4A- 8h, 16h and 24h). This is in line with previous studies where they reported that genes coding for peroxisomal β -oxidation proteins such as Pxa1p are repressed by glucose but are induced several fold in the presence of fatty acid containing medium such as oleate (Gurvitz and Rottensteiner, 2006; Karpichev et al., 2008; Karpichev and Small, 1998; Rottensteiner et al., 2002). Hence, it is difficult to draw conclusions from the data on Pxa1-tFT stability gained from cells grown on glucose medium because of the low levels of Pxa1-tFT present under these conditions. By contrast, Pxa1-tFT levels are well induced and easily detectable on oleate-plus medium (Figure 4A), demonstrating that Pxa1 turnover can be studied effectively under this condition using tFT approach.

Pex13-tFT on the other hand was expressed in cells grown on glucose and oleate-plus medium (Figure 4B). The levels were higher at 8 hours of growth on oleate containing media, after which Pex13-tFT levels started to decrease (Figure 4B- 16h and 24h). This pattern of Pex13-tFT induction in glucose and oleate-plus medium was similar to Pex13-mGFP levels in WT cells, as previously reported (Wroblewska et al., 2017), suggesting that tFT tagging does not affect the induction of Pex13p. In addition to Pxa1-tFT and Pex13-tFT, we also followed Pex11-tFT levels, since this PMP was observed to be the most stable fusion protein. Compared to Pxa1-tFT and Pex13-tFT, Pex11-tFT levels remained stable over time and the levels were slightly higher in oleate-plus medium compared to the levels in glucose (Figure 4C). Similar to Pex13-tFT, the induction of tFT tagged Pex11p is comparable with previous reports (Wroblewska et al., 2017), indicating that our PMP-tFT fusions behave as expected.

In addition to full-length PMP-tFT fusions (PMP-mCherry-sfGFP), an immunoreactive band at ~45 kDa was present in all the samples (Figure 4A, 4B and 4C, represented by *) while a band at ~33 kDa was also observed with samples containing Pex11-tFT and Pex13-tFT (Figure 4B and 4C, represented by **). Previously, Khmelinskii et al., reported that the 45 kDa band denotes a shorter mCherry-sfGFP product (Khmelinskii et al., 2016). This product is caused by the hydrolysis of the mature mCherry fluorophore during TCA lysate preparation, which leads to breakdown of the mCherry polypeptide. Furthermore, the immunoreactive band at ~ 33 kDa was shown to be produced by incomplete proteasomal degradation of tFT fusions and these fragments appeared to be relatively stable in the cell (Khmelinskii et al., 2016). The presence of additional bands in our western blot analysis (Figure 4 * and **) is in line with the previous data, indicating that PMP-tFT fusions behave in a way similar to other tFT fusion proteins. Overall, our data demonstrates that PMP-tFT fusions are well induced and their pattern of induction is as expected.

Figure 4: The induction of PMP-tFT fusions in cells growing on oleate-plus medium. Representative western blot of Pxa1-tFT (A), Pex13-tFT (B) and Pex11-tFT (C) levels in glucose grown cells (peroxisome repressing) shifted to oleate-plus medium (peroxisome inducing). Samples were collected from glucose (0 h) and oleate grown cells at indicated time points. Cells were lysed and samples were subjected to SDS-PAGE and immunoblotting with antibodies against mGFP, Pyruvate Carboxylase 1 (Pyc) or Glucose 6 Phosphate Dehydrogenase (G6PD). Pyc and G6PD acts as loading control. *denotes a mCherry-sfGFP product obtained from mCherry hydrolysis during TCA lysate preparation and ** denotes a tFT fragment produced due to incomplete proteasomal processing (see text for details). TCA samples were run on 7.5% SDS PAGE gel (A) and 12.5% SDS PAGE gel (B, C).

PMP-tFT fusions display spot like structures, suggesting peroxisomal localization

Next, we examined if cells expressing PMP-mCherry-sfGFP (PMP-tFT) fusions exhibit spot-like or punctate structures, which is a characteristic feature of PMP localization on the peroxisomal membrane (Mattiuzzi Usaj et al., 2015; Mix et al., 2018; Nyathi et al., 2010;

Palmieri et al., 2001). For this, we used fluorescence microscopy (FM) analysis to image Pxa1-tFT, Pex13-tFT and Pex11-tFT cells grown on glucose and oleate-plus medium. Together with the strains expressing PMP-tFT fusions, we used a WT strain lacking fluorescent markers, as control.

As expected, WT cells did not exhibit any spot-like structures in either the GFP or mCherry channel (Figure 5A) while several GFP and mCherry positive fluorescent spots were observed in Pxa1-tFT and Pex13-tFT cells grown on oleate-plus medium (Figure 5B and 5C – 8h and 16h), suggesting that these PMP-tFT fusions are localized to peroxisomes. Pxa1-tFT cells on the other hand exhibited only one or two faint spot-like structures on glucose (Figure 5B- 0h). This data validates our western blot analysis, where tFT tagged Pxa1p displayed low levels in glucose (Figure 4A- 0h). Furthermore, the reduction in the number of GFP and mCherry spots observed in Pex13-tFT cells at later time points in oleate-plus medium (Figure 5C- 16h), again confirms the western blotting data on Pex13-tFT levels (Figure 4B- 16h). Compared to Pxa1-tFT and Pex13-tFT cells which exhibited many fluorescent spots, fewer spot-like structures were observed with Pex11-tFT cells grown on oleate-plus medium and these structures were larger than in the other PMP-tFT containing strains tested (Figure 5D). This result is in contrast to the previous data on Pex11-mGFP localization, where several spot-like structures were observed in Pex11-mGFP cells grown on oleate (Mattiuzzi Usaj et al., 2015). Since Pex11p is involved in peroxisome fission (Marshall et al., 1995) and deletion of *PEX11* results in a reduced number of peroxisomes that are larger than in WT cells (Rottensteiner et al., 2003b), we suspect that fusing the tFT tag to the C-terminus of Pex11p disturbs Pex11p function.

Figure 5: PMP-tFT fusions display spot like structures in cells, suggesting correct peroxisomal localization. Fluorescence microscopy analysis of cells expressing Pxa1-tFT (B), Pex13-tFT (C) or Pex11-tFT (D) grown in glucose (0 h) and oleate-plus medium (8 and 16 h). WT strain lacking fluorescent markers was used as a control (A). Images were processed using Image J with optimal settings. sfGFP (200, 700) and mCherry (200, 400) settings were used to process images of WT and Pxa1-tFT. For Pex13-tFT: sfGFP (200, 800) and mCherry (200, 400) and for Pex11-tFT: sfGFP (200, 8000) and mCherry (200, 5000) settings were used. Scale bar represents 3 μ m.

The unstable PMP-tFT fusions have a fast turnover

Since Pex13-tFT and Pxa1-tFT are observed to be unstable, based on our tFT analysis (Figure 2B), it is strongly suggestive that these PMPs undergo protein degradation. Hence, our next step was to validate our tFT analysis using biochemical approaches to examine the degradation of Pex13-tFT and Pxa1-tFT. To achieve this, we performed chase analysis with the protein synthesis inhibitor cycloheximide (CHX), assessing the turnover of Pex13-tFT and Pxa1-tFT in cells grown on oleate-plus medium for ~12 hrs (O.D 1.5). We also followed Pex11-tFT turnover, since this Pex11-tFT displayed the highest mCherry/sfGFP ratio in our tFT analysis and was therefore expected to be stable compared to the other PMP-tFT fusions. As a control, Pxa1-tFT, Pex13-tFT and Pex11-tFT cells grown on oleate-plus medium for ~12 hrs were treated with DMSO (Figure 6A, 6B and 6C – Ctrl). Equal volume of samples from control and CHX treated cells were collected at indicated time points (0, 30, 60 and 120 mins) and used for western blotting (see materials and methods). We observed that Pxa1-tFT is rapidly degraded in CHX treated cells (Figure 6A- CHX) compared to Ctrl cells (Figure 6A- Ctrl). Pex13-tFT on the other hand was observed to be degraded in cells treated with DMSO (Figure 6B- Ctrl) or CHX (Figure 6B- CHX), with the degradation being faster in CHX treatment. Similar pattern

of degradation was observed with Pex11-tFT (Figure 6C- Ctrl and CHX). The reduction of Pex13-tFT and Pex11-tFT levels observed with Ctrl cells grown on oleate-plus medium (for 12h- 14h) can partly be explained by our previous western blot results, where tFT tagged Pex13p and Pex11p displayed lower levels at 16h (Figure 4B- 16h compared to 8h), suggesting that there could either be more degradation or reduced Pex13-tFT synthesis in Ctrl cells grown oleate-plus medium between 12h and 14h (Figure 6B- Ctrl). The increase in Pxa1-tFT levels observed with Ctrl cells after 120 mins (~ 14hrs in oleate-plus medium) again confirms our western blotting results, where Pxa1-tFT levels were observed to increase between 8h and 16h of growth in oleate-plus medium (Figure 4A 8h and 16h).

We quantified the protein levels in CHX treated cells and observed that both Pxa1-tFT and Pex13-tFT undergo rapid protein degradation (Figure 6A and 6B) whereas Pex11-tFT turnover is much slower (Figure 6C). Pxa1-tFT and Pex13-tFT displayed a half-life of around 15 mins and 30 mins respectively, compared to Pex11-tFT, which exhibited a half-life of around 60 mins (Figure 6D). Similar to our western blot analysis displayed in Figure 4, two immunoreactive bands at ~45 kDa and ~33 kDa were observed with CHX chase analysis of Pxa1-tFT, Pex13-tFT and Pex11-tFT (Figure 6A, 6B and 6C). The band at ~33 kDa, produced due to the incomplete proteasomal degradation of tFT fusions, is highly visible in the samples containing Pxa1-tFT (Figure 6A- indicated by **) and Pex13-tFT (Figure 6B- indicated by **) compared to Pex11-tFT (Figure 6C- indicated by **). This strongly suggests a role for proteasome in the degradation of Pxa1-tFT and Pex13-tFT.

Figure 6: Pxa1-tFT and Pex13-tFT are rapidly degraded. Cycloheximide (CHX) chase analysis conducted with cells expressing Pxa1-tFT (A), Pex13-tFT (B) and Pex11-tFT (C). Cells expressing PMP-tFT fusions were grown on oleate-plus medium to an OD of 1.5 and cells were treated with DMSO (Ctrl) or CHX. Equal volume of samples (6 OD) were collected at indicated time points (0, 30, 60 and 120 mins) for western blotting with antibodies against mGFP and Pyc. *denotes a shorter, mCherry-sfGFP product resulting from mCherry hydrolysis during TCA lysate preparation and ** denotes a fast migrating tFT fragment produced by incomplete proteasomal processing (Khmelniskii et al., 2016). D) Quantification of Pxa1-tFT, Pex13-tFT and Pex11-tFT levels after CHX addition. Protein levels were normalized to Pyc at the corresponding time point and to the protein levels at time point 0 or T0 (set to 1). Values represent the mean $\pm$ SD of three independent experiments.

Discussion

Imbalances in protein homeostasis, caused by either excessive or reduced protein degradation, often leads to severe disease (Heo and Rutter, 2011; Matus et al., 2011). Therefore, knowledge on which proteins are degraded, as well as the function of their degradation, is essential to

understand how protein degradation is linked to cellular health. Several methods can be used to study the degradation of proteins. Of these methods, the tandem fluorescent timer (tFT) approach has been successfully used to investigate protein turnover in *S. cerevisiae* cells *in vivo* (Khmelniskii et al., 2012). Here, we have validated the tFT approach to study the stability of a number of PMPs, involved in different peroxisomal functions. We have utilized a tFT consisting of sfGFP and mCherry and used the mCherry/sfGFP ratio to gain insight into the stability of PMPs under different conditions. Using this approach, we observed that individual PMP-tFT fusions in cells displayed varied stability profiles, ranging from unstable to highly stable, establishing that a tFT can be used to report on the stability of individual PMPs (Figure 2).

Due to the size and the fold of the tFT tag, it is very possible that tagging a protein with a tFT affects the stability and/or behaviour of the protein. In line with this, Kowalski et al., 2018 showed that mitochondrial proteins fused to a tFT become mislocalized to the cytosol and are subsequently degraded (Kowalski et al., 2018). Furthermore, this same study demonstrated that the tFT tagged mitochondrial protein COX12 was impaired in function. We noticed comparable behaviour with tFT tagged Pxa1p, one of the PMP-tFT fusions used in our analysis. Pxa1p plays a role in transporting fatty acids into peroxisomes, where they undergo β -oxidation. Interestingly, wild-type Pxa1p is reported to be stable with a half-life of ~9 hrs (Shani and Valle, 1996), while we observed that tFT tagged Pxa1p is unstable and rapidly degraded with a half-life of ~15 mins (Figure 6). Furthermore, we observed that *faa2* cells expressing Pxa1-tFT displayed reduced growth on oleate, suggesting that tFT tagged Pxa1p is impaired in function (Figure 5). This strongly suggests that fusion of a large tFT tag to Pxa1p affects the stability and function of Pxa1p, indicating that Pxa1-tFT could be degraded for quality control purposes, due to reduced functionality. Because PMPs are involved in peroxisomal biogenesis, any faulty PMPs present in the peroxisomal membrane need to be removed in order to maintain peroxisome homeostasis. However, little is known about PMP quality control. Our tFT approach therefore provides an interesting way in which the stability and degradation of PMPs that are prone to become faulty or non-functional can be identified and studied. In addition, the tFT approach can be used to identify factors involved in the degradation of faulty PMPs and gain insights into the mechanism of PMP quality control (see chapter 3 of this thesis).

While a tFT can be used to identify unstable PMPs, that are degraded for quality control purposes, the tFT approach has also the potential to unveil PMPs that can undergo degradation for purposes other than quality control. An example is Pex13-tFT, which retained its functionality yet is rapidly degraded, indicating a role for targeted degradation of Pex13-tFT (Figure 5). In line with this, the degradation of Pex13p observed in *H. polymorpha* was suggested to have a role in matrix protein import (Chen et al., 2018). The authors also demonstrated that Pex2p- a peroxisomal RING E3 protein is involved in the degradation of *H. polymorpha* Pex13p. Here, we used the tFT approach to show that *S. cerevisiae* Pex13p is

degraded like *H. polymorpha* Pex13p. This approach can be used further to investigate whether Pex2p has a role in *S. cerevisiae* Pex13p degradation and also identify which other factors may be involved in the degradation of Pex13p (see chapter 4 of this thesis).

In this report, we have shown that the tFT approach can be used to investigate PMP stability and degradation in live *S. cerevisiae* cells. However, there are some points that need to be taken into consideration when employing the tFT approach. Our western blot analysis demonstrates that, similar to other tFT fusions (Khmelniskii et al., 2016), certain PMP-tFT fusions exhibit two prominent immunoreactive bands at ~45 kDa (produced from mCherry hydrolysis during TCA processing) and ~33 kDa. The 33 kDa band, which is reported to be produced by incomplete proteasomal degradation of tFT fusions (Khmelniskii et al., 2016), is highly visible with unstable protein fusions (Pxa1-tFT and Pex13-tFT) compared to Pex11-tFT, a stable fusion protein (Figure 6). This in itself could be used as readout to identify PMP-tFTs processed by the proteasome. Furthermore, fusion of a tFT tag to PMPs may result in destabilization of the PMPs, as was observed with our Pxa1-tFT fusion. While the tFT approach could be used to destabilize PMPs or identify PMPs that are prone to become faulty, this might not be always be desirable and should be taken into consideration when using the tFT approach to investigate protein turnover.

In summary, the tFT approach is a valid and robust method that can be used to investigate PMP stability or turnover *in vivo*. As an added advantage, the tFT approach can be used in combination with Synthetic Genetic Array (SGA) technique to identify factors involved in the degradation of unstable PMP-tFT fusions identified from our analysis (Khmelniskii et al., 2014). By crossing unstable PMP-tFT fusions with a deletion library consisting of mutants inhibited in autophagy or the ubiquitin proteasome system (UPS), mutants that increase the stability of tFT fusions are the potential candidates involved in the degradation of the unstable PMP-tFT fusions, providing insights into the underlying mechanism and factors involved in PMP degradation. Such approaches have been used in chapters 3 and 4 of this thesis.

Materials and Methods

Yeast strains and plasmid

Saccharomyces cerevisiae strains and plasmids used in this study are listed in Table 1 and 2 respectively. The primers used in this study are mentioned in Table 3. Gene fragments, used for C-terminal tagging (gene integrations) or gene deletions, were amplified in a PCR reaction using Phusion DNA polymerase (Thermo Fisher Scientific). All gene integrations were confirmed by colony PCR using Phire Hot Start II DNA polymerase (Thermo Fisher Scientific). Gene deletions were checked by colony PCR as well as southern blotting analysis of

chromosomal DNA, using the ECL Direct Nucleic Acid Labelling and Detection system (Thermo Fisher Scientific), according to the protocol provided by the manufacturer.

Preparation of *S. cerevisiae* competent cells and transformations were performed as described previously (Knop et al., 1999). Cells were grown overnight at 30°C in 10 mL YPD medium. The OD₆₀₀ of the overnight culture was measured and cells were diluted to 0.15 OD in 50 mL YPD medium. After growth at 30°C to an OD of 0.9, cells were pelleted by centrifugation at 5000 rpm for 5 min, washed with sterile water twice and with 1M sorbitol once. Cells were then resuspended in 300 µL of 1 M Sorbitol, stored at -80°C or used directly for performing transformations. For each transformation - 50 µL of competent cells, 12-15 µL of PCR product, 40 µL pre-boiled salmon sperm DNA (boiled at 100°C for 10 min and immediately cooled on ice-water) and 300 µL Li-AC solution mix (800 µL mL 50% PEG-3350, 100 µL 100 mM Tris-HCl pH7.5 with 10 mM EDTA and 100 µL 1M Lithium acetate) were mixed together and incubated at 30°C for at least 30 mins. Followed by this 40 µL DMSO was added, heat shock was performed at 42°C for at least 15 mins and cells were immediately placed on ice for 1 mins. Cells were then collected by brief centrifugation, washed with 1mL YPD and finally resuspended in 4 mL YPD. After 2-3 hrs of incubation at 30°C, cells were pelleted down and plated on YPD plates containing appropriate antibiotics or on YND plates containing required amino acids. Plates were incubated at 30°C for 2 to 3 days.

Construction of *S. cerevisiae* Pxa1-tFT

In order to fuse tFT tag to the C-terminus of *PXA1*, first the tFT tagging module along with ~50bp flanking regions homologous to C-terminus of *PXA1* was amplified in a PCR reaction using plasmid pMaM168 as a template and primers Int-Pxa1-Fwd and Int-Pxa1-Rev. The tFT tagging cassette consists of full-length *mCherry* sequence, selection marker (*URA3* gene), two I-SceI cut sequences, *mCherry*^{ΔN} (sequences coding for *mCherry* lacking last 91 amino acids) and full-length sfGFP sequences (Fig 1C). The amplified PCR product was then transformed into the wild-type strain (yMaM330) that expresses I-SceI coding sequences under the control of *GALI* promoter (Table 1). Cells were then plated on YND plates containing 30 mg/L leucine, 20mg/L histidine and 20mg/L methionine and incubated at 30°C for minimum of 3 days. Correct integration of tFT module or the positive transformants of Pxa1-tFT were checked by colony PCR using primers IPxa1-cPCR-Fwd and IPxa1-cPCR-Rev

Construction of *S. cerevisiae* *faa2*.Pxa1-tFT and *pxa1 faa2*

Saccharomyces cerevisiae *faa2*.Pxa1-tFT strain was constructed by replacing Faa2 region in Pxa1-tFT strain with geneticin resistance gene. The deletion of *faa2* was performed as follows: First, a PCR fragment containing *kanMX* sequences (coding for geneticin marker) and ~50bp of the *FAA2* flanking regions were amplified from the plasmid pFA6a-kanMX using the primers Del-KFaa2-Fwd and Del-KFaa2-Rev. The resulting PCR cassette or *FAA2* deletion cassette

was transformed into Pxa1-tFT strain, cells were plated on YPD plates containing 150 µg/ml Geneticin (Invitrogen) and incubated at 30°C for 2-3 days. Positive transformants of *faa2.Pxa1-tFT*, grown on YPD selective plates, were selected and checked by colony PCR using primers DKFaa2-cPCR-Fwd and DKFaa2-cPCR -Rev. The obtained positive transformants were further checked by southern blotting to confirm *FAA2* deletion in Pxa1-tFT.

For the construction of *pxa1 faa2* strain, first *PXA1* was deleted in yMaM330 (wild-type) strain to create *pxa1* strain. Followed by this, *FAA2* was replaced by *URA3* gene in *pxa1* strain to create *pxa1 faa2* double deletion strain. To construct *pxa1* strain, a *PXA1* deletion cassette containing hygromycin (*hphMX*) resistance gene along with 45- 50bp homologous sequences in the *PXA1* region were amplified from the plasmid pHIPH-Pex14 mKate2 using the primers Del-Pxa1-Fwd and Del-Pxa1-Rev. The obtained *PXA1* deletion cassette was transformed into yMaM330 and cells were plated on YPD plates containing 300 µg/ml Hygromycin (Invitrogen). Hygromycin resistant transformants were selected and checked by colony PCR using primers DPxa1-cPCR-Fwd and DPxa1-cPCR-Rev. The deletion of *PXA1* in yMaM330 was further confirmed by southern blotting. With the newly obtained *pxa1* strain, *FAA2* deletion cassette was amplified from pMaM168 using the primers Del-UFaa2-Fwd and Del-UFaa2-Rev. The cassette containing *URA3* resistance gene and ~50bp *FAA2* flanking regions was transformed into *pxa1* and cells were plated on YND plates containing 30 mg/L leucine, 20mg/L histidine and 20mg/L methionine. The positive transformants of *FAA2* deletion in *pxa1* were checked by colony PCR using primers DUFaa2-cPCR-Fwd and DUFaa2-cPCR -Rev. In addition to colony PCR, the obtained positive transformants were further checked by southern blotting to confirm *FAA2* deletion in *pxa1*.

Culture conditions

Yeast cells were cultured overnight at 30°C on YM2 medium (6.7 g/L yeast nitrogen base without amino acids (YNB, Difco), 1% Caesin hydrolysate (Sigma-Aldrich), 60 mg/L uracil, 60 mg/L tryptophan) containing 2% glucose. Cells were then diluted to OD₆₀₀ of 0.1 in YM2 medium plus 0.3% glucose and grown for 7-9 hrs. To induce peroxisome proliferation, cells grown on YM2 medium plus 0.3% glucose were transferred to YNO-glucose or oleate-plus medium (6.7 g/L YNB, 1% casein hydrolysate, 60 mg/L uracil, 60 mg/L tryptophan, 0.5% tween, 0.1% oleate and 0.1% glucose). In oleate-plus medium cells were grown to an OD of 1.5, after which cells were harvested for TCA lysate preparation, fluorescence microscopy or cycloheximide (CHX) chase experiment. For CHX chase analysis, cells were treated with cycloheximide (CHX, Sigma-Aldrich), a protein synthesis inhibitor, to a final concentration of 6 mg/ml. Where indicated, equal amount of DMSO (control for CHX) was added to cells.

Preparation of yeast TCA lysates

TCA lysates were prepared using cells grown on oleate-plus medium to OD of 1.5. Cell extracts of TCA-treated cells were prepared for SDS-PAGE as described previously (Baerends et al., 2000b). SDS-PAGE and western blotting were performed by established methods. Equal amounts of protein were loaded per lane and blots were probed with antibodies against mGFP (Santa Cruz Biotech, sc-9996), pyruvate carboxylase 1 (Pyc) and glucose 6 phosphate dehydrogenase (G6PD). Secondary goat anti-rabbit (Thermo Fisher Scientific, 31460) antibody conjugated to alkaline phosphatase or goat anti-mouse (Thermo Fisher Scientific, 31430) antibody conjugated to horseradish peroxidase were used for detection. Pyc and G6PD was used as a loading control.

Quantification of Western blots

Western blots were scanned using a densitometer (Bio-Rad, GS-710) and protein levels were quantified using Image J software (<https://imagej.nih.gov/ij/>). For CHX chase experiments, the intensity of each band measured was normalized by dividing by the intensity of the corresponding Pyc band (loading control). The normalized values at time point 0 was set to 1.0 and values obtained after CHX addition at indicated time points are displayed as a fraction of 0 values. Standard deviations were calculated using Excel. The data presented are derived from three independent experiments.

Seamless marker excision and stability analysis of PMP-tFT fusions *in vivo*

Tandem fluorescent protein timers (tFT): mCherry and sfGFP were endogenously tagged to PMPs listed in Fig 1D as described (Khmelninskii et al., 2011). Cells expressing PMP-tFT fusions were grown to saturation (24 h at 30°C) in SC medium (6.7 g/L YNB, 2g/L amino acid dropout mix (-URA)) plus 2% glucose. After 24h of growth on SC plus 2% glucose, cells were then spotted on glucose plates (SC medium containing 2% glucose and 2% agar) using a RoToR pinning robot (Singer Instruments) and incubated for 24 h at 30°C. For seamless *URA3* marker excision, colonies were then pinned onto galactose plates (SC medium containing 2% galactose, 2% Raffinose and 2% agar) to induce expression of I-SceI and grown for 24 h at 30°C. Subsequent pinning onto glucose plates (i.e. SC medium lacking uracil) was used to assess the efficiency of marker excision. Pinning from galactose plates onto 5-FOA plates (SC medium plus 2% glucose and 1g/L 5-fluoroorotic acid (5-FOA) was used to remove cells that had not lost the *ScURA3* marker (Figure 2A–C). Followed by this cells were pinned on to glucose plates (6.7 g/L YNB, 2g/L amino acid dropout mix (-LEU), 2 % glucose and 2% agar) and oleate-plus plates (6.7 g/L YNB, 2g/L amino acid dropout mix (-LEU), 0.5% tween, 0.1% oleate, 0.1% glucose and 2% agar). Using Infinite M1000 Pro plate reader equipped with automatic plate loading stackers (Tecan) and custom temperature control chambers, mCherry intensity (587/10 nm excitation, 610/10 nm emission) and sfGFP intensity (488/10nm excitation, 510/10 nm

emission) were measured from colonies grown on glucose and oleate-plus plates. First set of measurements were taken after 24h (1 day) of growth and measurements were taken every 24h until 96h (4 days).

Whole colony fluorescence intensities (mCherry and sfGFP), obtained from PMP-tFT fusions in cells were corrected for auto fluorescence by subtracting the intensity measurements obtained with the control strain, yMaM344 (expressing non-fluorescent protein). The values obtained were then corrected for median effects by dividing the median of fluorescence intensities (mCherry and sfGFP) obtained after auto fluorescence correction from PMP-tFT fusions in cells. The corrected values of mCherry divided by the sfGFP values yielded a ratio, representing the stability of PMP-tFT fusions in cells. The heat map generated using the ratios are presented in Fig 2.

Fluorescence microscopy

All images were captured at room temperature using an inverted microscope (Axio Scope A1, 100×1.30 NA Plan-Neofluar objective, Carl Zeiss), Micro-Manager software and a digital camera (CoolSNAP HQ²; Photometrics). For wide field microscopy, GFP fluorescence was visualized with a 470/440-nm band pass excitation filter, a 495-nm dichromatic mirror, and 525/50-nm band pass emission filter. mCherry fluorescence was visualized with a 587/25-nm band pass excitation filter, a 605-nm dichromatic mirror, and 647/70-nm band pass emission filter.

For localization analysis, cells were grown on oleate-plus medium to an OD₆₀₀ of 1.5 and images were captured. The acquired images were processed using Image J software (<https://imagej.nih.gov/ij/>) with optimal settings. sfGFP (200, 700) and mCherry (200, 400) settings were used to process images of WT and Pxa1-tFT. For Pex13-tFT: sfGFP (200, 800) and mCherry (200, 400) and for Pex11-tFT: sfGFP (200, 8000) and mCherry (200, 5000) settings were used. Scale bar represents 3µm. For figure preparation, Adobe Photoshop CS6 and Adobe Illustrator CC 2018 was used.

Spot assay

Yeast strains were grown overnight at 30 °C on YM2 medium containing 2% glucose and shifted to YM2 medium plus 0.3% glucose. After 6h of growth in YM2 plus 0.3% glucose, cells were diluted to 0.1 OD and ten-fold serial dilutions were made up to 0.0001 OD. From every dilution, 3 µL yeast cells were spotted onto oleate plates containing 6.7 g/L YNB, 1 g/L SCSM–LEU dropout mix (Hopkins mixture, FOR MEDIUM), 180 mg/L leucine, 0.1% oleate, 0.05% Tween and 2% agar was added. Plates were incubated at 30°C for 7 or 9 days before imaging.

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TABLE 1- *Saccharomyces cerevisiae* strains used in this study

Strain	Description [genotype]	Reference
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(Khmelniskii et al., 2014) (Knop lab)
BY4742	<i>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Reference
yMaM330 [Wild type-WT]	<i>MATalpha his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 can1Δ::STE2pr-spHIS5 lyp1Δ::STE3pr-LEU2 leu2Δ::GAL1pr-I-SCEI-natNT2</i>	(Khmelniskii et al., 2014) (Knop lab)
yMaM344 [control strain]	<i>yMaM330, ura3Δ0::mCherryΔN-I-SceI site-CYC1 term-ScURA3-I-SceI site-mCherryΔN [URA3]</i>	(Khmelniskii et al., 2014) (Knop lab)
Pex2-tFT	<i>yMaM330, PEX2::mCherry-sfGFP [URA3]</i>	Knop lab
Pex3-tFT	<i>yMaM330, PEX3::mCherry-sfGFP [URA3]</i>	Knop lab
Pex10-tFT	<i>yMaM330, PEX10::mCherry-sfGFP [URA3]</i>	Knop lab
Pex11-tFT	<i>yMaM330, PEX11::mCherry-sfGFP [URA3]</i>	Knop lab
Pex12-tFT	<i>yMaM330, PEX12::mCherry-sfGFP [URA3]</i>	Knop lab
Pex13-tFT	<i>yMaM330, PEX13::mCherry-sfGFP [URA3]</i>	Knop lab
Pex14-tFT	<i>yMaM330, PEX14::mCherry-sfGFP [URA3]</i>	Knop lab
Pex17-tFT	<i>yMaM330, PEX17::mCherry-sfGFP [URA3]</i>	Knop lab
Pex22-tFT	<i>yMaM330, PEX22::mCherry-sfGFP [URA3]</i>	Knop lab
Pex25-tFT	<i>yMaM330, PEX25::mCherry-sfGFP [URA3]</i>	Knop lab
Pex27-tFT	<i>yMaM330, PEX27::mCherry-sfGFP [URA3]</i>	Knop lab
Ant1-tFT	<i>yMaM330, ANT1::mCherry-sfGFP [URA3]</i>	Knop lab
Pxa1-tFT	<i>yMaM330, PXA1::mCherry-sfGFP [URA3]</i>	This study
Inp1-tFT	<i>yMaM330, INP1::mCherry-sfGFP [URA3]</i>	Knop lab
Inp2-tFT	<i>yMaM330, INP2::mCherry-sfGFP [URA3]</i>	Knop lab

<i>faa2</i> .Pxa1-tFT	<i>faa2</i> deletion in <i>PXA1</i> -tFT, <i>faa2::kanMX</i>	This study
<i>pxa1</i>	<i>pxa1</i> deletion, <i>yMaM330</i> , <i>pxa1::hphMX</i>	This study
<i>pxa1faa2</i>	<i>faa2</i> deletion in <i>pxa1</i> , <i>faa2::URA3</i>	This study
<i>pex4</i>	<i>pex4</i> deletion strain, <i>BY4741</i> , <i>pex4::kanMX</i>	Knop lab
<i>pex13</i>	<i>pex13</i> deletion strain, <i>BY4742</i> , <i>pex13::kanMX</i>	Euroscarf

TABLE 2- Plasmids used in this study

Plasmid	Description	Use	Reference
pMaM168	pFA6a plasmid containing tFT module (<i>mCherry-I-SceI</i> site- <i>SpCYC1</i> term- <i>ScURA3</i> - <i>I-SceI</i> site- <i>mCherry</i> Δ <i>N-sfGFP</i>) ; <i>URA3</i> , <i>Amp^R</i>	1. C-terminal <i>tFT</i> tagging ; <i>PXA1</i> - <i>tFT</i> integration into <i>yMaM330</i> Gene disruption (<i>faa2</i>) by <i>URA3</i> marker	(Khmelinskii et al., 2014) (Knop lab)
pFA6a- kanMX6	pFA6a plasmid containing geneticin (<i>kanMX6</i>) resistance gene; <i>kanMX</i> , <i>Amp^R</i>	2. Gene disruption (<i>faa2</i>) by <i>kanMX6</i> marker	(Khmelinskii et al., 2014) (Knop lab)
pHIPH-Pex14mKate2	pHIPH containing <i>mKate2</i> and C-terminal of <i>PEX14</i> ; <i>hphMX</i> , <i>Amp^R</i>	Gene disruption (<i>pxa1</i>) by <i>hphMX</i> marker	(Chen et al., 2018)

TABLE 3- Oligonucleotides (primers) used in this study

Primer	Sequence (5' to 3')	Description
Int-Pxa1-Fwd	TGGGAAGATGAGAGGACG AAGCTACGGGAAAAGCTT GAAATTATTCGTACGCTGC AGGTCGAC	To amplify a fragment of 3211 bp from pMaM168, PCR fragment contains tFT module and ~50bp flanking regions homologous in the C-terminus of the Pxa1 region; used to construct Pxa1-tFT strain
Int-Pxa1-Rev	TAAATTATATTCGCTAAAT AAAATCTCTCCCTTTCTAG GGTGTTTTCAATCGATGAA TTCGAGCTCG	To amplify a fragment of 3211 bp from pMaM168, PCR fragment contains tFT module and ~50bp flanking regions

		homologous in the C-terminus of the Pxa1 region; used to construct Pxa1-tFT strain
IPxa1-cPCR-Fwd	AGATGCCATTGCCGATTG GA	Colony PCR- forward primer, to check positive transformants of Pxa1-tFT (expected size- 803 bp)
IPxa1-cPCR-Rev	AGATGAACTCGCCGTCCT GC	Colony PCR- reverse primer, to check positive transformants of Pxa1-tFT
Del-KFaa2-Fwd	GAATGCAGGTTACAAAAA ACGGATAAGAACAACTT GTTTCGAAATATGCGTACG CTGCAGGTCGAC	To amplify a fragment of 1607 bp from pFa6A-kanMX, PCR fragment contains kanMX resistance gene and ~50 bp flanking <i>Faa2</i> region; used to construct <i>faa2</i> (deletion) strain
Del-KFaa2-Rev	ACATCAAAGTTTTTCTAGT TTGAATGTGTTCCAAATCGT CATAAGTACCTAATCGATG AATTCGAGCTCG	To amplify a fragment of 1607 bp from pFa6A-kanMX, PCR fragment contains kanMX resistance gene and ~50 bp flanking <i>Faa2</i> region; used to construct <i>faa2</i> (deletion) strain
DKFaa2-cPCR-Fwd	CAGAACATCCTCCGACC GT	Colony PCR- forward primer, to check positive transformants of <i>faa2</i> (expected size- 790 bp)
DKFaa2-cPCR-Rev	AGAAACAACCTCTGGCGCA TCG	Colony PCR- forward primer, to check positive transformants of <i>faa2</i> (expected size- 790 bp)
Del-Pxa1-Fwd	ACCCGTTTCAGACAATCTG GAAGTCTTAGAACGCATA ACACAGAAATGCCACAC ACCATAGCTTCA	To amplify 1851 bp fragment from pHIPH-Pex14mKate2, PCR fragment contains 45-50 bp homologous sequence in Pxa1 region and Hygromycin resistance gene; used to construct <i>pxa1</i> strain
Del-Pxa1-Rev	ATAAATTATATTCGCTAAA TAAATCTCTCCCTTTCTA GGGTGTTTTACGTTTTCG ACACTGGATGGCGGC	To amplify 1851 bp fragment from pHIPH-Pex14mKate2, PCR fragment contains 45-50 bp homologous sequence in Pxa1 region and Hygromycin resistance gene; used to construct <i>pxa1</i> strain

DPxa1-cPCR-Fwd	GATCGGGCTTAACCGCAT	Colony PCR- forward primer, to check positive transformants of <i>pxa1</i> (expected size- 790 bp)
DPxa1-cPCR-Rev	ACCTCCTTTGGCCTTCAAT C	Colony PCR- forward primer, to check positive transformants of <i>pxa1</i> (expected size- 790 bp)
Del-UFaa2-Fwd	AATGCAGGTTACAAAAAC GGATAAGAACAACCTTGTT TCGAAATATGGGTCCGCAT ACCACCTTTTCA	To amplify 1232 bp fragment from pMaM168, PCR fragment contains <i>URA3</i> sequence and ~50 bp homologous sequence in Faa2 region; used to delete <i>FAA2</i> in <i>pxa1</i> strain
Del-UFaa2-Rev	CATCAAAGTTTTTCTAGTT TGAATGTGTTCCAAATCGTC ATAAGTACCTATGCGCGCC ACCGCATAGGGTA	To amplify 1232 bp fragment from pMaM168, PCR fragment contains <i>URA3</i> sequence and ~50 bp homologous sequence in Faa2 region; used to delete <i>FAA2</i> in <i>pxa1</i> strain
DUFaa2-cPCR-Fwd	CAGAACATCCTCCGGACC GT	Colony PCR- forward primer, to check positive transformants of <i>FAA2</i> deletion in <i>pxa1</i> (expected size- 477 bp)
DUFaa2-cPCR-Rev	TAGGATGAGTAGCAGCAC G	Colony PCR- forward primer, to check positive transformants of <i>FAA2</i> deletion in <i>pxa1</i> (expected size- 477 bp)

References

- Al-Saryi, N.A., M.Y. Al-Hejjaj, C.W.T. van Roermund, G.E. Hulmes, L. Ekal, C. Payton, R.J.A. Wanders, and E.H. Hetteema. 2017. Two NAD-linked redox shuttles maintain the peroxisomal redox balance in *Saccharomyces cerevisiae*. *Scientific reports*. 7:11868.
- Baerends, R.J., K.N. Faber, J.A. Kiel, I.J. van der Klei, W. Harder, and M. Veenhuis. 2000a. Sorting and function of peroxisomal membrane proteins. *FEMS Microbiol Rev*. 24:291-301.
- Baerends, R.J., K.N. Faber, A.M. Kram, J.A. Kiel, I.J. van der Klei, and M. Veenhuis. 2000b. A stretch of positively charged amino acids at the N terminus of *Hansenula polymorpha* Pex3p is involved in incorporation of the protein into the peroxisomal membrane. *J Biol Chem*. 275:9986-9995.
- Baker, A., D.J. Carrier, T. Schaedler, H.R. Waterham, C.W. van Roermund, and F.L. Theodoulou. 2015. Peroxisomal ABC transporters: functions and mechanism. *Biochem Soc Trans*. 43:959-965.
- Barry, J.D., E. Dona, D. Gilmour, and W. Huber. 2016. TimerQuant: a modelling approach to tandem fluorescent timer design and data interpretation for measuring protein turnover in embryos. *Development*. 143:174-179.
- Belle, A., A. Tanay, L. Bitincka, R. Shamir, and E.K. O'Shea. 2006. Quantification of protein half-lives in the budding yeast proteome. *Proc Natl Acad Sci U S A*. 103:13004-13009.
- Bellu, A.R., F.A. Salomons, J.A. Kiel, M. Veenhuis, and I.J. Van Der Klei. 2002. Removal of Pex3p is an important initial stage in selective peroxisome degradation in *Hansenula polymorpha*. *J Biol Chem*. 277:42875-42880.
- Brown, L.A., and A. Baker. 2003. Peroxisome biogenesis and the role of protein import. *Journal of cellular and molecular medicine*. 7:388-400.
- Chen, X., S. Devarajan, N. Danda, and C. Williams. 2018. Insights into the Role of the Peroxisomal Ubiquitination Machinery in Pex13p Degradation in the Yeast *Hansenula polymorpha*. *J Mol Biol*. 430:1545-1558.
- Colleaux, L., L. d'Auriol, M. Betermier, G. Cottarel, A. Jacquier, F. Galibert, and B. Dujon. 1986. Universal code equivalent of a yeast mitochondrial intron reading frame is expressed into *E. coli* as a specific double strand endonuclease. *Cell*. 44:521-533.
- Distel, B., R. Erdmann, S.J. Gould, G. Blobel, D.I. Crane, J.M. Cregg, G. Dodt, Y. Fujiki, J.M. Goodman, W.W. Just, J.A. Kiel, W.H. Kunau, P.B. Lazarow, G.P. Mannaerts, H.W. Moser, T. Osumi, R.A. Rachubinski, A. Roscher, S. Subramani, H.F. Tabak, T. Tsukamoto, D. Valle, I. van der Klei, P.P. van Veldhoven, and M. Veenhuis. 1996. A unified nomenclature for peroxisome biogenesis factors. *J Cell Biol*. 135:1-3.
- Dobrucki, J.W., D. Feret, and A. Noatynska. 2007. Scattering of exciting light by live cells in fluorescence confocal imaging: phototoxic effects and relevance for FRAP studies. *Biophysical journal*. 93:1778-1786.
- Dona, E., J.D. Barry, G. Valentin, C. Quirin, A. Khmelinskii, A. Kunze, S. Durdu, L.R. Newton, A. Fernandez-Minan, W. Huber, M. Knop, and D. Gilmour. 2013. Directional tissue migration through a self-generated chemokine gradient. *Nature*. 503:285-289.
- El Magraoui, F., A. Schrotter, R. Brinkmeier, L. Kunst, T. Mastalski, T. Muller, K. Marcus, H.E. Meyer, W. Girzalsky, R. Erdmann, and H.W. Platta. 2014. The cytosolic domain of Pex22p stimulates the Pex4p-dependent ubiquitination of the PTS1-receptor. *PLoS One*. 9:e105894.
- Elgersma, Y., L. Kwast, A. Klein, T. Voorn-Brouwer, M. van den Berg, B. Metzger, T. America, H.F. Tabak, and B. Distel. 1996. The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import PTS1-containing proteins. *J Cell Biol*. 135:97-109.

- Engelen, M., S. Kemp, and B.T. Poll-The. 2014. X-linked adrenoleukodystrophy: pathogenesis and treatment. *Current neurology and neuroscience reports*. 14:486.
- Erdmann, R., and G. Blobel. 1996. Identification of Pex13p a peroxisomal membrane receptor for the PTS1 recognition factor. *J Cell Biol*. 135:111-121.
- Fagarasanu, A., M. Fagarasanu, G.A. Eitzen, J.D. Aitchison, and R.A. Rachubinski. 2006. The peroxisomal membrane protein Inp2p is the peroxisome-specific receptor for the myosin V motor Myo2p of *Saccharomyces cerevisiae*. *Dev Cell*. 10:587-600.
- Fagarasanu, A., F.D. Mast, B. Knoblach, and R.A. Rachubinski. 2010. Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast. *Nat Rev Mol Cell Biol*. 11:644-654.
- Fagarasanu, M., A. Fagarasanu, Y.Y. Tam, J.D. Aitchison, and R.A. Rachubinski. 2005. Inp1p is a peroxisomal membrane protein required for peroxisome inheritance in *Saccharomyces cerevisiae*. *J Cell Biol*. 169:765-775.
- Gabaldon, T. 2010. Peroxisome diversity and evolution. *Philos Trans R Soc Lond B Biol Sci*. 365:765-773.
- Geva-Zatorsky, N., I. Issaeva, A. Mayo, A. Cohen, E. Dekel, T. Danon, L. Cohen, Y. Liron, U. Alon, and E. Eden. 2012. Using bleach-chase to measure protein half-lives in living cells. *Nat Protoc*. 7:801-811.
- Gurvitz, A., and H. Rottensteiner. 2006. The biochemistry of oleate induction: transcriptional upregulation and peroxisome proliferation. *Biochim Biophys Acta*. 1763:1392-1402.
- Heo, J.M., and J. Rutter. 2011. Ubiquitin-dependent mitochondrial protein degradation. *Int J Biochem Cell Biol*. 43:1422-1426.
- Hettema, E.H., C.W. van Roermund, B. Distel, M. van den Berg, C. Vilela, C. Rodrigues-Pousada, R.J. Wanders, and H.F. Tabak. 1996. The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. *Embo J*. 15:3813-3822.
- Hiltunen, J.K., A.M. Mursula, H. Rottensteiner, R.K. Wierenga, A.J. Kastaniotis, and A. Gurvitz. 2003. The biochemistry of peroxisomal beta-oxidation in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol Rev*. 27:35-64.
- Iizuka, R., M. Yamagishi-Shirasaki, and T. Funatsu. 2011. Kinetic study of de novo chromophore maturation of fluorescent proteins. *Anal Biochem*. 414:173-178.
- Islinger, M., S. Grille, H.D. Fahimi, and M. Schrader. 2012. The peroxisome: an update on mysteries. *Histochem Cell Biol*. 137:547-574.
- Karpichev, I.V., J.M. Durand-Heredia, Y. Luo, and G.M. Small. 2008. Binding characteristics and regulatory mechanisms of the transcription factors controlling oleate-responsive genes in *Saccharomyces cerevisiae*. *J Biol Chem*. 283:10264-10275.
- Karpichev, I.V., and G.M. Small. 1998. Global regulatory functions of Oaf1p and Pip2p (Oaf2p), transcription factors that regulate genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 18:6560-6570.
- Kawalek, A., C. Jagadeesan, and I.J. van der Klei. 2016. Impaired biosynthesis of the non-bilayer lipids phosphatidylethanolamine or cardiolipin does not affect peroxisome biogenesis and proliferation in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun*. 480:228-233.
- Khmelniskii, A., E. Blaszcak, M. Pantazopoulou, B. Fischer, D.J. Omnus, G. Le Dez, A. Brossard, A. Gunnarsson, J.D. Barry, M. Meurer, D. Kirmaier, C. Boone, W. Huber, G. Rabut, P.O. Ljungdahl, and M. Knop. 2014. Protein quality control at the inner nuclear membrane. *Nature*. 516:410-413.
- Khmelniskii, A., P.J. Keller, A. Bartosik, M. Meurer, J.D. Barry, B.R. Mardin, A. Kaufmann, S. Trautmann, M. Wachsmuth, G. Pereira, W. Huber, E. Schiebel, and M. Knop. 2012. Tandem fluorescent protein timers for in vivo analysis of protein dynamics. *Nature biotechnology*. 30:708-714.

- Khmelninskii, A., M. Meurer, N. Duishoev, N. Delhomme, and M. Knop. 2011. Seamless gene tagging by endonuclease-driven homologous recombination. *PLoS One*. 6:e23794.
- Khmelninskii, A., M. Meurer, C.T. Ho, B. Besenbeck, J. Fuller, M.K. Lemberg, B. Bukau, A. Mogk, and M. Knop. 2016. Incomplete proteasomal degradation of green fluorescent proteins in the context of tandem fluorescent protein timers. *Mol Biol Cell*. 27:360-370.
- Knop, M., K. Siegers, G. Pereira, W. Zachariae, B. Winsor, K. Nasmyth, and E. Schiebel. 1999. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast*. 15:963-972.
- Kowalski, L., P. Bragoszewski, A. Khmelninskii, E. Glow, M. Knop, and A. Chacinska. 2018. Determinants of the cytosolic turnover of mitochondrial intermembrane space proteins. *BMC biology*. 16:66.
- Lind, K., and J. Norbeck. 2009. A QPCR-based reporter system to study post-transcriptional regulation via the 3' untranslated region of mRNA in *Saccharomyces cerevisiae*. *Yeast*. 26:407-413.
- Lingard, M.J., M. Monroe-Augustus, and B. Bartel. 2009. Peroxisome-associated matrix protein degradation in *Arabidopsis*. *Proc Natl Acad Sci U S A*. 106:4561-4566.
- Lippincott-Schwartz, J., N. Altan-Bonnet, and G.H. Patterson. 2003. Photobleaching and photoactivation: following protein dynamics in living cells. *Nat Cell Biol*. Suppl:S7-14.
- Marshall, P.A., Y.I. Krimkevich, R.H. Lark, J.M. Dyer, M. Veenhuis, and J.M. Goodman. 1995. Pmp27 promotes peroxisomal proliferation. *J Cell Biol*. 129:345-355.
- Mattiazzi Usaj, M., M. Brloznik, P. Kaferle, M. Zitnik, H. Wolinski, F. Leitner, S.D. Kohlwein, B. Zupan, and U. Petrovic. 2015. Genome-Wide Localization Study of Yeast Pex11 Identifies Peroxisome-Mitochondria Interactions through the ERMES Complex. *J Mol Biol*. 427:2072-2087.
- Matus, S., L.H. Glimcher, and C. Hetz. 2011. Protein folding stress in neurodegenerative diseases: a glimpse into the ER. *Curr Opin Cell Biol*. 23:239-252.
- Merzlyak, E.M., J. Goedhart, D. Shcherbo, M.E. Bulina, A.S. Shcheglov, A.F. Fradkov, A. Gaintzeva, K.A. Lukyanov, S. Lukyanov, T.W. Gadella, and D.M. Chudakov. 2007. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nature methods*. 4:555-557.
- Mix, A.K., U. Cenci, T. Heimerl, P. Marter, M.L. Wirkner, and D. Moog. 2018. Identification and Localization of Peroxisomal Biogenesis Proteins Indicates the Presence of Peroxisomes in the Cryptophyte *Guillardia theta* and Other "Chromalveolates". *Genome biology and evolution*. 10:2834-2852.
- Morimoto, R.I. 2008. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes Dev*. 22:1427-1438.
- Morita, M., S. Matsumoto, A. Sato, K. Inoue, D.G. Kostsin, K. Yamazaki, K. Kawaguchi, N. Shimozawa, S. Kemp, R.J. Wanders, H. Kojima, T. Okabe, and T. Imanaka. 2018. Stability of the ABCD1 Protein with a Missense Mutation: A Novel Approach to Finding Therapeutic Compounds for X-Linked Adrenoleukodystrophy. *JIMD reports*.
- Mosser, J., A.M. Douar, C.O. Sarde, P. Kioschis, R. Feil, H. Moser, A.M. Poustka, J.L. Mandel, and P. Aubourg. 1993. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature*. 361:726-730.
- Nakayama, K.I., and K. Nakayama. 2006. Ubiquitin ligases: cell-cycle control and cancer. *Nature reviews. Cancer*. 6:369-381.
- Nyathi, Y., C. De Marcos Lousa, C.W. van Roermund, R.J. Wanders, B. Johnson, S.A. Baldwin, F.L. Theodoulou, and A. Baker. 2010. The *Arabidopsis* peroxisomal ABC transporter, comatose, complements the *Saccharomyces cerevisiae* pxa1 pxa2Delta mutant for metabolism of long-chain fatty acids and exhibits fatty acyl-CoA-stimulated ATPase activity. *J Biol Chem*. 285:29892-29902.

- Palmieri, L., H. Rottensteiner, W. Girzalsky, P. Scarcia, F. Palmieri, and R. Erdmann. 2001. Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter. *EMBO J.* 20:5049-5059.
- Pedelacq, J.D., S. Cabantous, T. Tran, T.C. Terwilliger, and G.S. Waldo. 2006. Engineering and characterization of a superfolder green fluorescent protein. *Nature biotechnology.* 24:79-88.
- Platta, H.W., F. El Magraoui, B.E. Baumer, D. Schlee, W. Girzalsky, and R. Erdmann. 2009. Pex2 and pex12 function as protein-ubiquitin ligases in peroxisomal protein import. *Mol Cell Biol.* 29:5505-5516.
- Pratt, J.M., J. Petty, I. Riba-Garcia, D.H. Robertson, S.J. Gaskell, S.G. Oliver, and R.J. Beynon. 2002. Dynamics of protein turnover, a missing dimension in proteomics. *Mol Cell Proteomics.* 1:579-591.
- Ribeiro, D., I. Castro, H.D. Fahimi, and M. Schrader. 2012. Peroxisome morphology in pathology. *Histol Histopathol.* 27:661-676.
- Rottensteiner, H., A. Hartig, B. Hamilton, H. Ruis, R. Erdmann, and A. Gurvitz. 2003a. *Saccharomyces cerevisiae* Pip2p-Oaf1p regulates PEX25 transcription through an adenine-less ORE. *Eur J Biochem.* 270:2013-2022.
- Rottensteiner, H., L. Palmieri, A. Hartig, B. Hamilton, H. Ruis, R. Erdmann, and A. Gurvitz. 2002. The peroxisomal transporter gene ANT1 is regulated by a deviant oleate response element (ORE): characterization of the signal for fatty acid induction. *Biochem J.* 365:109-117.
- Rottensteiner, H., K. Stein, E. Sonnenhol, and R. Erdmann. 2003b. Conserved function of pex11p and the novel pex25p and pex27p in peroxisome biogenesis. *Mol Biol Cell.* 14:4316-4328.
- Schimke, R.T., and D. Doyle. 1970. Control of enzyme levels in animal tissues. *Annu Rev Biochem.* 39:929-976.
- Schrader, M., N.A. Bonekamp, and M. Islinger. 2012. Fission and proliferation of peroxisomes. *Biochim Biophys Acta.* 1822:1343-1357.
- Schrader, M., and H.D. Fahimi. 2006. Peroxisomes and oxidative stress. *Biochim Biophys Acta.* 1763:1755-1766.
- Schrader, M., and H.D. Fahimi. 2008. The peroxisome: still a mysterious organelle. *Histochem Cell Biol.* 129:421-440.
- Shani, N., and D. Valle. 1996. A *Saccharomyces cerevisiae* homolog of the human adrenoleukodystrophy transporter is a heterodimer of two half ATP-binding cassette transporters. *Proc Natl Acad Sci U S A.* 93:11901-11906.
- Shani, N., P.A. Watkins, and D. Valle. 1995. PXA1, a possible *Saccharomyces cerevisiae* ortholog of the human adrenoleukodystrophy gene. *Proc Natl Acad Sci U S A.* 92:6012-6016.
- Smith, J.J., and J.D. Aitchison. 2009. Regulation of peroxisome dynamics. *Curr Opin Cell Biol.* 21:119-126.
- Smith, J.J., and J.D. Aitchison. 2013. Peroxisomes take shape. *Nat Rev Mol Cell Biol.* 14:803-817.
- Smith, J.J., M. Marelli, R.H. Christmas, F.J. Vizeacoumar, D.J. Dilworth, T. Ideker, T. Galitski, K. Dimitrov, R.A. Rachubinski, and J.D. Aitchison. 2002. Transcriptome profiling to identify genes involved in peroxisome assembly and function. *J Cell Biol.* 158:259-271.
- Subach, F.V., O.M. Subach, I.S. Gundorov, K.S. Morozova, K.D. Piatkevich, A.M. Cuervo, and V.V. Verkhusha. 2009. Monomeric fluorescent timers that change color from blue to red report on cellular trafficking. *Nature chemical biology.* 5:118-126.
- Subramani, S., A. Koller, and W.B. Snyder. 2000. Import of peroxisomal matrix and membrane proteins. *Annu Rev Biochem.* 69:399-418.
- Tersikh, A., A. Fradkov, G. Ermakova, A. Zaisky, P. Tan, A.V. Kajava, X. Zhao, S. Lukyanov, M. Matz, S. Kim, I. Weissman, and P. Siebert. 2000. "Fluorescent timer": protein that changes color with time. *Science.* 290:1585-1588.

- Tsuboi, T., T. Kitaguchi, S. Karasawa, M. Fukuda, and A. Miyawaki. 2010. Age-dependent preferential dense-core vesicle exocytosis in neuroendocrine cells revealed by newly developed monomeric fluorescent timer protein. *Mol Biol Cell*. 21:87-94.
- van Roermund, C.W., L. Ijlst, W. Majczak, H.R. Waterham, H. Folkerts, R.J. Wanders, and K.J. Hellingwerf. 2012. Peroxisomal fatty acid uptake mechanism in *Saccharomyces cerevisiae*. *J Biol Chem*. 287:20144-20153.
- Veenhuis, M., M. Mateblowski, W.H. Kunau, and W. Harder. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast*. 3:77-84.
- Wach, A., A. Brachat, C. Alberti-Segui, C. Rebischung, and P. Philippsen. 1997. Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast*. 13:1065-1075.
- Wang, X., S. Li, Y. Liu, and C. Ma. 2015. Redox regulated peroxisome homeostasis. *Redox biology*. 4:104-108.
- Waterham, H.R., S. Ferdinandusse, and R.J. Wanders. 2016. Human disorders of peroxisome metabolism and biogenesis. *Biochim Biophys Acta*. 1863:922-933.
- Williams, C. 2014. Going against the flow: a case for peroxisomal protein export. *Biochim Biophys Acta*. 1843:1386-1392.
- Williams, C., M. van den Berg, E. Geers, and B. Distel. 2008. Pex10p functions as an E3 ligase for the Ubc4p-dependent ubiquitination of Pex5p. *Biochem Biophys Res Commun*. 374:620-624.
- Williams, C., and I.J. van der Klei. 2013. Pexophagy-linked degradation of the peroxisomal membrane protein Pex3p involves the ubiquitin-proteasome system. *Biochem Biophys Res Commun*. 438:395-401.
- Wroblewska, J.P., L.D. Cruz-Zaragoza, W. Yuan, A. Schummer, S.G. Chuartzman, R. de Boer, S. Oeljeklaus, M. Schuldiner, E. Zalckvar, B. Warscheid, R. Erdmann, and I.J. van der Klei. 2017. *Saccharomyces cerevisiae* cells lacking Pex3 contain membrane vesicles that harbor a subset of peroxisomal membrane proteins. *Biochimica et biophysica acta. Molecular cell research*. 1864:1656-1667.
- Zhang, L., N.G. Gurskaya, E.M. Merzlyak, D.B. Staroverov, N.N. Mudrik, O.N. Samarkina, L.M. Vinokurov, S. Lukyanov, and K.A. Lukyanov. 2007. Method for real-time monitoring of protein degradation at the single cell level. *BioTechniques*. 42:446, 448, 450.

3

Chapter 3

Proteasome dependent quality control of the peroxisomal membrane protein Pxalp

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Author Contributions

CW conceived and supervised the project, with input from EH, SK and MK. CW, SD, MM, CVR and XC designed the experiments. SD and CW analysed the data with the help from other authors. SD performed biochemical and fluorescence microscopy experiments with the support from XC. SD and MM performed tFT screening experiments. CVR performed β -oxidation experiment. EH was involved in plasmid construction, SD and CW wrote the manuscript with the contribution from all the authors.

Abstract

Peroxisomes are eukaryotic organelles that function in numerous metabolic pathways and defects in peroxisome function can cause serious developmental brain disorders such as adrenoleukodystrophy (ALD). Peroxisomal membrane proteins (PMPs) play a crucial role in regulating peroxisome function. Therefore, PMP homeostasis is vital for peroxisome function. Recently, we established that certain PMPs are degraded by the Ubiquitin Proteasome System (UPS) yet little is known about how faulty/non-functional PMPs are targeted for degradation via quality control. Here we have investigated the degradation of Pxa1p, a fatty acid transporter in the yeast *Saccharomyces cerevisiae*. Pxa1p is a homologue of the human protein ALDP and mutations in ALDP result in the severe disorder ALD. By introducing two corresponding ALDP mutations into Pxa1 (Pxa1^{MUT}), fused to mGFP, we show that Pxa1^{MUT}-mGFP is rapidly degraded from peroxisomes in a proteasome-dependent manner, while wild type Pxa1-mGFP remains relatively stable. Furthermore, we identify a role for the ubiquitin ligase Ufd4p in Pxa1^{MUT}-mGFP degradation. Finally, we establish that inhibiting Pxa1^{MUT}-mGFP degradation results in a partial rescue of Pxa1p activity in cells. Together, our data demonstrate that faulty PMPs can undergo proteasome-dependent quality control. Furthermore, our observations may provide new insights into the role of ALDP degradation in ALD.

Keywords: protein degradation, proteasome, ALD, peroxisome, ufd4

Introduction

Peroxisomes are eukaryotic organelles that encompass a protein-rich matrix bound by a single membrane. Their morphology, abundance and function depends on species and developmental stage (Gabaldon, 2010). Some well-known peroxisomal functions include fatty acid oxidation and hydrogen peroxide detoxification, but many more exist (Smith and Aitchison, 2013). Their importance in human health is underlined by the severe diseases such as adrenoleukodystrophy (ALD) caused by defects in peroxisome function (Kemp et al., 2016).

Peroxisome function is largely determined by peroxisomal protein content. Most peroxisomal membrane proteins (PMPs) are synthesized in the cytosol and targeted directly to peroxisomes (Nuttall et al., 2011) although a subset of PMPs may be delivered to peroxisomes via the endoplasmic reticulum (Mayerhofer, 2016). PMPs regulate many aspects of peroxisome biology, including peroxisomal protein import (Fang et al., 2004), peroxisome numbers (Williams et al., 2015) and small molecule transport into peroxisomes (van Roermund et al., 2012). For peroxisome function, PMP homeostasis is vital; this includes the regulation of targeting but also protein quality control processes and protein degradation. Recently, we demonstrated that the PMPs Pex3p and Pex13p in the yeast *Hansenula polymorpha* are actively down-regulated (Chen et al., 2018; Williams and van der Klei, 2013). Pex3p degradation initiates selective autophagy of peroxisomes while Pex13p degradation is linked to peroxisomal matrix protein import. Both Pex3p and Pex13p are degraded by the Ubiquitin Proteasome System (UPS) (Bett, 2016). In this system, ubiquitin (Ub) is activated by a ubiquitin-activating enzyme (E1), then transferred to the active site cysteine of an ubiquitin-conjugating enzyme (E2) and finally, Ub is attached to the substrate with the aid of an ubiquitin ligase (E3) (Metzger and Weissman, 2010; Schulman and Harper, 2009; Ye and Rape, 2009). Three classes of E3s exist. HECT E3 ligases accept Ub onto an active site cysteine and then transfer Ub to a substrate (Metzger et al., 2012; Weber et al., 2019), whereas RING E3 ligases act as bridge between E2 and substrate, allowing Ub transfer to occur (Deshaies and Joazeiro, 2009; Metzger et al., 2014). The third class of E3 ligase, known as RING-in-between-RING (RBR) E3s, contain a RING domain, followed by an RBR domain and finally a RING-like domain (Wenzel et al., 2011). These RBR E3s, like HECT E3s, accept Ub from an E2 onto a cysteine in the RING-like domain before transferring it to the substrate (Dove et al., 2016).

The examples of Pex3p and Pex13p demonstrate that targeted down-regulation of PMPs does occur. However, because peroxisomes are involved in various oxidative metabolic reactions, they can generate large amounts of reactive oxygen species (ROS) such as hydrogen peroxide (Schrader and Fahimi, 2006). Because ROS are toxic compounds which cause damage to biomolecules (Costa et al., 2007), it is highly likely that PMPs residing in the ROS rich environment of the peroxisome undergo oxidative damage. Therefore, PMP quality control is likely to play a vital role in peroxisome biology yet to date little is known on how misfolded, non-functional or faulty PMPs are targeted for degradation via quality control. Here we have investigated PMP quality control in the yeast *Saccharomyces cerevisiae* using Pxa1p as

substrate. Pxa1p is a half-ABC transporter, containing six transmembrane helices and a nucleotide-binding domain (NBD). The hetero-dimerization of Pxa1p with Pxa2p, another half-ABC transporter, is required for transporting acyl-CoA such as Oleoyl-CoA into peroxisomes (Hettema et al., 1996; Shani et al., 1996). Pxa1p is the homologue of human adrenoleukodystrophy protein (ALDP) (Shani et al., 1995). Mutations in ALDP cause ALD (Engelen et al., 2014) and many ALDP mutants are rapidly degraded, likely by the proteasome (Morita et al., 2019). This has led to the suggestion that blocking ALDP degradation might constitute a feasible therapeutic approach to treat ALD (Morita et al., 2019; Zhang et al., 2011). However, to date little is known about the mechanisms underlying ALDP instability or degradation.

Using a mutant form of Pxa1p (Pxa1^{MUT}) that mimics ALD-causing ALDP mutations, fused to mGFP, we show that Pxa1^{MUT}-mGFP is rapidly degraded from peroxisomes. Furthermore, we show that Pxa1^{MUT}-mGFP degradation is dependent on the proteasome while our data also demonstrate a role for the E3 ligase Ufd4p in Pxa1^{MUT}-mGFP degradation. Finally, we show that inhibiting Pxa1^{MUT}-GFP degradation partially restores Pxa1p function *in vivo*. Taken together, our study demonstrates that faulty PMPs can undergo proteasome-mediated degradation. Furthermore, our observations on Pxa1^{MUT}-mGFP degradation may provide new insights into the role of ALDP stability in ALD.

Results

Pxa1^{MUT}-mGFP undergoes proteasome-mediated degradation from peroxisomes

Unlike wild-type (WT) ALDP, many mutant versions of ALDP are unstable and are rapidly degraded (Takahashi et al., 2007; Yamada et al., 1997), suggesting that quality control of faulty peroxisomal fatty acid transporters occurs. To investigate this in *S. cerevisiae*, we chose to study the degradation of the yeast ALDP homologue Pxa1p. For this, we selected two ALD causing mutations in ALDP, a commonly occurring glycine to serine substitution at position 512 (p.Gly512Ser) together with a lysine to arginine substitution at position 513 (p.Lys513Arg), both present in the walker A motif of the NBD (www.adrenoleukodystrophy.info). The p.Gly512Ser mutation causes reduced protein stability *in vivo* (Feigenbaum et al., 1996) although importantly, it does not abolish ATP-hydrolysis activity *in vitro* (Roerig et al., 2001). The p.Lys513Arg mutation also causes protein instability *in vivo* (www.adrenoleukodystrophy.info). The corresponding ALDP mutations (p.Gly650Ser and p.Lys651Arg) were both introduced into yeast *PXA1*. We chose to introduce two mutations to maximize the destabilizing effect to Pxa1p. The Gly650Ser/Lys651Arg mutant form of Pxa1p is referred to as Pxa1^{MUT}. We choose to fuse Pxa1^{MUT} to mGFP (Pxa1^{MUT}-mGFP), which allowed us to follow Pxa1^{MUT} using both biochemical and microscopy based techniques. Furthermore, the DNA encoding for Pxa1^{MUT}-mGFP was integrated into the genome of the *S. cerevisiae* strains used and therefore represents the only copy of *PXA1* in the cells.

Since Pxa1p functions in transporting fatty acyl-CoA, such as Oleoyl-CoA, into peroxisomes for β -oxidation (Hettema et al., 1996; Shani et al., 1996), we performed all our experiments using cells grown on oleate containing media, to better understand the turnover of Pxa1^{MUT}-mGFP under conditions when the protein is required. For this purpose, *S. cerevisiae* cells were pre-cultivated on glucose medium and shifted to oleate medium, to induce the expression of the proteins required for peroxisomal β -oxidation (Gurvitz and Rottensteiner, 2006; Karpichev and Small, 1998; Smith et al., 2002). However, because we performed experiments using deletion mutants that are unable to utilize oleate as carbon source (see below), we used oleate medium containing 0.1% glucose throughout the study, to allow results to be comparable. Therefore unless otherwise stated, oleate medium always contained 0.1% glucose.

First, we investigated whether Pxa1^{MUT}-mGFP is properly targeted to peroxisomes. For this, we used fluorescence microscopy to examine the localization of Pxa1^{MUT}-mGFP in cells grown on oleate. Pex3-mKate2 (Figure 1A) or DsRed-SKL (Figure S1A) was used as a marker for peroxisomes. As control, we used cells producing an mGFP-tagged version of WT Pxa1p (Pxa1-mGFP). We observed that mGFP spots co-localized with mKate2 spots in strains expressing Pxa1-mGFP (Figure 1A, row 4) or Pxa1^{MUT}-mGFP (Figure 1A, row 6) while line profile data indicate a strong correlation between the normalized fluorescent intensity of mGFP and mKate2 (Figure 1A, row 4 and row 6, right), indicating that both Pxa1-fusions correctly target to peroxisomes. Likewise, mGFP spots co-localized with DsRed spots in cells expressing Pxa1^{MUT}-mGFP and DsRed-SKL (Figure S1A), confirming that Pxa1^{MUT}-mGFP correctly targets to peroxisomes. We also observed larger, more diffuse spots in both mGFP and mKate2 channels (Figure 1A, red arrows). However, these were also seen in WT cells lacking fluorescent markers (Figure 1A, row 1) and likely represent auto-fluorescence.

The mGFP signal in cells expressing Pxa1^{MUT}-mGFP was lower and fewer GFP spots were visible compared to those expressing Pxa1-mGFP (Figure 1A, row 6), which is consistent with the reduced protein levels of Pxa1^{MUT}-mGFP compared to those of Pxa1-mGFP (Figure 1B, 1C and S1C). Lower protein levels could suggest either reduction in protein production or reduced protein stability. To examine the stability of Pxa1^{MUT}-mGFP we performed chase analysis using the protein synthesis inhibitor cycloheximide (CHX). For this purpose, cells were pre-cultivated on glucose, transferred to oleate containing media (peroxisome inducing conditions) and grown for 11 hrs until an OD₆₀₀ of ~1.5. After 11 hrs of growth in oleate containing media, cells were treated with either CHX or DMSO (control), cells were grown further for 6 hrs (Pxa1-mGFP) or 2hrs (Pxa1^{MUT}-mGFP) on inducing medium and samples were collected for western blotting.

Figure 1: Pxa1^{MUT}-mGFP targets to peroxisomes but displays reduced protein levels

- A. Pxa1-mGFP and Pxa1^{MUT}-mGFP localization in cells co-expressing Pex3-mKate2 (row 4 and 6). Cells pre-cultivated on glucose were grown on oleate/glucose media to an OD₆₀₀ of 1.5 and fluorescence images were taken. Here, a WT strain lacking fluorescent markers (row 1) together with WT strains expressing only Pex3-mKate2 (row 2), Pxa1-mGFP (row 3) or Pxa1^{MUT}-mGFP (row 5) act as controls. Blue arrows- GFP spots, yellow arrows- mKate2 spots and red arrows- background fluorescence. The line profiles (taken from the spots indicated with blue or yellow lines in images of WT.Pxa1-mGFP + Pex3-mKate2 and Pxa1^{MUT}-mGFP + Pex3-mKate2 cells) indicate normalized fluorescent intensity along a line drawn through the peroxisomes (dotted yellow line). Scale bar: 3 μ m.
- B. Cells expressing Pxa1-mGFP or Pxa1^{MUT}-mGFP were grown on oleate/glucose media to an OD₆₀₀ of 1.5, lysed and samples were subjected to SDS-PAGE and immunoblotting with antibodies against mGFP and Pyc.
- C. Bar chart displaying Pxa1-mGFP and Pxa1^{MUT}-mGFP levels, normalized to the loading control Pyc. Protein levels in Pxa1-mGFP cells were set to 1. Values represent the mean $\pm$ SD of three independent experiments. Here, asterisks represent statistically significant decrease in Pxa1^{MUT}-mGFP levels compared to Pxa1-mGFP levels. For quantification, blots in Figure S1C were used.

We observed an increase in Pxa1-mGFP and Pxa1^{MUT}-mGFP levels in DMSO-treated cells over time (Figure 2A, DMSO), consistent with the observation that Pxa1-mGFP levels continue to increase until ~36 hrs after transfer of cells to induction medium (Figure S1D). Significantly, Pxa1^{MUT}-mGFP was rapidly degraded and displayed a significantly shorter half-life (~15 mins) compared to Pxa1-mGFP (~360 mins or 6 hrs) (Figure 2A, CHX and S1E) in

cells treated with CHX, indicating that the mutations introduced into Pxa1p result in protein instability.

A

B

C

D

Figure 2: Pxa1^{MUT}-mGFP is rapidly degraded from peroxisomes

- A. Cycloheximide (CHX) chase analysis on cells expressing Pxa1-mGFP and Pxa1^{MUT}-mGFP. (Left) Cells pre-cultivated on glucose media were grown on oleate/glucose media (inducing condition) to an OD₆₀₀ of 1.5. After treatment with DMSO (Ctrl) or CHX, cells were grown further on inducing medium and samples were collected at the indicated time points for immunoblotting with antibodies against mGFP and Pyc. (Right) Quantification of Pxa1-mGFP and Pxa1^{MUT}-mGFP levels after CHX addition. Protein levels were normalized to Pyc at the corresponding time point and to the protein levels at time point 0 (set to 1). Values represent the mean $\pm$ SD of three independent experiments. For quantification, blots in Figure S1E were used.
- B. Co-localization analysis of Pxa1-mGFP (Left) and Pxa1^{MUT}-mGFP (Right) with the peroxisomal marker Pex3-mKate2 after CHX treatment. Cells expressing Pxa1-mGFP or Pxa1^{MUT}-mGFP together with Pex3-mKate2 were grown on oleate/glucose media to an OD₆₀₀ of 1.5 and treated with CHX. Fluorescence images were taken from cells grown on oleate/glucose media at the indicated time points (mins) after CHX addition. Blue arrows- mGFP spots, yellow arrows- mKate2 spots and red arrows- background fluorescence. Scale bar: 3 μ m. Line profiles were generated as described in Figure 1.
- C. Box plot quantification of mGFP and mKate2 fluorescence intensity at the peroxisomal membrane in WT cells producing Pex3-mKate2 and Pxa1-mGFP (Left) or Pxa1^{MUT}-mGFP (Right) after CHX treatment. Fluorescence intensities were measured in cells (n=40) using ImageJ as described in the materials and methods section. The box represents intensity values from the 25th percentile to the 75th percentile. The orange area represents intensity values from the 25th -50th percentile and the grey area represents intensity values from the 50th -75th percentile. Whiskers indicate maximum and minimum values.
- D. Average ratio $\pm$ SD per cell (n = 40) of mGFP to mKate intensities in WT cells producing Pex3-mKate2 and Pxa1-mGFP (Left) or Pxa1^{MUT}-mGFP (Right). Numbers above the columns (Left) depict the p-value. Asterisks (Right) denote significance between ratios at different time points, ** P < 0.01- statistically significant (Right) and P > 0.05- not significant (Left).

Next, we sought to determine whether Pxa1^{MUT}-mGFP degradation occurs after targeting to peroxisomes. For this, we imaged CHX treated WT cells co-expressing Pex3-mKate2 and Pxa1-mGFP or Pxa1^{MUT}-mGFP. We observed that the number of mGFP positive spots reduced over time in cells expressing Pxa1^{MUT}-mGFP (Figure 2B, right panel) while cells expressing Pxa1-mGFP displayed many more mGFP positive spots after 120 mins of CHX treatment (Figure 2B, left panel). The number of mKate2 spots remained stable after CHX treatment in both strains (Figure 2B). These observations suggest that Pxa1^{MUT}-mGFP is degraded from peroxisomes. To validate this further, we quantified mGFP and mKate2 fluorescence intensities on peroxisomes in our fluorescent images. We observed that, as expected, the mKate2 intensity remained stable over time in the two strains (Figure 2C). Likewise, the mGFP intensity on peroxisomes in CHX-treated Pxa1-mGFP cells remained stable (Figure 2C, left panel) but decreased rapidly in Pxa1^{MUT}-mGFP cells treated with CHX (Figure 2C, right panel). In addition, the mGFP/mKate2 ratio in CHX treated Pxa1^{MUT}-mGFP cells decreased rapidly over time but remained stable in Pxa1-mGFP cells after CHX addition (Figure 2D). The rate of the decrease in mGFP intensity in Pxa1^{MUT}-mGFP cells treated with CHX appears different from that obtained using western blotting (Figure 2A), likely due to the

high background observed in the mGFP channel (Figure 1A, row 1 and 2). Together, these data strongly suggest that Pxa1^{MUT}-mGFP is rapidly degraded from peroxisomes.

Since ALDP mutants are thought to be degraded via the proteasome (Takahashi et al., 2007), we first examined whether the proteasome has a role in Pxa1^{MUT}-mGFP degradation, through the use of a mixture of the proteasome inhibitors MG132 and Bortezomib (Figure 3A and S1F). We assessed Pxa1^{MUT}-mGFP turnover in cells lacking Pdr5p (the major drug efflux transporter (Golin et al., 2007)), to enhance the uptake of inhibitors (Dederer et al., 2019; Fleming et al., 2002). In addition, we examined Pxa1^{MUT}-mGFP degradation in cells lacking Atg12p (Figure 3B and S1G), which is involved in autophagy (Hanada et al., 2007). From our CHX chase analysis, we observed that Pxa1^{MUT}-mGFP degradation is inhibited in *pdr5* cells after the addition of proteasome inhibitors (Figure 3A, CHX+ MG132+Bortezomib) compared to non-treated cells (Figure 3A, CHX). These data, together with the observation that the deletion of *ATG12* had no effect on Pxa1^{MUT}-mGFP turnover (Figure 3B and S1G) demonstrates that the proteasome is involved in Pxa1^{MUT}-mGFP degradation. Next, we checked whether ubiquitination has a role in Pxa1^{MUT}-mGFP degradation by expressing a mutant form of ubiquitin (Ub^{K48R}) in Pxa1^{MUT}-mGFP cells, to inhibit poly-ubiquitin chain formation on the substrate and subsequent degradation by the proteasome (Thrower et al., 2000). We observed that Pxa1^{MUT}-mGFP turnover is reduced in cells expressing Ub^{K48R}, compared to Ub expressing cells (Figure 3C and S1G). However, we were unable to detect ubiquitinated Pxa1^{MUT}-mGFP (Figure 3D). Possibly ubiquitinated Pxa1^{MUT}-mGFP is below the level of detection because of the high speed by which Pxa1^{MUT}-mGFP is degraded. In summary, these data demonstrate that Pxa1^{MUT}-mGFP undergoes rapid, proteasome-mediated degradation from peroxisomes.

Figure 3: Inhibiting the proteasome but not autophagy disturbs Pxa1^{MUT}-mGFP degradation

- A. Graph representing Pxa1^{MUT}-mGFP levels in *pdr5* cells under proteasome inhibitor (PI) conditions. *pdr5* cells expressing Pxa1^{MUT}-mGFP pre-cultivated on glucose were grown on oleate/glucose media for 10 h. After incubation with DMSO or proteasome inhibitors (MG132 and Bortezomib) for 90 mins, CHX was added to DMSO (represented as CHX) and PI treated cells (denoted as CHX+MG132+Bortezomib). TCA samples for western blotting were collected from cells grown on oleate/glucose media at the indicated time points after treatment with inhibitors. Protein levels were normalized to Pyc at the corresponding time point and to the protein levels at time point 0 (set to 1). Values represent the mean $\pm$ SD of three independent experiments. For quantification, blots in Figure S1F were used.
- B. Pxa1^{MUT}-mGFP levels in WT and *atg12* cells after CHX treatment. Cells pre-cultivated on glucose were grown on oleate/glucose media to an OD₆₀₀ of 1.5 and treated with CHX. TCA samples were collected from cells grown on oleate/glucose at the indicated time points after CHX treatment and probed with western blotting. Protein levels were normalized to Pyc at the corresponding time point and to the protein levels at time point 0 (set to 1). For quantification, blots in Figure S1G were used.
- C. Quantification of Pxa1^{MUT}-mGFP levels in WT cells and cells expressing Myc-tagged ubiquitin (Ub) or ubiquitin mutant (Ub^{K48R}) after treatment with CHX. The experiment and quantification of levels were performed as mentioned in Figure 3B. Values represent the mean $\pm$ SD of three independent experiments. For quantification, blots in Figure S1G were used.
- D. Immunoprecipitation performed using anti-GFP antibodies on lysates from *pdr5* cells, *pdr5*.Pxa1^{MUT}-mGFP cells, *pdr5*.Pxa1^{MUT}-mGFP/MycUb cells and *pdr5*.MycUb cells. Cells were pre-cultivated on glucose,

transferred to oleate/glucose media for 10 hrs and incubated with proteasome inhibitors (MG132 and Bortezomib) for 90 mins. Pxa1^{MUT}-mGFP was immunoprecipitated under native conditions using GFP-trap magnetic beads. Load and elute fractions were subjected to immunoblotting with antibodies against the Myc-tag (to detect Myc-ubiquitin) or the GFP-tag (to detect Pxa1^{MUT}-mGFP).

Identifying components required for Pxa1-tFT degradation

To gain the first insights into mechanisms of Pxa1^{MUT}-mGFP quality control, we set out to identify components required for Pxa1^{MUT}-mGFP degradation using a tandem fluorescent protein timer (tFT). A tFT is a fusion of two fluorescent proteins; the slow maturing mCherry and the rapid maturing sfGFP (Figure 4A). When tagged to a protein of interest, the mCherry/sfGFP ratio provides information on protein stability. tFT fusions undergoing fast turnover are degraded prior to mCherry maturation, resulting in a low mCherry/sfGFP ratio, whereas the mCherry/sfGFP ratio increases for proteins with slower turnover (Khmelniskii et al., 2012). Similar to Pxa1^{MUT}-mGFP, Pxa1-tFT levels in cells were much lower compared to Pxa1-mGFP levels (Figure 4B) while Pxa1-tFT was rapidly degraded in cells treated with CHX (Figure 4C and S1H). The turnover of tFT-tagged Pex11p, a stable PMP (Chen et al., 2018), was slower in comparison (Figure 4C and S1H). Furthermore, the GFP immunoreactive band at around 33kDa observed with samples containing Pxa1-tFT (Figure 4B and S1H, marked **) is derived from incomplete tFT processing by the proteasome (Khmelniskii et al., 2016), suggesting a role for the proteasome in Pxa1-tFT turnover. From these data, we conclude that, similar to Pxa1^{MUT}-mGFP, Pxa1-tFT undergoes proteasome-mediated degradation and for this reason, we did not create a Pxa1-tFT fusion containing the Gly650Ser/Lys651Arg mutations.

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Figure 4: Identifying factors involved in Pxa1^{MUT}-tFT degradation by tandem fluorescent timer

A. Schematic representation of the Pxa1-tandem fluorescent timer (tFT) fusion. Pxa1p is fused to two fluorescent proteins with different maturation kinetics: rapidly maturing sfGFP and slow maturing mCherry. The fluorescent intensity ratio of mCherry to sfGFP provides information of Pxa1p stability.

- B. WT cells together with cells expressing Pxa1-mGFP, Pxa1-tFT or Pxa1^{MUT}-mGFP were initially grown on glucose, shifted to oleate/glucose media and grown until an OD₆₀₀ of 1.5. After lysis, samples were probed by immunoblotting with antibodies against mGFP and Pyc. *denotes a shorter, mCherry-sfGFP product resulting from mCherry hydrolysis during TCA treatment (Gross et al., 2000; Khmelinskii et al., 2016) and ** denotes a tFT fragment produced by incomplete proteasomal processing (Khmelinskii et al., 2016).
- C. The stability of tFT tagged Pxa1p and Pex11p was assayed using CHX chase analysis. Cells pre-cultivated on glucose were grown on oleate/glucose media an OD₆₀₀. 1.5. After treatment with CHX, cells were further grown on oleate/glucose media and samples collected at the indicated points were subjected for western blotting. Protein levels obtained at each time point were normalized to Pyc at the corresponding time point and to the protein levels at time point 0 (set to 1). Values represent the mean ± SD of three independent experiments. For quantification, blots in Figure S1H were used.
- D. Screen for components involved in Pxa1-tFT stability. WT and mutant cells expressing Pxa1-tFT were grown on oleate/glucose plates and fluorescent intensities were measured. The resulting mCherry/sfGFP ratio on the indicated days (1-3) were used to calculate the Z-score (see materials and methods). Z-score colour coded from blue (decrease) to red (increase), represents changes in Pxa1-tFT stability. Mutant strains with Z-score >1.0 (indicated by *) on two of the three days tested were defined as potentially interesting.
- E. Western blot analysis of Pxa1-tFT levels in WT, *atg12*, *pex2* and *pex4* cells grown on oleate/glucose media to an OD₆₀₀ of 1.5. After lysis, samples were probed by immunoblotting with antibodies against mGFP and Pyc. *denotes a shorter, mCherry-sfGFP product resulting from mCherry hydrolysis during TCA lysate preparation (Khmelinskii et al., 2016). The apparent discrepancy between the molecular weight of Pxa1-tFT in this panel and in Figure 4B is because samples here were run onto a 7.5% acrylamide SDS-PAGE gel while samples in 4B were loaded onto a 10% acrylamide SDS-PAGE gel, resulting in a different run profile.

Next, we investigated the stability of Pxa1-tFT in a library of 132 strains that lack a protein involved in protein degradation or which contained a mutant version of a protein involved in protein degradation (in case the deletion was lethal) using a synthetic genetic array (Schuldiner et al., 2005; Tong and Boone, 2006). This library represents a large fraction of the factors known to have a role in the degradation of proteins in yeast (see Table S2 for details on which strains were included in the library). The effect of the mutations on the stability of Pxa1-tFT was examined using mCherry/sfGFP intensity ratios obtained from whole colonies after one, two and three days of growth on oleate/glucose plates. These mCherry/sfGFP ratios were used to calculate a heat map of Z-scores (Figure 4D), which depicts the stability of Pxa1-tFT in each strain. A strain displaying similar Pxa1-tFT stability as in WT cells would have a Z-score of 0 while strains with enhanced stability would exhibit a Z-score >0 (see materials and methods). Mutant strains that displayed an increase in Z-score >1.0 on two of the three days tested were considered as potential candidates that increased Pxa1-tFT stability. Pxa1-tFT stability was increased in cells lacking functional proteasomes (*pre2*, *pre6*, *rpn10*, *rpt6-25* and *ubp6* cells) but not in cells lacking Atg12p (Figure 4D), confirming a role for the proteasome in Pxa1-tFT stability. These results demonstrate that our tFT analysis is a valid way to identify factors potentially involved in Pxa1^{MUT}-mGFP degradation.

Pxa1-tFT stability was increased in strains deleted for genes coding for the peroxisomal E2 Pex4p or one of the three peroxisomal E3 ligases Pex2p, Pex10p and Pex12p (Agne et al., 2003) (Figure 4D). Consistent with this, Pxa1-tFT protein levels were enhanced in *pex2* and *pex4* cells (Figure 4E), thereby confirming our tFT data. Roles for these proteins in PMP ubiquitination/degradation have already been reported (Chen et al., 2018; Sargent et al., 2016; Williams and van der Klei, 2013), suggesting that common mechanisms may govern PMP ubiquitination and degradation. However, Pxa1-tFT stability was also enhanced in cells lacking a number of de-ubiquitinating enzymes (DUBs), which could suggest a role for these DUBs in the deubiquitination of Pxa1^{MUT}-mGFP prior to proteasomal degradation. In addition, cells lacking the E3s Nam7p, Dma2p, Tull1p and Ufd4p, the E3 co-factor proteins Ela1p and Skp2p and the ubiquitin-binding protein Dsk2p (Supplementary Table S2), factors not previously associated with peroxisomes, also displayed enhanced Pxa1-tFT stability. Overall, our tFT analysis identified factors potentially involved in the degradation of faulty Pxa1p.

Components involved in Pxa1-tFT stability play a role in Pxa1^{MUT}-mGFP degradation

Previously we demonstrated that the peroxisomal ubiquitination machinery is required for the degradation of the PMPs Pex3p and Pex13p (Chen et al., 2018; Williams and van der Klei, 2013). Because deletion of *PEX4*, *PEX2*, *PEX10* or *PEX12* results in Pxa1-tFT stabilization (Figure 4D and 4E), we were interested to investigate whether they also had a role in Pxa1^{MUT}-mGFP degradation. Indeed, Pxa1^{MUT}-mGFP levels were enhanced in cells deleted for *PEX2* or *PEX4* (Figure 5A and S2A) while Pxa1^{MUT}-mGFP turnover was also inhibited in these cells (Figure 5B and S2B), validating our tFT data as well as indicating a role for these peroxisomal proteins in Pxa1^{MUT}-mGFP degradation. Furthermore, Pxa1^{MUT}-mGFP localised to peroxisomes in these strains (Figure 5C), indicating that stabilisation did not arise from mistargeting to other cell compartments. However, because cells deleted for *PEX2* or *PEX4* also lack functional peroxisomes (Burkhart et al., 2014), we investigated the impact of peroxisome function alone on Pxa1^{MUT}-mGFP turnover by assessing the stability and turnover of Pxa1^{MUT}-mGFP in *pex5* cells, which also lack functional peroxisomes (Van der Leij et al., 1993). Our data demonstrate that Pxa1^{MUT}-mGFP degradation was inhibited in *pex5* cells (Figure 5A, B and S2B), suggesting that Pxa1^{MUT}-mGFP degradation may be linked to peroxisome function. Again, Pxa1^{MUT}-mGFP targeting was not impaired in *pex5* cells (Figure 5C). We observed that Pex3-mKate2 levels are comparable in WT, *pex2*, *pex4* and *pex5* cells (Figure S2C), suggesting that the effect of these deletion strains is not general for all PMPs. Nevertheless, these data indicate that studying Pxa1^{MUT}-mGFP turnover in strains deficient in peroxisome function could be challenging because any effects may potentially be indirect.

Therefore, we turned to investigate the role of candidates identified in our tFT analysis not previously associated with peroxisome function, including Ufd4p, Nam7p, Ela1p, Dsk2p, Skp2p, Tull1p and Dma2p. Interestingly, we observed that Pxa1^{MUT}-mGFP levels were significantly higher in cells lacking Ufd4p (Figure 6A and S2A). Deletion of *ELA1*, *DSK2*,

SKP2, *TUL1* or *DMA2* did not impact significantly on Pxa1^{MUT}-mGFP levels (Figure 6A and S2A) and were not investigated further. Furthermore, Pxa1^{MUT}-mGFP levels were significantly lower in the *NAM7* deletion strain compared to the WT (Figure 6A and S2A). However, because we were specifically interested in UPS mutants that increased the stability of Pxa1^{MUT}-mGFP, the role of *NAM7* in Pxa1^{MUT}-mGFP stability was not investigated further.

Significantly, we observed that *ufd4* cells retain the ability to grow on media containing oleate as sole carbon source (Figure 6B), indicating that they contain functional peroxisomes and hence that Pxa1^{MUT}-mGFP stabilization in this strain does not stem from a deficiency in peroxisome function. Since Ufd4p is a cytosolic E3 ligase that regulates the degradation of faulty proteins (Theodoraki et al., 2012), but it has not been linked to peroxisome function, we chose to investigate the role of Ufd4p in Pxa1^{MUT}-mGFP degradation further. We performed CHX chase analysis and observed that Pxa1^{MUT}-mGFP degradation is inhibited in *ufd4* cells compared to WT cells (Figure 6C and S2B). Furthermore, using fluorescence microscopy in combination with CHX chase assays, we investigated the localization and turnover of Pxa1^{MUT}-mGFP in *ufd4* cells. As can be seen in Figure 6D (t=0, left panel), mGFP spots co-localize with mKate2 spots in *ufd4* cells. In addition, line profile data indicate strong correlation between the normalized fluorescent intensity of mGFP and mKate2, demonstrating that Pxa1^{MUT}-mGFP is indeed localised to peroxisomes in *ufd4* cells (Figure 6D). The peroxisomal localisation of Pxa1^{MUT}-mGFP in *ufd4* cells was confirmed using DsRed-SKL as a peroxisomal marker (Figure S1A). Significantly, cells deleted for *UFD4* display increased numbers of mGFP spots after 120 mins of CHX treatment, compared to the WT strain (Figure 6D, right panel; t=120 mins). Furthermore, mGFP spots in *ufd4* cells co-localize with mKate2 spots after 120 mins of CHX treatment (Figure 6D, left panel), demonstrating that Pxa1^{MUT}-mGFP accumulates on peroxisomes in these cells. Together, these data establish that the E3 ligase Ufd4p is involved in the proteasome-dependent degradation of Pxa1^{MUT}-mGFP.

Figure 5: Pxa1^{MUT}-mGFP degradation is inhibited in peroxisome deficient strains

- A. Bar chart displaying Pxa1^{MUT}-mGFP levels in WT, *pex2* and *pex4* strains (Left) and WT, *atg12* and *pex5* strains (Right). Cells pre-cultivated on glucose were grown on oleate/glucose to an OD₆₀₀ of ~1.5 and TCA samples were collected for western blotting. For quantification of Pxa1^{MUT}-mGFP levels, blots in Figure S2A were used. Pxa1^{MUT}-mGFP levels in WT were set to 1, values represent the mean $\pm$ SD of three independent experiments. Asterisks represent statistically significant increase of Pxa1^{MUT}-mGFP levels in mutant strains compared to in WT cells. ** P < 0.01- statistically significant and P > 0.05- not significant (NS).
- B. CHX chase of Pxa1^{MUT}-mGFP in WT, *pex2* and *pex4* strains (Top) and WT, *atg12* and *pex5* strains (Bottom). Cells were grown on oleate/glucose media to an OD₆₀₀ of 1.5, treated with CHX and samples were collected from oleate/glucose grown cells at the indicated time points after CHX treatment. Protein levels were

normalized to Pyc at the corresponding time point and to the protein levels at time point 0 (set to 1). Values represent the mean $\pm$ SD of three independent experiments. For quantification, blots in Figure S2B were used.

C. Co-localization analysis of Pxa1^{MUT}-mGFP with the peroxisomal marker Pex3-mKate2 in WT, *pex2*, *pex4* and *pex5* cells. The WT strain lacking fluorescent markers was used as control. Fluorescence images were taken from cells grown on oleate/glucose media to an OD₆₀₀ of 1.5. Blue arrows- GFP spots, yellow arrows- mKate2 spots and red arrows- background fluorescence. Scale bar: 3 μ m

Figure 6: Ufd4, a cytosolic E3 ligase is involved in Pxa1^{MUT}-mGFP degradation

- A. Pxa1^{MUT}-mGFP levels in WT, *ufd4*, *nam7*, *ela1*, *dsk2*, *skp1*, *tul1* or *dma2* cells. TCA samples for western blotting were collected from cells pre-cultivated on glucose and grown on oleate/glucose to an OD₆₀₀ of ~1.5. For quantification of Pxa1^{MUT}-mGFP levels, blots in Figure S2A were used. Pxa1^{MUT}-mGFP levels in WT were set to 1, values represent the mean $\pm$ SD of three independent experiments. Numbers above the columns depict the p-value.
- B. Ten-fold serial dilutions of WT and *pex4* cells together with WT and *ufd4* cells producing Pxa1^{MUT}-mGFP were spotted on oleate plates and grown at 30° C for 7 days.

C. CHX chase of Pxa1^{MUT}-mGFP in WT and *ufd4* strains. Cells were pre-cultivated on glucose, grown on oleate/glucose to an OD₆₀₀ 1.5, treated with CHX and samples were collected from oleate/glucose grown cells at the indicated time points after CHX addition. Protein levels were normalized to Pyc at the corresponding time point and to the protein levels at time point 0 (set to 1). Values represent the mean $\pm$ SD of three independent experiments. For quantification, blots in Figure S2B were used.

Co-localization analysis of Pxa1^{MUT}-mGFP with the peroxisomal marker Pex3-mKate2 in *ufd4* (Top panel) or WT (Bottom panel, taken from Figure 2A, for comparison) cells, after CHX treatment. Cells were grown as indicated in Figure 6C and images were taken from cells grown on oleate/glucose media at the indicated time points (mins) after CHX treatment. Blue arrows- GFP spots, yellow arrows- mKate2 spots and red arrows- background fluorescence, Scale bar: 3 μ m. Line profiles were generated as described in Figure 1. The circular structures visible in GFP images of *ufd4*.Pxa1^{MUT}-mGFP cells (top panel) expressing Pxa1^{MUT}-mGFP can also be seen in images of WT.Pxa1^{MUT}-mGFP cells (Figure S1B, top lane) and likely represent large peroxisomes.

Inhibiting Pxa1^{MUT}-mGFP degradation enhances peroxisomal β -oxidation

Around 70% of the mutant forms of ALDP are unstable and are degraded (Kemp et al., 2001; Takahashi et al., 2007). However, many of these mutants retain a certain level of functionality (Roerig et al., 2001). This has led to the hypothesis that blocking ALDP degradation could enhance ALDP activity in cells and be beneficial for ALD patients (Morita et al., 2019; Yamada et al., 1997; Zhang et al., 2011). Our data clearly indicate that Pxa1^{MUT}-mGFP builds up at peroxisomes when its degradation is inhibited in *ufd4* cells. Hence, we reasoned that we could use our experimental setup as proof of principle and measured the capacity of WT and *ufd4* cells expressing Pxa1-mGFP or Pxa1^{MUT}-mGFP to perform β -oxidation (Figure 7A). Pxa1^{MUT}-mGFP in WT cells displayed reduced activity in comparison to Pxa1-mGFP (Figure 7A) although higher than *pxa1* cells, indicating that importantly, Pxa1^{MUT}-mGFP retains a degree of functionality. Significantly, *ufd4* cells expressing Pxa1^{MUT}-mGFP exhibited an increase in β -oxidation activity of around 15% compared to the activity of WT.Pxa1^{MUT}-mGFP cells (Figure 7A). We also observed that β -oxidation appeared enhanced in *ufd4* cells expressing Pxa1-mGFP compared to WT cells, although statistical analysis suggested that this increase was not significant (Figure 7A). To investigate further, we determined the levels of Pxa1-mGFP in WT and *ufd4* cells expressing Pxa1-mGFP (Figure 7B), observing that these levels were indeed increased in *ufd4* cells. Significantly, Pex14p levels were not affected by *ufd4* deletion (Figure 7B), indicating that the effect is likely to be specific for Pxa1-mGFP.

Overall, our results provide proof of principle that blocking Pxa1^{MUT}-mGFP degradation can partially restore Pxa1^{MUT}-mGFP function in cells, supporting the view that inhibiting ALDP degradation may be a promising therapeutic avenue to explore to target ALD.

Figure 7: Inhibiting Pxa1^{MUT}-mGFP degradation enhances β-oxidation activity *in vivo*.

- A. Bar chart displaying β-oxidation activity in WT and *ufd4* cells expressing Pxa1-mGFP or Pxa1^{MUT}-mGFP. β-oxidation activity was measured in cells grown overnight on oleate/glucose media. β-oxidation in *pxa1* and *fox1* cells acts as control. The activity in WT.Pxa1-mGFP cells was taken as a reference [100%]. Values represent the mean ± SD of four independent experiments. Numbers above the columns depict the p-value calculated to determine whether changes to β-oxidation activity in the different strains, are significant.
- B. Bar chart displaying Pxa1-mGFP and Pxa1^{MUT}-mGFP levels in WT and *ufd4* cells. Pex14p levels in WT and *ufd4* cells acts as a control. Cells pre-cultivated on glucose were grown on oleate/glucose to an OD₆₀₀ of 1.5, cells were lysed and samples were probed by immunoblotting. For quantification, blots in Figure S2C were used. Pxa1-mGFP levels in WT.Pxa1-mGFP cells were set to 1. Similarly, Pex14 levels in WT.Pxa1-mGFP cells were used as a reference and set to 1. Values represent the mean ± SD of three independent experiments. Numbers above the columns depict the p-value calculated to determine whether changes to Pxa1/Pxa1^{MUT}-mGFP or Pex14p levels in the different strains, when compared to the corresponding WT strain, are significant.

Discussion

Here we have demonstrated that a faulty version of the peroxisomal fatty acid transporter Pxa1p is targeted for proteasome-dependent degradation. Pxa1^{MUT}-mGFP, our chosen substrate, is rapidly degraded from peroxisomes. An important question remains how Pxa1^{MUT}-mGFP is recognized for degradation? The mutations introduced into Pxa1p (Gly650Ser/Lys651Arg) are present in the conserved walker A motif of the NBD (Walker et al., 1982). Mutation of either these conserved lysine or glycine residues compromises both the ATP hydrolysis and substrate translocation activity of ABC transporters (Byeon et al., 1995; Schneider et al., 1994; Zoghbi and Altenberg, 2014), including ALDP (Roerig et al., 2001). Hence, the substitutions we introduced into Pxa1^{MUT}-mGFP likely reduce ATP hydrolysis and hence transport activity, which in turn could allow Pxa1^{MUT}-mGFP to be recognized for degradation. However, Pxa1p forms a heterodimer with Pxa2p and deletion of *PXA2* decreases the stability of Pxa1p (Shani

and Valle, 1996), indicating that Pxa2p binding is required for Pxa1p stability. Therefore, it is equally possible that the substitutions in Pxa1^{MUT}-mGFP inhibit binding to Pxa2p, which in turn allows Pxa1^{MUT}-mGFP to be recognized for degradation. Interestingly, Byeon et al. reported that mutating the walker A lysine in muscle adenylate kinase results in a conformational change in the NBD (Byeon et al., 1995) while recent work on the bacterial transporter MJ0796 demonstrated that walker A lysine mutations impact on both ATP hydrolysis and homo-dimerization (Zoghbi and Altenberg, 2014). Hence, we speculate that the introduced mutations in Pxa1^{MUT}-mGFP result in a conformational change in Pxa1p, which could allow the protein to be recognized for degradation. Identifying which factors are involved in the recognition of faulty peroxisomal fatty acid transporters will provide valuable insights into the mechanisms underlying their degradation.

Our data show that the addition of proteasome inhibitors significantly reduce Pxa1^{MUT}-mGFP turnover, demonstrating a role for the proteasome in Pxa1^{MUT}-mGFP degradation (Figure 3A). While the data on the ubiquitin mutant (Ub^{K48}) suggests that ubiquitination is involved in Pxa1^{MUT}-mGFP degradation (Figure 3C), our attempts to detect ubiquitinated forms of Pxa1^{MUT}-mGFP proved unsuccessful (Figure 3D). Notably, ubiquitinated forms of ALDP carrying the R617H or H667D mutations could also not be detected (Takahashi et al., 2007), even though these mutants were likely degraded by the proteasome. There could be two possible explanations for this: Pxa1^{MUT}-mGFP is ubiquitinated but that the ubiquitinated form of Pxa1^{MUT}-mGFP is below the limit of detection, or that the ubiquitination of another protein could facilitate the degradation of Pxa1^{MUT}-mGFP. The latter mode of degradation has been proposed for several substrates of the proteasome. Dang et al., reported that instead of A3G, its binding partner viral infectivity factor (Vif) undergoes poly-ubiquitination and this could be critical for A3G proteasomal degradation (Dang et al., 2008), proposing that poly-ubiquitinated Vif might act as an adaptor protein to bring A3G to the proteasome for degradation (Prakash et al., 2009). Another protein that may be degraded via a similar mechanism is the retinoblastoma tumour suppressor protein (Rb) (Gonzalez et al., 2001). Rb is thought to be targeted for proteasomal degradation by the ubiquitination of its binding partner, human papillomavirus protein E7 (Berezutskaya and Bagchi, 1997; Gonzalez et al., 2001). Pxa2p forms a heterodimer with Pxa1p (Hettema et al., 1996; Shani et al., 1996), but we do not suspect that Pxa2p ubiquitination is required for Pxa1^{MUT}-mGFP degradation because loss of Pxa2p does not inhibit Pxa1p degradation but instead decreases the stability of Pxa1p (Shani and Valle, 1996). Clearly further work is required to investigate how ubiquitin contributes to the proteasome-mediated degradation of Pxa1^{MUT}-mGFP.

Based on the results of our tFT screening, several proteins not previously associated with peroxisomal function were identified that could play a role in Pxa1-tFT stability (Figure 4C). Of these, only loss of Ufd4p, a cytosolic E3 ligase involved in the degradation of faulty proteins (Theodoraki et al., 2012), significantly increased the stability of Pxa1^{MUT}-mGFP (Figure 6A), indicating that Ufd4p is involved in Pxa1^{MUT}-mGFP degradation. Although future

work needs to investigate the mechanisms by which Ufd4p controls Pxa1^{MUT}-mGFP degradation, we consider it significant that a cytosolic E3 ligase could regulate the turnover of a faulty PMP because this indicates that general cellular quality control pathways can facilitate the degradation of faulty PMPs. While the data we present here concern the degradation of a mutant form of Pxa1p, we consider it unlikely that the sole purpose of PMP quality control is the degradation of mutant PMPs; we consider it more likely that damaged PMPs undergo quality control. As previously mentioned, the ROS rich environment of the peroxisome could result in the oxidative damage of proteins, which could suggest that damaged Pxa1p undergoes PMP quality control. In line with this, although Pxa1-mGFP is much more stable than Pxa1^{MUT}-mGFP in our CHX experiments, degradation of Pxa1-mGFP does occur (Figure 2A) while Pxa1-mGFP levels are higher in *ufd4* cells compared to WT cells (Figure 7B). Therefore, it is plausible that Pxa1^{MUT}-mGFP is targeted for degradation via quality control mechanisms that usually acts upon damaged Pxa1p because Pxa1^{MUT}-mGFP is impaired in function.

Deletion of *UFD4* does not fully inhibit the degradation of Pxa1^{MUT}-mGFP. Similarly, Pxa1^{MUT}-mGFP degradation appears only partly reduced in cells lacking the peroxisomal E3 ligase Pex2p. This could suggest that two pathways target Pxa1^{MUT}-mGFP for degradation, one requiring Ufd4p and the other involving Pex2p or perhaps that Pex2p and Ufd4p collaborate to facilitate Pxa1^{MUT}-mGFP degradation. Ufd4p can team up with Ubr1p, a RING E3, to facilitate the degradation of Mgt1p, a DNA repair demethylase (Hwang et al., 2009). However, the impact of peroxisome function on Pxa1^{MUT}-mGFP degradation remains to be determined, meaning that further data on the role of Pex2p (and other members of the peroxisomal ubiquitination machinery) are required to validate this hypothesis. Nevertheless, a model that depicts Pex2p and Ufd4p teaming up to degrade faulty PMPs remains an attractive one.

Mutations in gene sequences can result in formation of faulty proteins, which could pose serious threats to the cell, for two reasons. One reason is the loss of protein activity. However, there is also gain-of-toxic function that is unrelated to the protein's function and which has become increasingly relevant to human disease (Winklhofer et al., 2008). Faulty proteins often expose hydrophobic regions normally buried within the core (Gibson and Ellory, 2002) that may take part in unwanted protein-protein interactions, resulting in protein aggregation (Dobson, 2004). To minimize such harmful effects, cell employs protein quality control systems to remove faulty proteins (Fredrickson and Gardner, 2012). For instance, mutations at position F508 in Cystic fibrosis transmembrane conductance regulator (CFTR), which increase the tendency of CFTR to aggregate but do not inhibit activity completely (Du et al., 2015; Luciani et al., 2010), result in rapid protein degraded (Ward et al., 1995). However, the rapid degradation of mutant CFTR leaves cells devoid of CFTR molecules, eventually resulting in the severe condition cystic fibrosis (Thomas et al., 1992) and several lines of research have focused on blocking CFTR degradation as potential treatment for cystic fibrosis (Zhang et al., 2012). Similarly, many ALDP mutant proteins that cause ALD are unstable, leading to the suggestion that blocking ALDP degradation could represent a novel treatment for ALD (Morita

et al., 2019). Indeed, a subset of ALDP mutant proteins in patient cells become stable under low-temperature culture conditions and exhibit proper peroxisomal localization (Morita et al., 2019) while they also display increased residual β -oxidation activity (Zhang et al., 2011). Though unstable, a significant proportion of ALDP mutants retain a certain degree of function (Morita et al., 2019; Roerig et al., 2001). In line with this, our data demonstrate that Pxa1^{MUT}-mGFP cells display increased β -oxidation compared to *pxa1* cells (Figure 7A), indicating that Pxa1^{MUT}-mGFP is partly functional. Furthermore, stabilizing Pxa1^{MUT}-mGFP, through the deletion of *UFD4*, enhances peroxisomal β -oxidation in cells, which would indeed support the notion that blocking ALDP mutant degradation might constitute a feasible therapeutic approach to treat ALD. However, Yamada et al. indicated that proteasome inhibitors enhance the stability of certain ALDP mutants but did not increase peroxisomal β -oxidation in the corresponding cells (Yamada et al., 1997). While these results may seem contradictory to those reported here, it is important to note that the location where the mutants build up upon stabilization is critical in determining whether stabilization will result in enhanced activity. Yamada and co-workers did not address this in their study (Yamada et al., 1997) and it is possible that these ALDP mutants are stabilized elsewhere in the cell. Hence, further work on the mechanism of ALDP degradation is therefore needed to understand at which point blocking ALDP mutant degradation could positively affect ALDP activity. Nevertheless, our data demonstrate that *UFD4* deletion results in a build-up of Pxa1^{MUT}-mGFP at the peroxisomal membrane and significantly, a human homolog of Ufd4p, known as TRIP12 (Park et al., 2009), is involved in the degradation of a range of different proteins (Hanoun et al., 2014; Kajiro et al., 2011; Larrieu et al., 2020). It would therefore be interesting to investigate whether TRIP12 has a role in ALDP degradation and if so, whether blocking TRIP12 dependent ALDP mutant degradation improves β -oxidation in ALD patients.

In summary, we have demonstrated that a faulty peroxisomal fatty acid transporter undergoes proteasome-mediated degradation and identify Ufd4p as playing an important role in facilitating this degradation. In addition, these results may help to shed new light on the role of ALDP degradation in ALD.

Materials and Methods

Construction of plasmids and *S. cerevisiae* strains

S. cerevisiae transformations were performed with the Lithium acetate method, as described previously (Knop et al., 1999). *S. cerevisiae* strains and plasmids used in this study are listed in Table 1 and 2 respectively. The primers used in the study are listed in Table S1. Phusion DNA polymerase (Thermo Scientific) was used for the amplification of gene fragments.

The *S. cerevisiae* WT.Pxa1-mGFP strain was constructed as follows. PCR was performed on pHIPZ-mGFP using the Pxa1-mGFP Fw and Pxa1-mGFP Rev primers to amplify the C-terminal region of *PXA1* together with the Zeocin resistance cassette (*bleMX6*) and the

mGFP coding sequences and the obtained PCR fragment was transformed into the yMaM330 strain. The plasmid pGW053 was constructed as follows: the genomic region of the *PXA1* gene was amplified by PCR using Forward primer VIP1080, 485 bp upstream of open reading frame and Reverse primer VIP1081, 192 bp downstream of ORF. This was inserted into Ycplac111 using gap repair between EcoRI and HindIII sites, resulting in pGW046. This plasmid was used as template for site directed mutagenesis to generate Pxa1 G650S/K651R using VIP672 and VIP673, producing pGW053. For the construction of *S. cerevisiae* WT.Pxa1^{MUT}-mGFP, first a recombinant plasmid pHIPZ-Pxa1^{MUT}-mGFP was constructed. PCR was performed on pGW053 using Pxa1^{MUT} Fw and Pxa1^{MUT} Rev primers to amplify the C-terminal region of *PXA1* (carrying the two mutations *G650S/K651R*) and to introduce PciI and BglII sites. The resulting DNA fragment, digested with PciI and BglII, was cloned into PciI/ BglII cut pHIPZ-mGFP to generate pHIPZ-Pxa1^{MUT}-mGFP. PCR was then performed on pHIPZ-Pxa1^{MUT}-mGFP using Int_Pxa1^{MUT} Fw and Int_Pxa1^{MUT} Rev to amplify the Pxa1^{MUT}-mGFP fragment and the obtained PCR product was then transformed into the yMaM330 strain. The obtained PCR fragment was also transformed into *pdr5*, *atg12* and *ufd4* strains to *pdr5*.Pxa1^{MUT}-mGFP, *atg12*.Pxa1^{MUT}-mGFP and *ufd4*.Pxa1^{MUT}-mGFP respectively. The positive transformants of WT.Pxa1^{MUT}-mGFP, *pdr5*.Pxa1^{MUT}-mGFP, *atg12*.Pxa1^{MUT}-mGFP and *ufd4*.Pxa1^{MUT}-mGFP were checked both by colony PCR and sequencing. In addition, PCR fragment was also transformed into *nam7*, *ela1*, *dk2*, *skp2*, *dma2* and *tul1* cells to generate the mutant strains expressing Pxa1^{MUT}-mGFP.

The *S. cerevisiae* Pxa1-tFT strain was generated as follows. PCR was performed on pMaM168 to amplify C-terminal region of *PXA1* and tFT tagging module using primers Pxa1-tFT Fw and Pxa1-tFT Rev. The generated PCR product was then transformed into yMaM330. Strains expressing Pex3-mKate2 were constructed as follows. Pex3-mKate2 cassette consisting of C-terminal region of *PEX3*, hygromycin resistance gene (*hphMX*) and mKate2 coding sequences was amplified from pHIPH-Pex14mKate2 using primers Pex3-mKate Fw and Pex3-mKate Rev. The generated PCR fragment was transformed into WT, WT.Pxa1-mGFP, WT.Pxa1^{MUT}-mGFP and *ufd4*.Pxa1^{MUT}-mGFP. Furthermore, the PCR fragment was also transformed into *pex2*. Pxa1^{MUT}-mGFP, *pex4*. Pxa1^{MUT}-mGFP and *pex5*. Pxa1^{MUT}-mGFP strains, which were generated as described below

S. cerevisiae WT.Pxa1^{MUT}-mGFP and *ufd4*.Pxa1^{MUT}-mGFP strains expressing DsRed-SKL were constructed as follows. First, the PCR cassette (consisting of *TEF* promoter and DsRed-SKL coding sequences) was digested NotI/SalI and cloned into NotI/SalI cut pHIPX7-DsRed-SKL to create pHIPH7-DsRed-SKL. The PCR cassette containing *TDH3* promoter sequence was amplified from pP_{TDH3}-GFP-SKL was digested with NotI/BamHI and cloned into NotI/BamHI cut pHIPH7-DsRed-SKL, to create pHIPH8-DsRed-SKL. The resulting plasmid after linearization with MunI was transformed into WT.Pxa1^{MUT}-mGFP and *ufd4*.Pxa1^{MUT}-mGFP strains to generate WT.Pxa1^{MUT}-mGFP. DsRedSKL and *ufd4*.Pxa1^{MUT}-mGFP. DsRed-

SKL respectively. In these strains, the expression of DsRed-SKL was under the control of *TDH3* promoter.

S. cerevisiae WT.Pxa1^{MUT}-mGFP+MycUb and WT.Pxa1^{MUT}-mGFP+MycUbK48R strains were constructed as follows. First, the Yeast episomal plasmids (Y_{EP}) expressing MycUb and MycUbK48R from the CUP1 promoter were constructed: the *CUP1* promoter coding sequence was amplified from pCGCN-FAA4 using primers CUP1_Fw and CUP1_Rev, digested with NotI/BamHI and cloned into NotI/BamHI cut pRDV1 (MycUb) or pRDV2 (MycUbK48R). The resulting vectors were then used to amplify Pcup1-MycUb and Pcup1-MycUbK48R sequences individually using primers CUP1_Fw and MycUb/UbK48R_Rev and these PCR fragments were digested with NotI and SacI and cloned into NotI/ SacI cut pRG226 (Addgene, 64529). The Y_{EP}-P_{CUP1}-MycUbK48R plasmid was transformed into the WT.Pxa1^{MUT}-mGFP strain to generate WT.Pxa1^{MUT}-mGFP+MycUbK48R. The Y_{EP}-P_{CUP1}-MycUb plasmid was transformed individually into WT.Pxa1^{MUT}-mGFP, *pdr5*.Pxa1^{MUT}-mGFP and *pdr5* strains to generate WT.Pxa1^{MUT}-mGFP+MycUb, *pdr5*.Pxa1^{MUT}-mGFP + MycUb and *pdr5* + MycUb respectively.

Construction of *S. cerevisiae* deletion strains

The *pxa1* deletion strain was constructed by replacing the *PXA1* region with the hygromycin resistance gene (*hphMX*). A PCR fragment containing the hygromycin resistance gene and *PXA1* flanking regions were amplified from pHIPH-Pex14mKate2 using primers DPxa1_Fw and DPxa1_Rev. The resulting *PXA1* deletion cassette was transformed into yMaM330. For the construction of *pex5*.Pxa1^{MUT}-mGFP, the *PEX5* deletion cassette (geneticin resistance gene and *PEX5* flanking regions) amplified from pFa6AkanMX6 using primers DPex5_Fw and DPex5_Rev was transformed in WT.Pxa1^{MUT}-mGFP.

S. cerevisiae *pex2* and *pex4* strains expressing Pxa1^{MUT}-mGFP or Pxa1-tFT were constructed as follows. The *pex2* and *pex4* deletion cassettes consisting of geneticin resistance cassette (*kanMX*) and *PEX2* or *PEX4* flanking regions were amplified from pFa6AkanMX6 using DPex2_Fw and DPex2_Rev (for *PEX2* deletion cassette) and DPex4_Fw and DPex4_Rev (for *PEX4* deletion cassette). The obtained deletion cassettes were then transformed individually into WT.Pxa1^{MUT}-mGFP and Pxa1-tFT strains to generate *pex2*.Pxa1^{MUT}-mGFP, *pex4*.Pxa1^{MUT}-mGFP, *pex2*.Pxa1-tFT and *pex4*.Pxa1-tFT strains.

For the construction of *atg12*.Pxa1-tFT strain, deletion cassette consisting of geneticin resistance cassette (*kanMX*) and *ATG12* flanking regions was amplified from pFa6AkanMX6 using primers DATg12_Fw and DATg12_Rev (for *ATG12* deletion cassette). The obtained deletion cassette was then transformed into Pxa1-tFT to generate *atg12*.Pxa1-tFT strain.

All gene integrations were confirmed by colony PCR using Phire Hot Start II DNA polymerase (Thermo Fisher Scientific) and gene deletions were checked by colony PCR as well as southern blotting analysis of chromosomal DNA, using the ECL Direct Nucleic Acid

Labelling and Detection system (Thermo Fisher Scientific), according to the protocol provided by the manufacturer.

Growth conditions

The *Escherichia coli* strain DH5 α was used for cloning purposes. *E. coli* cells were grown in LB supplemented with 100 μ g/ml Ampicillin at 37 °C. Yeast transformants were selected on YPD plates containing 2% agar and 100 μ g/ml Zeocin (Invitrogen), 150 μ g/ml Geneticin (Invitrogen) or 300 μ g/ml Hygromycin (Invitrogen) or on YND plates containing 6.7 g/L yeast nitrogen base without amino acids (YNB, Difco), 5g/L D- glucose monohydrate (Sigma-Aldrich), 2% agar and 30 mg/L Leucine, 20 mg/L Histidine and 20 mg/L Methionine or 30 mg/L Uracil, when required.

For all the experiments, yeast cells were initially cultured at 30°C on YM2 medium (6.7 g/L YNB, 1% casein hydrolysate (Sigma-Aldrich), 60 mg/L uracil, 60 mg/L tryptophan) containing either 2% or 0.3% (w/v) glucose. To induce peroxisome proliferation, cells grown on YM2 medium plus 0.3% glucose were transferred to YNO-glucose medium (6.7 g/L YNB, 1% casein hydrolysate, 60 mg/L uracil, 60 mg/L tryptophan, 0.05% tween, 0.1% oleate and 0.1% glucose). For strains expressing Myc tagged ubiquitin: MycUb and MycUb^{K48R} under the control of *CUP1* promoter and uracil coding gene, *URA3* (Table 1), cells were pre-cultured at 30°C on YM2 medium (lacking uracil) plus 0.3% glucose and then grown on YNO-glucose medium (lacking uracil) to an OD₆₀₀ of 1.5. To inhibit protein synthesis, cells were treated with cycloheximide (CHX, Sigma-Aldrich) to a final concentration of 6 mg/ml. To inhibit proteasome, cells were treated with 70 μ M of MG1232 (Sigma-Aldrich) and 70 μ M of Bortezomib (Selleckchem). Where indicated, an equal amount of DMSO (control for CHX) was added to cells. For spot assays, yeast strains were grown overnight on YM2 medium containing 2% glucose and shifted to YM2 medium plus 0.3% glucose. After 6h of growth in YM2 plus 0.3 glucose, yeast cells were spotted onto oleate plates containing 6.7 g/L YNB, 0.1% yeast extract, 0.1% oleate, 0.05% Tween, 2% agar and when required uracil (30 mg/L), leucine (60 mg/L), methionine (20 mg/L) and histidine (20 mg/L) was added. Plates were incubated at 30°C for 7 days before imaging.

Preparation of yeast TCA lysates

Cell extracts of TCA-treated cells were prepared for SDS-PAGE as described previously (Baerends et al., 2000). SDS-PAGE and western blotting were performed by established methods. Equal volume of samples were loaded per lane and blots were probed with antibodies against mGFP (Santa Cruz Biotech, sc-9996), pyruvate carboxylase 1 (Pyc), Pex14p, rabbit polyclonal antisera raised against the Myc tag (Santa Cruz Biotech, sc-789) or HA tag (Sigma, H6908). Secondary goat anti-rabbit (Thermo Fisher Scientific, 31460) or goat anti-mouse

(Thermo Fisher Scientific, 31430) antibodies conjugated to horseradish peroxidase or alkaline phosphatase were used for detection. Pyc was used as a loading control.

Quantification of Western blots

Western blots were scanned using a densitometer (Bio-Rad, GS-710) and protein levels were quantified using Image J software (<https://imagej.nih.gov/ij/>). The intensity of each band measured was normalized by dividing by the intensity of the corresponding Pyc band (loading control). The normalized Pxa1^{MUT}-mGFP values were compared to Pxa1-mGFP values, which was set to 1. The levels of Pxa1^{MUT}-mGFP in mutant cells are displayed relative to Pxa1^{MUT}-mGFP levels in WT (set to 1). For CHX experiments, the normalized values at time point 0 (T0) was set to 1.0 and values obtained after CHX addition at indicated time points are displayed as a fraction of T0 values. Standard deviations were calculated using Excel. Significance was determined using two tailed student's t-test. * represents P-values < 0.05, ** represents P-values < 0.01, and *** represents P-values < 0.001. The data presented are derived from three independent experiments.

tFT screening with UPS deletion library

Tandem fluorescent protein timers (tFT): mCherry and sfGFP were endogenously tagged to Pxa1 as described (Khmelniskii et al., 2011). By the use of a pinning robot (Singer Instruments), Pxa1-tFT was crossed to UPS deletion library or an array of haploid strains consisting of knockout, temperature sensitive (*ts*) and Decreased Abundance by mRNA perturbation (Damp) alleles of individual components of the Ubiquitin Proteasome System (UPS) (Li et al., 2011; Schuldiner et al., 2005; Winzeler et al., 1999). In addition, Pxa1-tFT was crossed to a control strain expressing *kanMX* selection marker from the *ura3Δ* locus, referred to as wild-type (WT). Screens were performed in 1536 format, with four technical replicates of each cross arranged next to each other. The selection of diploids, sporulation and the selection of haploids carrying Pxa1-tFT and a genetic perturbation, was performed by sequential pinning on appropriate selective media as described (Baryshnikova et al., 2010), followed by seamless marker excision (Khmelniskii et al., 2011). Using an Infinite M1000 Pro plate reader equipped with automatic plate loading stacker (Tecan) and custom temperature control chamber, mCherry intensity (587/10 nm excitation, 610/10 nm emission) and sfGFP intensity (488/10nm excitation, 510/10 nm emission) was measured from colonies grown on a modified YNO-glucose medium containing (6.7 g/L YNB, 2g/L amino acid dropout mix (-LEU), 0.05% tween, 0.1% oleate, 0.1% glucose and 2% agar). First set of measurements were taken after 24 h of growth and measurements were taken every 24 h until 72 h (3 days).

Whole colony fluorescence intensities (mCherry and sfGFP), obtained from UPS mutant crossed to Pxa1-tFT, were corrected for auto fluorescence by subtracting the measurements of a corresponding mutant crossed to the control strain, yMaM344 (expressing non-fluorescent protein). The median value was calculated out of the four technical replicates. The corrected

values of mCherry divided by the sfGFP values yielded a ratio, representing the stability of Pxa1-tFT in the UPS mutants. Similarly, the mCherry/sfGFP ratio of Pxa1-tFT in WT was corrected for auto fluorescence. Using the ratios obtained on day one, two and three a Z- score for each day was calculated by the formula. A Z-score indicates how many standard deviations a value is from the mean of all values. Z-score can be calculated from the formula: $Z = (X - \mu) / \sigma$. In the formula, Z is the Z-score, X is the value of the element, μ is the population mean, and σ is the standard deviation. A negative Z-score indicates that the value of the element is below average while positive Z-score indicates the value is above average. To identify UPS mutants which potentially increased the stability of Pxa1-tFT, Z-score (**Z**) was calculated using the formula

$$Z = \frac{\text{mCherry/sfGFP ratio in a UPS mutant (X)} - \text{mean mCherry/sfGFP ratio in WT and UPS mutants (\mu)}}{\text{Standard deviation of mCherry/sfGFP ratio in WT and UPS mutants (\sigma)}}$$

A strain (UPS mutant) with a Z-score of 0 indicates that Pxa1-tFT stability is similar to the WT as well to the population mean (WT and UPS mutants). While, the mutants with Z-score of 1.0 and 2.0 indicates that the mCherry/sfGFP ratio in a corresponding mutant is different from the WT and population mean by 1.0 and 2.0 standard deviations. Hence, mutant stains displaying a Z-score >0 indicate enhanced Pxa1-tFT stability. UPS mutants with an increase in Z-score >1.0 on two of the three days tested were considered as potential candidates that significantly increased Pxa1-tFT stability. The heat map generated using the Z-scores (obtained from mCherry/sfGFP ratio of Pxa1-tFT in UPS mutants and the WT) is presented in Figure 2D.

Fluorescence microscopy

All images were captured at room temperature using an inverted microscope (Axio Scope A1, 100×1.30 NA Plan-Neofluar objective, Carl Zeiss), Micro-Manager software and a digital camera (CoolSNAP HQ²; Photometrics). For wide field microscopy, GFP fluorescence was visualized with a 470/440-nm band pass excitation filter, a 495-nm dichromatic mirror, and 525/50-nm band pass emission filter. mCherry fluorescence was visualized with a 587/25-nm band pass excitation filter, a 605-nm dichromatic mirror, and 647/70-nm band pass emission filter.

For co-localization analysis, cells were pre-grown on YM2 medium containing glucose, transferred to YNO-glucose (low fluorescence) medium and grown until an OD₆₀₀ of 1.5 after which fluorescence microscopy images were captured. For CHX chase analysis, cells grown in the same way as indicated above were treated with cycloheximide (CHX) and images were taken from cells grown on oleate/glucose at indicated time points (mins) after CHX treatment (Figure 2B). The acquired images were processed using Image J software (<https://imagej.nih.gov/ij/>) with optimal settings: mGFP (200, 500), mKate2 (200, 600) and

DsRed (200, 500). For co-localization analysis in WT, *pex2*, *pex4* and *pex5* cells expressing Pxa1^{MUT}-mGFP and Pex3-mKate2 (Figure 5C), images were processed using optimal settings: mGFP (200, 800) and mKate2 (200, 600). For quantifying the co-localization of Pxa1-mGFP or Pxa1^{MUT}-mGFP in cells expressing peroxisomal markers (Pex3-mKate2 or DsRed-SKL) (Figure 1, 2, 6 and S1), a line was drawn along GFP and mKate2/ DsRed-SKL spots using ImageJ and the intensity was measured. Using this data, normalized fluorescent intensity of green and red spots were calculated and plotted against the distance of the line (from the beginning to the end).

For quantification of the Pex3-mKate2 and Pxa1-mGFP or Pxa1^{MUT}-mGFP fluorescence intensities in CHX treated cells (Figure 2C), a rectangular area was drawn using ImageJ to envelope the region containing the mGFP/mKate2 spot and pixel intensity inside the area was measured. Since the cells expressing Pxa1^{MUT}-mGFP contain very few mGFP positive spots at the later time points of CHX treatment, the rectangular area selected contained the peroxisomal marker or Pex3-mKate2 spots and the corresponding mGFP intensity was measured. The maximum fluorescence intensity of mGFP measured on peroxisomes was corrected for the background intensity and a box plot was made using Microsoft Excel. The box represents values from the 25 percentile to the 75 percentile; the horizontal line through the box represents the median value. Whiskers indicate maximum and minimum values. The mKate2 intensity was calculated and displayed in a similar way (Figure 2C). To calculate the mGFP/mKate2 intensity ratio, the maximum intensity of Pxa1-mGFP or Pxa1^{MUT}-mGFP, obtained at different time points after CHX treatment, was divided by the corresponding maximum intensity of Pex3-mKate2 in each cell. The dataset was subjected to the two-tail t-test. * represents P-values < 0.05, ** represents P-values < 0.01 and *** represents P-values < 0.001.

Immunoprecipitation assay

Immunoprecipitation was performed on cells grown on YNO-glucose medium for 10hrs and incubated with proteasome inhibitors (MG132 and Bortezomib) for 90 mins. Around 14 OD units of each strain were harvested by centrifugation. Immunoprecipitation using GFP-Trap® _M beads was performed according to manufacturer's protocol (Chromotek) with slight modifications. Cells were washed once with 1 mL cold lysis or RIPA buffer (50 mM Tris/Cl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 0.5% Deoxycholate) and resuspended in 0.75 mL of lysis buffer containing 2.5 µg/mL leupeptin and 1 mM PMSF (added just prior to use). Yeast cells were then lysed using glass beads (~ 30 mins) and samples were centrifuged at 8000 rpm at 4°C for 10 mins to remove cellular debris and unbroken cells. A fraction of supernatant (Load fraction) was collected and stored for SDS-PAGE analysis. The remaining supernatant fractions were then incubated with 30 µl GFP-Trap® _M beads (Chromotek) for 2 hrs at 4°C under constant mixing. The beads were magnetically separated until the supernatant was clear. The supernatant was discarded and beads were washed five times using 500 µl ice cold wash buffer (50 mM Tris/Cl pH 7.5, 150 mM NaCl and 2 mM

EDTA). The GFP-Trap®_M beads were then resuspended in 60 µl 4x SDS-Sample buffer and boiled for 10 minutes at 95°C. Finally, the beads were separated magnetically and the supernatant (Elute fraction) was collected. The load and elute fractions were subjected to SDS-PAGE and immunoblotting with antibodies against GFP and Myc tagged epitope.

β- Oxidation assay

Yeast strains were grown on glucose medium containing 5 g/L D glucose, and 6.7 g/L YNB. When required; histidine, leucine, uracil, or methionine was added. To induce peroxisome proliferation cells were grown for at least 24 hours on 5 g/L glucose medium, and then transferred to YPO medium (1.07 g/L oleate, 2.16 g/L Tween-80, 5 g/L peptone, 3 g/L yeast extract, 25 mM potassium phosphate buffer (pH=6)). Cells were grown overnight in YPO medium to induce β-oxidation. β-Oxidation assay in intact cells were performed as described previously by (van Roermund et al., 2008) with slight modifications. The β-oxidation capacity was measured in 50 mM MES, pH 6.0 and 9 g/L NaCl supplemented with 10 µM 1-¹⁴C-oleate. Subsequently, ¹⁴C-CO₂ was trapped with 2 M NaOH and used to quantify the rate of fatty acid oxidation. Results are presented as percentages relative to the rate of oxidation of wild-type cells expressing Pxa1-mGFP (set to 100). The data presented are from four independent experiments and significance is calculated using two-tailed student's t test. * represents P-values < 0.05, ** represents P-values < 0.01, and *** represents P-values < 0.001.

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Author's Contributions

CW conceived and supervised the project, with input from EH, SK and MK. CW, SD, MM, CVR and XC designed the experiments. SD and CW analysed the data with the help from other authors. SD performed biochemical and fluorescence microscopy experiments with the support from XC. SD and MM performed tFT screening experiments. CVR performed β-oxidation experiment. EH was involved in plasmid construction, SD and CW wrote the manuscript with the contribution from all the authors.

Conflict of interest

The authors declare no conflict of interest

Table 1. Yeast strains used in this study

Strain	Description [genotype]	Reference
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(Khmelniskii et al., 2014) (Knop lab)
BJ1991	<i>MATa, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2</i>	(van Roermund et al., 2008)
yMaM330 [Wild type- WT]	<i>MATalpha his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 can1Δ::STE2pr-spHIS5 lyp1Δ::STE3pr-LEU2 leu2Δ::GAL1pr- I-SCEI-natNT2</i>	(Khmelniskii et al., 2014) (Knop lab)
WT.Pex3-mKate2	<i>yMaM330, PEX3::mKate2-hphMX</i>	This study (Figure 1)
WT.PXA1-mGFP	<i>yMaM330, PXA1::mGFP-bleMX6</i>	This study (Figure 1, 2, 4, 7, S1 and S2)
WT.PXA1 ^{MUT} -mGFP	<i>yMaM330, PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 1, 2, 3, 4, 5, 6, 7, S1 and S2)
WT. PXA1 ^{MUT} -mGFP + PEX3 mKate2	<i>yMaM330, WT. PXA1^{MUT}-mGFP + PEX3::mKate2-hphMX</i>	This study (Figure 1)
WT. PXA1-mGFP + PEX3 mKate2	<i>yMaM330, WT. PXA1-mGFP + PEX3::mKate2-hphMX</i>	This study (Figure 1 and Figure 2)
WT. PXA1 ^{MUT} -mGFP + DsRed-SKL	<i>yMaM330, WT. PXA1^{MUT}-mGFP + SKL::DsRed-hphMX</i>	This study (Figure S1)
Pxa1-tFT	<i>yMaM330, PXA1::mCherry-I-SceI site-SpCYC1 term-ScURA3-I-SceI site-mCherryΔN-sfGFP</i>	This study (Figure 4 and S1F)
Pex11-tFT	<i>yMaM330, PEX11::mCherry-I-SceI site-SpCYC1 term-ScURA3-I-SceI site-mCherryΔN-sfGFP</i>	Knop lab (Figure 4 and S1F)
<i>UPS deletion library</i>	<i>BY4741, goi deletions::kanMX</i>	(Khmelniskii et al., 2014) Knop lab
yMaM344	<i>yMaM330, ura3Δ0::mCherryΔN-I-SceI site-SpCYC1 term-ScURA3-I-SceI site-mCherryΔN</i>	(Khmelniskii et al., 2014) (Knop lab)
<i>atg12</i>	<i>atg12 deletion strain, BY4741, atg12::kanMX</i>	Knop lab
<i>pex2</i>	<i>pex2 deletion strain, BY4741, pex2::kanMX</i>	Knop lab
<i>pex4</i>	<i>pex4 deletion strain, BY4741, pex4::kanMX</i>	Knop lab

<i>ufd4</i>	<i>ufd4</i> deletion strain, BY4741, <i>ufd4::kanMX</i>	Knop lab
<i>pdr5</i>	<i>pdr5</i> deletion strain, BY4741, <i>pdr5::kanMX</i>	Knop lab
<i>nam7</i>	<i>nam7</i> deletion strain, BY4741, <i>nam7::kanMX</i>	Knop lab
<i>ela1</i>	<i>ela1</i> deletion strain, BY4741, <i>ela1::kanMX</i>	Knop lab
<i>dsk2</i>	<i>dsk2</i> deletion strain, BY4741, <i>dsk2::kanMX</i>	Knop lab
<i>skp2</i>	<i>skp2</i> deletion strain, BY4741, <i>skp2::kanMX</i>	Knop lab
<i>dma2</i>	<i>dma2</i> deletion strain, BY4741, <i>dma2::kanMX</i>	Knop lab
<i>tul1</i>	<i>tul1</i> deletion strain, BY4741, <i>tul1::kanMX</i>	Knop lab
<i>atg12.PXA1-tFT</i>	<i>yMaM330, PXA1-tFT + atg12::kanMX</i>	This study (Figure 4)
<i>pex2.PXA1-tFT</i>	<i>yMaM330, PXA1-tFT + pex2::kanMX</i>	This study (Figure 4)
<i>pex4.PXA1-tFT</i>	<i>yMaM330, PXA1-tFT + pex4::kanMX</i>	This study (Figure 4)
<i>pdr5.PXA1^{MUT}-mGFP</i>	BY4741, <i>pdr5 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 2 and S1)
<i>atg12.PXA1^{MUT}-mGFP</i>	BY4741, <i>atg12 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 2, 4, S1 and S2)
<i>pex2.PXA1^{MUT}-mGFP</i>	<i>yMaM330, WT. PXA1^{MUT}-mGFP + pex2 deletion pex2::kanMX</i>	This study (Figure 5 and S2)
<i>pex2.PXA1^{MUT}-mGFP + PEX3 mKate2</i>	<i>yMaM330, pex2 PXA1^{MUT}-mGFP + PEX3::mKate2-hphMX</i>	This study (Figure 5)
<i>pex4.PXA1^{MUT}-mGFP</i>	<i>yMaM330, WT. PXA1^{MUT}-mGFP + pex4 deletion pex4::kanMX</i>	This study (Figure 5 and S2)
<i>pex4.PXA1^{MUT}-mGFP + PEX3 mKate2</i>	<i>yMaM330, pex4 PXA1^{MUT}-mGFP + PEX3::mKate2-hphMX</i>	This study (Figure 5)
<i>pex5.PXA1^{MUT}-mGFP</i>	<i>yMaM330, WT. PXA1^{MUT}-mGFP + pex5 deletion pex5::kanMX</i>	This study (Figure 5 and S2)
<i>pex5.PXA1^{MUT}-mGFP + PEX3 mKate2</i>	<i>yMaM330, pex5 PXA1^{MUT}-mGFP + PEX3::mKate2-hphMX</i>	This study (Figure 5)
<i>ufd4.PXA1^{MUT}-mGFP</i>	BY4741, <i>ufd4 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 5, 6, 7 and S2)
<i>ufd4.PXA1^{MUT}-mGFP + PEX3 mKate2</i>	<i>ufd4 PXA1^{MUT}-mGFP + PEX3::mKate2-hphMX</i>	This study (Figure 6)

<i>nam7.PXA1^{MUT}-mGFP</i>	<i>BY4741, nam7 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 6)
<i>ela1.PXA1^{MUT}-mGFP</i>	<i>BY4741, ela1 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 6)
<i>dsk2.PXA1^{MUT}-mGFP</i>	<i>BY4741, dsk2 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 6)
<i>skp2.PXA1^{MUT}-mGFP</i>	<i>BY4741, skp2 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 6)
<i>dma2.PXA1^{MUT}-mGFP</i>	<i>BY4741, dma2 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 6)
<i>tul1.PXA1^{MUT}-mGFP</i>	<i>BY4741, tul1 + PXA1G650S/K651R::mGFP-bleMX6</i>	This study (Figure 6)
<i>ufd4.PXA1^{MUT}-mGFP + DsRed-SKL</i>	<i>ufd4 PXA1^{MUT}-mGFP + SKL::DsRed-hphMX</i>	This study (Figure S1)
<i>ufd4.PXA1-mGFP</i>	<i>BY4741, ufd4 + PXA1::mGFP-bleMX6</i>	This study (Figure 7 and S2)
<i>fox1</i>	<i>BJ1991, fox1::kanMX</i>	(van Roermund et al., 2008).
<i>pxa1</i>	<i>yMaM330, pxa1 deletion, pxa1::hphMX</i>	This study (Figure 7)
<i>WT.PXA1^{MUT}-mGFP+MycUb^{K48R}</i>	<i>yMaM330, WT PXA1^{MUT}-mGFP + P_{CUP1}-MycUbK48R [yeast episomal plasmid, Y_{EP}-Pcup1-MycUbK48R]</i>	This study (Figure 3 and S1)
<i>WT.PXA1^{MUT}-mGFP+ MycUb</i>	<i>WT PXA1^{MUT}-mGFP + P_{CUP1}-MycUb [yeast episomal plasmid, Y_{EP}-Pcup1-MycUb]</i>	This study (Figure 3 and S1)
<i>pdr5+ MycUb</i>	<i>pdr5 + [yeast episomal plasmid, Y_{EP}-Pcup1-MycUb]</i>	This study (Figure 2 and S1)
<i>pdr5.PXA1^{MUT}-mGFP + MycUb</i>	<i>pdr5.PXA1^{MUT}-mGFP + [yeast episomal plasmid, Y_{EP}-Pcup1-MycUb]</i>	This study (Figure 2 and S1)

Table 2. Plasmids used in this study

Plasmid	Description	Use	Reference
pGW053	YCplac111 plasmid containing <i>PXA1</i> carrying two mutations <i>G650S/K651R</i> ; <i>LEU2</i> , <i>Amp^R</i>	Amplification of C-terminal <i>PXA1</i> (with <i>G650S/K651R</i>)	This study
pHIPZ- mGFP fusinator	pHIPZ containing <i>mGFP</i> ; <i>bleMX6</i> , <i>Amp^R</i>	1. Vector for cloning 2. C-terminal <i>mGFP</i> tagging to <i>PXA1</i> ; genome integration	(Saraya et al., 2010)
pHIPZ- Pxa1 ^{MUT} - mGFP	pHIPZ- <i>mGFP</i> containing C-terminal <i>PXA1</i> with two mutations <i>G650S/K651R</i> [<i>PXA1^{MUT}</i>]; <i>bleMX6</i> , <i>Amp^R</i>	C-terminal <i>mGFP</i> tagging to <i>PXA1^{MUT}</i> ; genome integration	This study
pHIPH- Pex14mKate2	pHIPH containing <i>mKate2</i> and C-terminal of <i>PEX14</i> ; <i>hphMX</i> , <i>Amp^R</i>	1. C-terminal <i>mKate2</i> tagging to <i>PEX3</i> ; genome integration 2. Gene disruption (<i>pxa1</i>) by <i>hphMX</i> marker	(Chen et al., 2018)
pFA6a- kanMX6	pFA6a plasmid containing geneticin (<i>kanMX6</i>) resistance gene; <i>kanMX</i> , <i>Amp^R</i>	Gene disruption (<i>atg12</i> , <i>pex2</i> , <i>pex4</i> and <i>pex5</i>) by <i>kanMX6</i> marker	(Khmelinskii et al., 2014)
pMaM168	pFA6a plasmid containing tFT module (<i>mCherry-I-SceISite-SpCYC1term-ScURA3-I-SceISite-mCherryΔN-sfGFP</i>); <i>URA3</i> , <i>Amp^R</i>	1. C-terminal <i>tFT</i> tagging; <i>PXA1-tFT</i> and <i>PEX11-tFT</i> integration into yMaM330	(Khmelinskii et al., 2014)
pRG226	Yeast episomal plasmid (<i>Y_{EP}</i>); <i>URA3</i> , 2μ ori	Vector for cloning	Addgene, 64529
pCGCN- FAA4	Plasmid containing <i>CUP1</i> promoter and <i>mGFP</i> ; <i>bleMX6</i> , <i>Amp^R</i>	Amplification of <i>CUP1</i> promoter; cloning	(Saraya et al., 2010)
pRDV1	Plasmid containing <i>Myc</i> tagged ubiquitin (<i>Ub</i>)	Amplification of <i>MycUb</i> ; vector for cloning	(Williams and van der Klei, 2013)

	under control of <i>DHAS</i> promoter; <i>hphMX</i> , <i>Amp^R</i>		
pRDV2	Plasmid containing <i>Myc</i> tagged ubiquitin mutant (<i>UbK48R</i>) under the control of <i>DHAS</i> promoter; <i>hphMX</i> , <i>Amp^R</i>	Amplification of <i>MycUbK48R</i> ; Vector for cloning	(Williams and van der Klei, 2013)
Y _{EP} -P _{CUP1} - <i>MycUb</i>	Yeast episomal plasmid (Y _{EP}) containing <i>CUP1</i> promoter and <i>MycUb</i> ; <i>URA3</i> , <i>Amp^R</i>	Integration into WT. <i>PXA1^{MUT}-mGFP</i> strain	This study
Y _{EP} -P _{CUP1} - <i>MycUbK48R</i>	Yeast episomal plasmid (Y _{EP}) containing <i>CUP1</i> promoter and <i>MycUbK48R</i> ; <i>URA3</i> , <i>Amp^R</i>	Integration into WT and WT. <i>PXA1^{MUT}-mGFP</i>	This study
pHIPX7- <i>DsRedSKL</i>	Plasmid containing <i>DsRed-SKL</i> under the control of <i>TEF</i> promoter; <i>LEU2</i> , <i>Amp^R</i>	Vector for cloning	(Krikken et al., 2009)
pHIPH7- <i>DsRed-SKL</i>	Plasmid containing <i>DsRed-SKL</i> under the control of <i>TEF</i> promoter; <i>hphMX</i> , <i>kan^R</i>	Vector for cloning	This study
pP _{TDH3} -GFP- <i>SKL</i>	Plasmid containing GFP- <i>SKL</i> under the control of <i>TDH3</i> promoter; <i>LEU2</i> , <i>Amp^R</i>	Amplification of <i>TDH3</i> promoter; cloning	(Kumar et al., 2016)
pHIPH8- <i>DsRedSKL</i>	Plasmid containing <i>DsRed-SKL</i> under the control of <i>TDH3</i> promoter, <i>hphMX</i> <i>Amp^R</i>	Integration into WT. <i>PXA1^{MUT}-mGFP</i> and <i>ufd4. PXA1^{MUT}-mGFP</i> strain	This study

Table 3. Primers used in this study

Primer	Sequence	Description
VIP1080	ACGACGGCCAGTGAATTCCCCACTCCTGCT ATCTTACG	To amplify <i>PXA1</i> gene; forward primer
VIP1081	CATGATTACGCCAAGCTTGACCAACAAGT TATAGTAG	To amplify <i>PXA1</i> gene; reverse primer
VIP672	GGCCAAATGGTTGCTCTAGAAGTTCAATTC AACGC	Site directed mutagenesis primer used to introduce G650S/K651R mutation into <i>PXA1</i> ; forward primer
VIP673	GCGTTGAATTGAACTTCTAGAGCAACCATT TGGCC	Site directed mutagenesis primer used to introduce G650S/K651R mutation into <i>PXA1</i> ; reverse primer
Pxa1-mGFP Fw	TGGGAAGATGAGAGGACGAAGCTACGGGA AAAGCTTGAAATTATTGTGAGCAAGGGCGA GGAGCT	To amplify Pxa1-mGFP module from pHIPZ-mGFP; forward primer
Pxa1-mGFP Rev	TAAATTATATTCGCTAAATAAAATCTCTCCC TTTCTAGGGTGTTTCACTGTACAGAAAAA AAAGAAAAATTTG	To amplify Pxa1-mGFP module from pHIPZ-mGFP; reverse primer
Pxa1 ^{MUT} Fw	GTAGACATGTTCCAATTCTATACAAGACTT ATCG	To amplify Pxa1 ^{MUT} from pGW053 and clone into pHIPZ-mGFP; forward primer
Pxa1 ^{MUT} Rev	GTGCAGATCTAATAATTTCAAGCTTTTCCCG TAG	To amplify Pxa1 ^{MUT} from pGW053 and clone into pHIPZ-mGFP; reverse primer
Int_Pxa1 ^{MUT} Fw	TCTGAAGGCAAATGATATAAAGTTACCA	To amplify Pxa1 ^{MUT} and mGFP sequences from pHIPZ- Pxa1 ^{MUT} -mGFP; forward primer
Int_Pxa1 ^{MUT} Rev	TAAATTATATTCGCTAAATAAAATCTCTCCC TTTCTAGGGTGTTTCACTGTACAGAAAAA AAAGAAAAATTTG	To amplify Pxa1 ^{MUT} and mGFP sequences from pHIPZ- Pxa1 ^{MUT} -mGFP; reverse primer
Pex3-mKate Fw	AGCGCCAGCGTATACAGCAACTTTGGCGTC TCCAGCTCGTTTTCCTTCAAGCCTATGGTTT CTGAAGTCACTCAAGGA	To amplify Pex3-mKate cassette from pHIP4-Pex14mKate2; forward primer
Pex3-mKate Rev	TACGCTATATATATATATATTCTGGTGTGAG TGTCAGTACTTATTACAGAGATTACGTTTTCG ACACTGGATGGCGGC	To amplify Pex3-mKate cassette from pHIP4-Pex14mKate2; reverse primer

Pxa1-tFT Fw	TGGGAAGATGAGAGGACGAAGCTACGGGA AAAGCTTGAAATTATTCGTACGCTGCAGGT CGAC	To amplify Pxa1-tFT module from pMaM168; forward primer
Pxa1-tFT Rev	TAAATTATATTCGCTAAATAAAATCTCTCCC TTTCTAGGGTGTTCCTCAATCGATGAATTCGA GCTCG	To amplify Pxa1-tFT module from pMaM168; reverse primer
DPex2_Fw	GATTGCCGAGATAACTGAGAAGACGTAATC TAAAGGCAACGCAGCATGCGTACGCTGCAG GTCGAC	To amplify PEX2 deletion cassette from pFA6akanMX6; forward primer
DPex2_Rev	ATAGTATACACATATATAGAGATACAAGCG AGGGAACGGGGCCCTTTAATCGATGAATTC GAGCTCG	To amplify PEX2 deletion cassette from pFA6akanMX6; reverse primer
DPex4_Fw	TGGAGACAACATAAAATATACATAATCATC GCCTTTATACATAATATGCGTACGCTGCAG GTCGAC	To amplify PEX4 deletion cassette from pFA6akanMX6; forward primer
DPex4_Rev	AGGGCCCATTTGTTTGCCATTGCAACACATC CATCCTACGTGGTATCAATCGATGAATTCG AGCTCG	To amplify PEX4 deletion cassette from pFA6akanMX6; reverse primer
DAtg12_Fw	ACATCCCTAACTGTATATTCTACAGTAGAGT GAACCAATGACAGTATGCGTACGCTGCAGG TCGAC	To amplify ATG12 deletion cassette from pFA6akanMX6; forward primer
DAtg12_Rev	ATCGACTGTAGGTTTCTTCTTAGACCATTTC CAGCGCCCGGGTATTTAATCGATGAATTCG AGCTCG	To amplify ATG12 deletion cassette from pFA6akanMX6; reverse primer
DPex5_Fw	TCCTATTTTGGATATATATACATCAATAAA CAATATATCATAACACATGCGTACGCTGCA GGTCGAC	To amplify PEX5 deletion cassette from pFA6akanMX6; forward primer
DPex5_Rev	ATGAATTTGGGCAGTGATGCGAGAACATAA AATTGCGGAGAACCATATCAATCGATGAAT TCGAGCTCG	To amplify PEX5 deletion cassette from pFA6akanMX6; reverse primer
DPxa1_Fw	ACCCGTTTCAGACAATCTGGAAGTCTTAGA ACGCATAACACAGAAATGCCACACACCATT AGCTTCA	To amplify PXA1 deletion cassette from pHIPH4-Pex14mKate2; forward primer
DPxa1_Rev	ATAAATTATATTCGCTAAATAAAATCTCTCC CTTCTAGGGTGTTCCTACGTTTCGACACT GGATGGCGGC	To amplify PXA1 deletion cassette from pHIPH4-Pex14mKate2; reverse primer
CUP1_Fw	GCATGCGGCCGCCCTTTATTTTCAGGCTGAT	To amplify CUP1 promoter sequence; forward primer

CUP1_R ev	GTGCGGATCCTTTATGTGATGATTGATTGAT TGAT	To amplify CUP1 promoter sequence; reverse primer
MycUb/ UbK48R _Rev	GTGCGAGCTCTCAACCACCTCTTAGTCTTAA G	To amplify MycUb or MycUbK48R sequence
TDH3- NotI-Fw	GCATCAGCGGCCGCCACGCTTTTTCAGTTCG AGT	To amplify TDH3 sequence with Not-I site from pP _{TDH3} -GFP-SKL
TDH3- BamHI- Rev	GCGCGCGGATCCTTTGTTTGTTTATGTGTGT TTAT	To amplify TDH3 sequence with BamHI site from pP _{TDH3} -GFP-SKL

Supplementary figures

Figure S1: Pxa1^{MUT}-mGFP undergoes UPS mediated degradation

- A. Co-localization analysis of Pxa1^{MUT}-mGFP with the peroxisomal marker DsRed-SKL in WT (middle panels) and *ufd4* (bottom panels) cells. The WT strain lacking fluorescent markers (top panels) was used as control. Cells were pre-grown on glucose media and shifted to oleate/glucose media. Fluorescence images were taken from cells grown under peroxisome inducing conditions to an OD₆₀₀ of ~1.5. Blue arrows- GFP spots, yellow arrows- DsRed spots and red arrows- background fluorescence. Scale bar: 3 μ m.
- B. Co-localization analysis of Pxa1^{MUT}-mGFP with the peroxisomal marker Pex3-mKate2 in WT (Top panels) and *ufd4* (bottom panels) cells treated with CHX. These are additional FM images of Figure 6D time T=0, showing that circular GFP-positive structures are visible in both in WT and *ufd4* cells (see Figure 6). Blue arrows- GFP spots, yellow arrows- mKate2 spots and red arrows- background fluorescence, scale bar:3 μ m. Line profiles were made as described in Figure 1.
- C. Western blots used for the quantification of Pxa1-mGFP and Pxa1^{MUT}-mGFP levels in Figure 1B.
- D. WT cells expressing Pxa1-mGFP were initially grown on glucose, shifted to oleate containing media and TCA samples were collected at indicated time points. After lysis, samples were probed by immunoblotting with antibodies against mGFP and glucose phosphate dehydrogenase (G6PD; loading control).
- E. CHX chase analysis of Pxa1-mGFP and Pxa1^{MUT}-mGFP, blots used for quantification in Figure 2A.
- F. CHX chase analysis of Pxa1^{MUT}-mGFP in *pdr5* cells in the presence or absence of the proteasome inhibitors MG132 and Bortezomib. Blots used for quantification presented in Figure 3A.
- G. CHX chase analysis of Pxa1^{MUT}-mGFP in *atg12* cells (top panel) and cells expressing Myc-tagged ubiquitin (Ub; middle panel) or ubiquitin mutant (Ub^{K48R}; bottom panel). Blots used for quantification in Figure 3B and 3C, respectively.
- H. CHX chase analysis of Pex11-tFT and Pxa1-tFT, blots used for quantification in Figure 4C. Top bands in each panel represent full-length PMP-tFT fusions, *denotes a shorter, mCherry-sfGFP product resulting from mCherry hydrolysis during TCA lysate preparation and ** denotes a tFT fragment produced by incomplete proteasomal processing (Gross et al., 2000; Khmelinskii et al., 2016).

Figure S2: Pxa1^{MUT}-mGFP degradation in peroxisome and non-peroxisome mutant strains

- A. Blots of Pxa1^{MUT}-mGFP levels in cells lacking functional peroxisomes. (Right) WT, *pex2* and *pex4* cells and (Middle) WT, *atg12* and *pex5* cells, blots used for quantification in Figure 5A. (Left) Blots of Pxa1^{MUT}-mGFP levels in mutant strains *ufd4*, *nam7*, *ela1*, *dsk2*, *skp1*, *tul1* and *dma2*, blots used for quantification in Figure 6A.
- B. CHX chase analysis of Pxa1^{MUT}-mGFP in WT and mutants (*pex2*, *pex4*, *ufd4* and *pex5*), blots used for quantification in Figure 5B and 6C.
- C. Pex3-mKate2 levels in WT and peroxisome mutant strains expressing Pxa1^{MUT}-mGFP. Bar graph (Right) showing Pex3-mKate2 levels in WT, *pex2*, *pex4* and *pex5* cells, quantified from western blots (Left). Cells were grown as indicated in Figure S1A, lysed and samples were subjected to SDS-PAGE and immunoblotting with antibodies against RFP and Pyc. Pex3-mKate2 levels were normalized to the loading control Pyc. Protein levels in WT cells were set to 1. Values represent the mean $\pm$ SD of three independent experiments. Numbers above the columns depict the p-value.
- D. Pxa1-mGFP and Pxa1^{MUT}-mGFP levels in WT and *ufd4* cells, blots used for quantification in Figure 7B.

References

- Agne, B., N.M. Meindl, K. Niederhoff, H. Einwachter, P. Rehling, A. Sickmann, H.E. Meyer, W. Girzalsky, and W.H. Kunau. 2003. Pex8p: an intraperoxisomal organizer of the peroxisomal import machinery. *Mol Cell*. 11:635-646.
- Baerends, R.J., K.N. Faber, A.M. Kram, J.A. Kiel, I.J. van der Klei, and M. Veenhuis. 2000. A stretch of positively charged amino acids at the N terminus of Hansenula polymorpha Pex3p is involved in incorporation of the protein into the peroxisomal membrane. *J Biol Chem*. 275:9986-9995.
- Baryshnikova, A., M. Costanzo, S. Dixon, F.J. Vizeacoumar, C.L. Myers, B. Andrews, and C. Boone. 2010. Synthetic genetic array (SGA) analysis in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Methods Enzymol*. 470:145-179.
- Berezutskaya, E., and S. Bagchi. 1997. The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26 S proteasome. *Journal of Biological Chemistry*. 272:30135-30140.
- Bett, J.S. 2016. Proteostasis regulation by the ubiquitin system. *Essays in biochemistry*. 60:143-151.
- Burkhart, S.E., Y.T. Kao, and B. Bartel. 2014. Peroxisomal ubiquitin-protein ligases peroxin2 and peroxin10 have distinct but synergistic roles in matrix protein import and peroxin5 retrotranslocation in Arabidopsis. *Plant Physiol*. 166:1329-1344.
- Byeon, L., Z. Shi, and M.D. Tsai. 1995. Mechanism of adenylate kinase. The "essential lysine" helps to orient the phosphates and the active site residues to proper conformations. *Biochemistry*. 34:3172-3182.
- Chen, X., S. Devarajan, N. Danda, and C. Williams. 2018. Insights into the Role of the Peroxisomal Ubiquitination Machinery in Pex13p Degradation in the Yeast Hansenula polymorpha. *J Mol Biol*. 430:1545-1558.
- Costa, V., A. Quintanilha, and P. Moradas-Ferreira. 2007. Protein oxidation, repair mechanisms and proteolysis in Saccharomyces cerevisiae. *IUBMB life*. 59:293-298.
- Dang, Y., L.M. Siew, and Y.H. Zheng. 2008. APOBEC3G is degraded by the proteasomal pathway in a vif-dependent manner without being polyubiquitylated. *Journal of Biological Chemistry*. 283:13124-13131.
- Dederer, V., A. Khmelinskii, A.G. Huhn, V. Okreglak, M. Knop, and M.K. Lemberg. 2019. Cooperation of mitochondrial and ER factors in quality control of tail-anchored proteins. *eLife*. 8.

- Deshaias, R.J., and C.A.P. Joazeiro. 2009. RING Domain E3 Ubiquitin Ligases. *Annual Review of Biochemistry*. 78:399-434.
- Dobson, C.M. 2004. Principles of protein folding, misfolding and aggregation. *Semin Cell Dev Biol*. 15:3-16.
- Dove, K.K., B. Stieglitz, E.D. Duncan, K. Rittinger, and R.E. Klevit. 2016. Molecular insights into RBR E3 ligase ubiquitin transfer mechanisms. *Embo Reports*. 17:1221-1235.
- Du, K., P.H. Karp, C. Ackerley, J. Zabner, S. Keshavjee, E. Cutz, and H. Yeger. 2015. Aggregates of mutant CFTR fragments in airway epithelial cells of CF lungs: New pathologic observations. *J Cyst Fibros*. 14:182-193.
- Engelen, M., S. Kemp, and B.T. Poll-The. 2014. X-linked adrenoleukodystrophy: pathogenesis and treatment. *Current neurology and neuroscience reports*. 14:486.
- Fang, Y., J.C. Morrell, J.M. Jones, and S.J. Gould. 2004. PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. *J Cell Biol*. 164:863-875.
- Feigenbaum, V., G. Lombard-Platet, S. Guidoux, C.O. Sarde, J.L. Mandel, and P. Aubourg. 1996. Mutational and protein analysis of patients and heterozygous women with X-linked adrenoleukodystrophy. *Am J Hum Genet*. 58:1135-1144.
- Fleming, J.A., E.S. Lightcap, S. Sadis, V. Thoroddsen, C.E. Bulawa, and R.K. Blackman. 2002. Complementary whole-genome technologies reveal the cellular response to proteasome inhibition by PS-341. *Proc Natl Acad Sci U S A*. 99:1461-1466.
- Fredrickson, E.K., and R.G. Gardner. 2012. Selective destruction of abnormal proteins by ubiquitin-mediated protein quality control degradation. *Semin Cell Dev Biol*. 23:530-537.
- Gabaldon, T. 2010. Peroxisome diversity and evolution. *Philos Trans R Soc Lond B Biol Sci*. 365:765-773.
- Gibson, J.S., and J.C. Ellory. 2002. Membrane transport in sickle cell disease. *Blood cells, molecules & diseases*. 28:303-314.
- Golin, J., S.V. Ambudkar, and L. May. 2007. The yeast Pdr5p multidrug transporter: How does it recognize so many substrates? *Biochemical and Biophysical Research Communications*. 356:1-5.
- Gonzalez, S.L., M. Stremlau, X. He, J.R. Basile, and K. Munger. 2001. Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. *Journal of Virology*. 75:7583-7591.
- Gross, L.A., G.S. Baird, R.C. Hoffman, K.K. Baldrige, and R.Y. Tsien. 2000. The structure of the chromophore within DsRed, a red fluorescent protein from coral. *Proceedings of the National Academy of Sciences of the United States of America*. 97:11990-11995.
- Gurvitz, A., and H. Rottensteiner. 2006. The biochemistry of oleate induction: transcriptional upregulation and peroxisome proliferation. *Biochim Biophys Acta*. 1763:1392-1402.
- Hanada, T., N.N. Noda, Y. Satomi, Y. Ichimura, Y. Fujioka, T. Takao, F. Inagaki, and Y. Ohsumi. 2007. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J Biol Chem*. 282:37298-37302.
- Hanoun, N., S. Fritsch, O. Gayet, V. Gigoux, P. Cordelier, N. Dusetti, J. Torrisani, and M. Dufresne. 2014. The E3 Ubiquitin Ligase Thyroid Hormone Receptor-interacting Protein 12 Targets Pancreas Transcription Factor 1a for Proteasomal Degradation. *Journal of Biological Chemistry*. 289:35593-35604.
- Hettema, E.H., C.W. van Roermund, B. Distel, M. van den Berg, C. Vilela, C. Rodrigues-Pousada, R.J. Wanders, and H.F. Tabak. 1996. The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. *Embo J*. 15:3813-3822.
- Hwang, C.S., A. Shemorry, and A. Varshavsky. 2009. Two proteolytic pathways regulate DNA repair by cotargeting the Mgt1 alkylguanine transferase. *Proc Natl Acad Sci U S A*. 106:2142-2147.
- Kajiro, M., M. Tsuchiya, Y. Kawabe, R. Furumai, N. Iwasaki, Y. Hayashi, M. Katano, Y. Nakajima, N. Goto, T. Watanabe, A. Murayama, H. Oishi, M. Ema, S. Takahashi, H. Kishimoto, and J. Yanagisawa. 2011. The E3 Ubiquitin Ligase Activity of Trip12 Is Essential for Mouse Embryogenesis. *Plos One*. 6.

- Karpichev, I.V., and G.M. Small. 1998. Global regulatory functions of Oaf1p and Pip2p (Oaf2p), transcription factors that regulate genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 18:6560-6570.
- Kemp, S., I.C. Huffnagel, G.E. Linthorst, R.J. Wanders, and M. Engelen. 2016. Adrenoleukodystrophy - neuroendocrine pathogenesis and redefinition of natural history. *Nature reviews. Endocrinology.* 12:606-615.
- Kemp, S., A. Pujol, H.R. Waterham, B.M. van Geel, C.D. Boehm, G.V. Raymond, G.R. Cutting, R.J. Wanders, and H.W. Moser. 2001. ABCD1 mutations and the X-linked adrenoleukodystrophy mutation database: role in diagnosis and clinical correlations. *Hum Mutat.* 18:499-515.
- Khmelniskii, A., E. Blaszczak, M. Pantazopoulou, B. Fischer, D.J. Omnus, G. Le Dez, A. Brossard, A. Gunnarsson, J.D. Barry, M. Meurer, D. Kirrmaier, C. Boone, W. Huber, G. Rabut, P.O. Ljungdahl, and M. Knop. 2014. Protein quality control at the inner nuclear membrane. *Nature.* 516:410-413.
- Khmelniskii, A., P.J. Keller, A. Bartosik, M. Meurer, J.D. Barry, B.R. Mardin, A. Kaufmann, S. Trautmann, M. Wachsmuth, G. Pereira, W. Huber, E. Schiebel, and M. Knop. 2012. Tandem fluorescent protein timers for in vivo analysis of protein dynamics. *Nature biotechnology.* 30:708-714.
- Khmelniskii, A., M. Meurer, N. Duishoev, N. Delhomme, and M. Knop. 2011. Seamless gene tagging by endonuclease-driven homologous recombination. *PLoS One.* 6:e23794.
- Khmelniskii, A., M. Meurer, C.T. Ho, B. Besenbeck, J. Fuller, M.K. Lemberg, B. Bukau, A. Mogk, and M. Knop. 2016. Incomplete proteasomal degradation of green fluorescent proteins in the context of tandem fluorescent protein timers. *Mol Biol Cell.* 27:360-370.
- Knop, M., K. Siegers, G. Pereira, W. Zachariae, B. Winsor, K. Nasmyth, and E. Schiebel. 1999. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast.* 15:963-972.
- Krikken, A.M., M. Veenhuis, and I.J. van der Klei. 2009. *Hansenula polymorpha* pex11 cells are affected in peroxisome retention. *FEBS J.* 276:1429-1439.
- Kumar, S., R. Singh, C.P. Williams, and I.J. van der Klei. 2016. Stress exposure results in increased peroxisomal levels of yeast Pnc1 and Gpd1, which are imported via a piggy-backing mechanism. *Biochim Biophys Acta.* 1863:148-156.
- Laarieu, D., M. Brunet, C. Vargas, N. Hanoun, L. Ligat, L. Dagnon, H. Lulka, R.M. Pommier, J. Selves, B.E. Jady, L. Bartholin, P. Cordelier, M. Dufresne, and J. Torrisani. 2020. The E3 ubiquitin ligase TRIP12 participates in cell cycle progression and chromosome stability. *Scientific reports.* 10.
- Li, Z., F.J. Vizeacoumar, S. Bahr, J. Li, J. Warringer, F.S. Vizeacoumar, R. Min, B. Vandersluis, J. Bellay, M. Devit, J.A. Fleming, A. Stephens, J. Haase, Z.Y. Lin, A. Baryshnikova, H. Lu, Z. Yan, K. Jin, S. Barker, A. Datti, G. Giaever, C. Nislow, C. Bulawa, C.L. Myers, M. Costanzo, A.C. Gingras, Z. Zhang, A. Blomberg, K. Bloom, B. Andrews, and C. Boone. 2011. Systematic exploration of essential yeast gene function with temperature-sensitive mutants. *Nature biotechnology.* 29:361-367.
- Luciani, A., V.R. Villella, S. Esposito, N. Brunetti-Pierri, D. Medina, C. Settembre, M. Gavina, L. Pulze, I. Giardino, M. Pettoello-Mantovani, M. D'Apolito, S. Guido, E. Masliah, B. Spencer, S. Quarantino, V. Raia, A. Ballabio, and L. Maiuri. 2010. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nature Cell Biology.* 12:863-875.
- Mayerhofer, P.U. 2016. Targeting and insertion of peroxisomal membrane proteins: ER trafficking versus direct delivery to peroxisomes. *Biochim Biophys Acta.* 1863:870-880.
- Metzger, M.B., V.A. Hristova, and A.M. Weissman. 2012. HECT and RING finger families of E3 ubiquitin ligases at a glance. *Journal of Cell Science.* 125:531-537.
- Metzger, M.B., J.N. Pruneda, R.E. Klevit, and A.M. Weissman. 2014. RING-type E3 ligases: Master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Bba-Mol Cell Res.* 1843:47-60.
- Metzger, M.B., and A.M. Weissman. 2010. Working on a chain: E3s ganging up for ubiquitylation. *Nat Cell Biol.* 12:1124-1126.
- Morita, M., S. Matsumoto, A. Sato, K. Inoue, D.G. Kostsin, K. Yamazaki, K. Kawaguchi, N. Shimozawa, S. Kemp, R.J. Wanders, H. Kojima, T. Okabe, and T. Imanaka. 2019. Stability of the ABCD1 Protein with a

- Missense Mutation: A Novel Approach to Finding Therapeutic Compounds for X-Linked Adrenoleukodystrophy. *JIMD reports*. 44:23-31.
- Nuttall, J.M., A. Motley, and E.H. Hettema. 2011. Peroxisome biogenesis: recent advances. *Curr Opin Cell Biol*. 23:421-426.
- Park, Y., S.K. Yoon, and J.B. Yoon. 2009. The HECT Domain of TRIP12 Ubiquitinates Substrates of the Ubiquitin Fusion Degradation Pathway. *Journal of Biological Chemistry*. 284:1540-1549.
- Prakash, S., T. Inobe, A.J. Hatch, and A. Matouschek. 2009. Substrate selection by the proteasome during degradation of protein complexes. *Nature chemical biology*. 5:29-36.
- Roerig, P., P. Mayerhofer, A. Holzinger, and J. Gartner. 2001. Characterization and functional analysis of the nucleotide binding fold in human peroxisomal ATP binding cassette transporters. *FEBS Lett*. 492:66-72.
- Saraya, R., M.N. Cepinska, J.A. Kiel, M. Veenhuis, and I.J. van der Klei. 2010. A conserved function for Inp2 in peroxisome inheritance. *Biochim Biophys Acta*. 1803:617-622.
- Sargent, G., T. van Zutphen, T. Shatseva, L. Zhang, V. Di Giovanni, R. Bandsma, and P.K. Kim. 2016. PEX2 is the E3 ubiquitin ligase required for pexophagy during starvation. *J Cell Biol*. 214:677-690.
- Schneider, E., S. Wilken, and R. Schmid. 1994. Nucleotide-induced conformational changes of MalK, a bacterial ATP binding cassette transporter protein. *J Biol Chem*. 269:20456-20461.
- Schrader, M., and H.D. Fahimi. 2006. Peroxisomes and oxidative stress. *Biochim Biophys Acta*. 1763:1755-1766.
- Schuldiner, M., S.R. Collins, N.J. Thompson, V. Denic, A. Bhamidipati, T. Punna, J. Ihmels, B. Andrews, C. Boone, J.F. Greenblatt, J.S. Weissman, and N.J. Krogan. 2005. Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile. *Cell*. 123:507-519.
- Schulman, B.A., and J.W. Harper. 2009. Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nat Rev Mol Cell Bio*. 10:319-331.
- Shani, N., A. Sapag, P.A. Watkins, and D. Valle. 1996. An *S. cerevisiae* peroxisomal transporter, orthologous to the human adrenoleukodystrophy protein, appears to be a heterodimer of two half ABC transporters: Pxa1p and Pxa2p. *Ann N Y Acad Sci*. 804:770-772.
- Shani, N., and D. Valle. 1996. A *Saccharomyces cerevisiae* homolog of the human adrenoleukodystrophy transporter is a heterodimer of two half ATP-binding cassette transporters. *Proc Natl Acad Sci U S A*. 93:11901-11906.
- Shani, N., P.A. Watkins, and D. Valle. 1995. PXA1, a possible *Saccharomyces cerevisiae* ortholog of the human adrenoleukodystrophy gene. *Proc Natl Acad Sci U S A*. 92:6012-6016.
- Smith, J.J., and J.D. Aitchison. 2013. Peroxisomes take shape. *Nat Rev Mol Cell Biol*. 14:803-817.
- Smith, J.J., M. Marelli, R.H. Christmas, F.J. Vizeacoumar, D.J. Dilworth, T. Ideker, T. Galitski, K. Dimitrov, R.A. Rachubinski, and J.D. Aitchison. 2002. Transcriptome profiling to identify genes involved in peroxisome assembly and function. *J Cell Biol*. 158:259-271.
- Takahashi, N., M. Morita, T. Maeda, Y. Harayama, N. Shimozaawa, Y. Suzuki, H. Furuya, R. Sato, Y. Kashiwayama, and T. Imanaka. 2007. Adrenoleukodystrophy: subcellular localization and degradation of adrenoleukodystrophy protein (ALDP/ABCD1) with naturally occurring missense mutations. *Journal of neurochemistry*. 101:1632-1643.
- Theodoraki, M.A., N.B. Nilleghoda, J. Saini, and A.J. Caplan. 2012. A network of ubiquitin ligases is important for the dynamics of misfolded protein aggregates in yeast. *J Biol Chem*. 287:23911-23922.
- Thomas, P.J., Y.H. Ko, and P.L. Pedersen. 1992. Altered Protein Folding May Be the Molecular-Basis of Most Cases of Cystic-Fibrosis. *FEBS Letters*. 312:7-9.
- Thrower, J.S., L. Hoffman, M. Rechsteiner, and C.M. Pickart. 2000. Recognition of the polyubiquitin proteolytic signal. *EMBO J*. 19:94-102.
- Tong, A.H., and C. Boone. 2006. Synthetic genetic array analysis in *Saccharomyces cerevisiae*. *Methods Mol Biol*. 313:171-192.
- Van der Leij, I., M.M. Franse, Y. Elgersma, B. Distel, and H.F. Tabak. 1993. PAS10 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A*. 90:11782-11786.

- van Roermund, C.W., L. Ijlst, W. Majczak, H.R. Waterham, H. Folkerts, R.J. Wanders, and K.J. Hellingwerf. 2012. Peroxisomal fatty acid uptake mechanism in *Saccharomyces cerevisiae*. *J Biol Chem.* 287:20144-20153.
- van Roermund, C.W., W.F. Visser, L. Ijlst, A. van Cruchten, M. Boek, W. Kulik, H.R. Waterham, and R.J. Wanders. 2008. The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. *Faseb J.* 22:4201-4208.
- Walker, J.E., M. Saraste, M.J. Runswick, and N.J. Gay. 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J.* 1:945-951.
- Ward, C.L., S. Omura, and R.R. Kopito. 1995. Degradation of Cfr by the Ubiquitin-Proteasome Pathway. *Cell.* 83:121-127.
- Weber, J., S. Polo, and E. Maspero. 2019. HECT E3 Ligases: A Tale With Multiple Facets. *Frontiers in physiology.* 10.
- Wenzel, D.M., A. Lissounov, P.S. Brzovic, and R.E. Klevit. 2011. UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature.* 474:105-U136.
- Williams, C., L. Opalinski, C. Landgraf, J. Costello, M. Schrader, A.M. Krikken, K. Knoops, A.M. Kram, R. Volkmer, and I.J. van der Klei. 2015. The membrane remodeling protein Pex11p activates the GTPase Dnm1p during peroxisomal fission. *Proceedings of the National Academy of Sciences.* 112:6377-6382.
- Williams, C., and I.J. van der Klei. 2013. Pexophagy-linked degradation of the peroxisomal membrane protein Pex3p involves the ubiquitin-proteasome system. *Biochem Biophys Res Commun.* 438:395-401.
- Winklhofer, K.F., J. Tatzelt, and C. Haass. 2008. The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. *EMBO Journal.* 27:336-349.
- Winzler, E.A., D.D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J.D. Boeke, H. Bussey, A.M. Chu, C. Connelly, K. Davis, F. Dietrich, S.W. Dow, M. El Bakkoury, F. Foury, S.H. Friend, E. Gentalen, G. Giaever, J.H. Hegemann, T. Jones, M. Laub, H. Liao, N. Liebundguth, D.J. Lockhart, A. Lucau-Danila, M. Lussier, N. M'Rabet, P. Menard, M. Mittmann, C. Pai, C. Rebischung, J.L. Revuelta, L. Riles, C.J. Roberts, P. Ross-MacDonald, B. Scherens, M. Snyder, S. Sookhai-Mahadeo, R.K. Storms, S. Veronneau, M. Voet, G. Volckaert, T.R. Ward, R. Wysocki, G.S. Yen, K. Yu, K. Zimmermann, P. Philippsen, M. Johnston, and R.W. Davis. 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science.* 285:901-906.
- Yamada, T., N. Shinnoh, and T. Kobayashi. 1997. Protease inhibitors suppress the degradation of mutant adrenoleukodystrophy proteins but do not correct impairment of very long chain fatty acid metabolism in adrenoleukodystrophy fibroblasts. *Neurochem Res.* 22:233-237.
- Ye, Y.H., and M. Rape. 2009. Building ubiquitin chains: E2 enzymes at work. *Nat Rev Mol Cell Bio.* 10:755-764.
- Zhang, W.Q., N. Fujii, and A.P. Naren. 2012. Recent advances and new perspectives in targeting CFTR for therapy of cystic fibrosis and enterotoxin-induced secretory diarrheas. *Future Med Chem.* 4:329-345.
- Zhang, X., C. De Marcos Lousa, N. Schutte-Lensink, R. Ofman, R.J. Wanders, S.A. Baldwin, A. Baker, S. Kemp, and F.L. Theodoulou. 2011. Conservation of targeting but divergence in function and quality control of peroxisomal ABC transporters: an analysis using cross-kingdom expression. *Biochem J.* 436:547-557.
- Zoghbi, M.E., and G.A. Altenberg. 2014. ATP binding to two sites is necessary for dimerization of nucleotide-binding domains of ABC proteins. *Biochem Biophys Res Commun.* 443:97-102.

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4

Chapter 4

Investigating Pex13p degradation in the yeast *Saccharomyces cerevisiae*

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Author contributions

CW conceived and supervised the project with the input from MK. CW, XC, SD, MM and MK designed the experiments. XC, SD and CW analysed the data. XC performed biochemical and FM experiments. SD performed the spot assay, SD and MM performed tFT analysis and data processing. All authors discussed the results. XC and CW wrote the manuscript, with contributions from all authors.

Abstract

Pex13p is a member of docking complex required for the import of peroxisomal matrix proteins. We previously demonstrated that Pex13p is rapidly degraded via the ubiquitination proteasome system (UPS) in a Pex2p-dependent manner in the yeast *Hansenula polymorpha*. However, whether Pex13p degradation is conserved in other species and which additional factors may be involved remains unclear. In this study, we demonstrate that UPS-mediated Pex13p degradation occurs in the yeast *Saccharomyces cerevisiae* and that the mechanisms of Pex13p degradation are similar to in *H. polymorpha*. Additionally, inactivation of Cdc48p, an ATPase involved in degrading mitochondrial and ER membrane proteins, does not result in stabilization of Pex13p *in vivo*, indicating that Pex13p degradation likely occurs via a different mechanism than other organellar membrane proteins. Furthermore, we utilize a tandem fluorescent protein timer approach to identify additional factors involved in Pex13p degradation. Our data demonstrate that cytosolic E2 and E3 enzymes play a role in Pex13p degradation. Taken together, our data provide further evidence that Pex13p degradation is conserved throughout evolution while they also uncover novel components of the UPS that are involved in Pex13p degradation. The implications of our findings are discussed.

Keywords: Peroxisome, protein degradation, ubiquitination, PMP, Pex13p

Introduction

Peroxisomes are cellular compartments in eukaryotic cells that house metabolic pathways. Common functions of peroxisomes include the β -oxidation of fatty acids and the decomposition of oxygen reactive species, although many species- and cell-specific peroxisomal functions are known (Gabaldon, 2010). Defects in peroxisome function can cause a spectrum of inherited developmental brain disorders (Walker et al, 2002). Peroxisome function depends on which peroxisomal membrane proteins (PMP) and peroxisomal matrix proteins (MAT) are present in the peroxisome. Both PMPs and MATs are post-translationally transported to peroxisomes with the aid of receptor proteins. MATs can be targeted to peroxisomes in one of two different ways: MATs with a C-terminal peroxisomal targeting signal type-1 (PTS1) sequence can be recognized by the cytosolic receptor Pex5p, while MATs containing an N-terminal PTS2 signal can be recognized by Pex7p (Braverman et al, 1997; Mukai et al, 2002). PTS2 protein import also requires the action of additional co-receptor proteins (Sichting et al, 2003). After recognition, the receptor-cargo complex binds to the docking complex on the peroxisomal membrane, consisting of Pex14p and Pex13p (Elgersma et al, 1996; Johnson et al, 2001). After cargo translocation and release, a process that is poorly understood (Girzalsky et al, 2010), the receptor Pex5p is ubiquitinated at the peroxisome by the peroxisomal ubiquitination machinery, consisting of the ubiquitin-conjugating enzyme Pex4p and the RING finger ubiquitin ligases Pex2p, Pex10p and Pex12p (Platta et al, 2007; Williams et al, 2008), which allows it to be recycled back to the cytosol, with the aid of Pex1p and Pex6p, two AAA-ATPases (Platta et al, 2008). The co-receptor proteins that function with Pex7p in the import of PTS2 proteins can also be ubiquitinated in similar way during the import cycle (El Magraoui et al, 2013).

Previously, we demonstrated that *Hansenula polymorpha* Pex13p is degraded via the ubiquitination proteasome system (UPS), and its degradation requires the peroxisomal ubiquitination machinery mentioned above (Chen et al, 2018). The UPS-mediated degradation of proteins occurs in a stepwise fashion (Hershko, 1996). Ubiquitin, a globular protein of ~8kDa, is first activated by a ubiquitin activating enzyme (E1) consuming ATP as energy. The activated ubiquitin was then transferred to the Cysteine residue of a ubiquitin conjugating enzyme (E2). Ubiquitin can then be either passed on to the active Cysteine of a HECT-class ubiquitin ligase (E3) and subsequently transferred to a specific substrate, or be transferred to a substrate directly from the E2 with the help of a RING E3 ligase (Scheffner et al, 1995). Attachment of ubiquitin to a substrate usually occurs via lysine residues on the substrate (Rodriguez, 1996), although ubiquitin attachment to cysteine, serine and threonine residues has been reported (Wang et al, 2007; Williams et al, 2007). In yeast, around 11 E2s and more than 60 E3s are involved in the ubiquitination process (Finley et al, 2012; Ravid & Hochstrasser, 2007). While ubiquitination serves many functions, UPS-mediated protein degradation often requires the attachment of a chain of ubiquitin molecules (referred to as poly-ubiquitination). In this case, ubiquitin itself becomes a substrate for ubiquitination and a common linkage

involved in UPS-mediated protein degradation is via Lysine-48 (K48) on ubiquitin (Hershko & Ciechanover, 1998).

In this manuscript, we have investigated the stability of Pex13p in the yeast *Saccharomyces cerevisiae*, to establish how conserved the Pex13p degradation process is across evolution. We demonstrate that Pex13p undergoes rapid degradation in wild type *S. cerevisiae* cells and also establish that Pex13p degradation is inhibited when poly-ubiquitin chain formation is blocked. Furthermore, Pex13p turnover is inhibited in *pex2* and *pex4* cells, indicating that the mechanism by which *H. polymorpha* and *S. cerevisiae* Pex13p is degraded is likely to be conserved. Furthermore, we show that the function of Cdc48p, an AAA-ATPase involved in the degradation of ER and mitochondrial membrane proteins, is not required for Pex13p degradation. Finally, we use a high throughput screening approach, combined with a tandem fluorescent timer (tFT) (Khmelniskii et al, 2014; Khmelniskii et al, 2012), to identify additional proteins involved in Pex13p degradation. Our tFT and subsequent biochemical analysis identifies a role for cytosolic E2 and E3 enzymes in Pex13p degradation, providing a solid platform for future studies aimed at understanding the molecular mechanisms and underlying functions of Pex13p degradation.

Results

The rapid degradation of Pex13p is conserved in *S. cerevisiae*

Previously we demonstrated that Pex13p in the yeast *H. polymorpha* undergoes rapid degradation via the UPS (Chen et al, 2018). In addition, UPS-mediated Pex13p degradation was reported to occur in plants (Pan et al, 2016), although this degradation event remain controversial (Ling et al, 2017; Pan & Hu, 2018). Therefore, we decided to investigate whether Pex13p degradation occurs in other organisms and chose the yeast *S. cerevisiae* for this. We utilized Pex13p fused to mGFP, which allowed us to detect the protein on western blot using anti-GFP antibodies as well as to follow the subcellular localization of Pex13p using fluorescence microscopy. Cells expressing Pex13-mGFP are able to grow on media containing oleic acid as sole carbon source (Figure 1A), a condition that requires peroxisome function for growth, indicating that Pex13-mGFP is a functional protein.

To investigate Pex13p turnover, we treated cells expressing Pex13-mGFP with cycloheximide (CHX). Treatment of cells with CHX blocks protein production and it is commonly used to investigate the kinetics of protein degradation. Cells were grown on oleic acid containing media, to stimulate peroxisome proliferation. Pex13-mGFP levels rapidly decreased after addition of CHX while Pex14p levels remained largely unaltered (Figure 1B, D & E), indicating that Pex13-mGFP undergoes protein degradation, similar to our data in the yeast *H. polymorpha* (Chen et al, 2018). Next, we investigated the role of the UPS in Pex13-mGFP degradation. To achieve this, we co-expressed the K48R mutant form of ubiquitin (Ub) in cells expressing Pex13-mGFP. This Ub-K48R mutant inhibits the formation of poly-

ubiquitination chain on substrates and therefore inhibits UPS-mediated protein degradation (Thrower et al, 2000). Our data demonstrate that Pex13-mGFP turnover is significantly reduced in cells expressing Ub-K48R compared to wild-type ($P < 0.01$), indicating that the UPS is involved in its degradation (Figure 1C & D). These data demonstrate that, similar to in *H. polymorpha*, Pex13p undergoes rapid, UPS-mediated degradation in *S. cerevisiae*.

Figure 1: Rapid degradation of Pex13-mGFP via the UPS occurs in *S. cerevisiae*.

A Spot assay to test the growth of cells expressing Pex13-tFT or Pex13-mGFP. *S. cerevisiae* cells were spotted onto oleic acid plates and cultured at 30°C for 7 days.

- B WT cells expressing Pex13-mGFP were grown on oleic acid containing media for 12 hrs and treated with DMSO (Ctrl) or Cycloheximide (CHX). TCA samples were taken at the indicated time (hrs) after DMSO/CHX addition and probed by SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p (indicated Pyc).
- C Representative western blots of Ub-K48R cells expressing Pex13-mGFP derived from cells grown and treated as in A. Samples were probed with SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- D Quantification of Pex13-mGFP levels in WT and Ub-K48R cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Asterisks denote statistically significant increases in protein levels compared to those in WT samples (* $P < 0.05$, ** $P < 0.01$).
- E Quantification of Pex14p levels in WT and Ub-K48R cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments.

The peroxisomal ubiquitination machinery is required for Pex13p degradation in *S. cerevisiae*

Previously, we demonstrated that Pex13p in the yeast *H. polymorpha* was stabilized when components of the peroxisomal ubiquitination machinery, such as the E3 ligase Pex2p or the E2 Pex4p are absent (Chen et al, 2018). Therefore, we investigated whether the mechanism by which Pex13p degradation occurs was also conserved in *S. cerevisiae*. We introduced Pex13-mGFP into *pex2* or *pex4* cells and followed Pex13p-mGFP degradation in CHX-treated cells grown on oleic acid containing media (Figure 2). Compared to WT, Pex13-mGFP turnover was clearly inhibited in *pex2* (Figure 2A & B) and *pex4* (Figure 2D & E) cells ($P < 0.001$), suggesting that Pex2p and Pex4p play a role in Pex13p degradation. Next, we investigated the subcellular localization of Pex13-mGFP in WT and *pex2* cells grown on oleic acid containing media. Pex13-mGFP co-localizes with Pex3-mKate (a stable peroxisomal membrane protein, used as the peroxisomal membrane marker) on the peroxisomal membrane in *pex2* cells (Figure 3A & B) and mGFP intensity is significantly increased in *pex2* cells (Figure 3A-D). Furthermore, Pex13-mGFP levels are significantly elevated in *pex2* cells compared to wild-type cells (Figure 3E & F), again indicating Pex13-mGFP degradation is inhibited in *pex2* cells. These data strongly suggest that Pex13p degradation in both *H. polymorpha* and *S. cerevisiae* proceeds via a similar mechanism.

Figure 2: Pex13-mGFP degradation is inhibited in cells lacking members of the peroxisomal ubiquitination machinery

- A Representative western blots of *pex2* cells expressing Pex13-mGFP derived from cells grown and treated as in (Figure 1B). Samples were probed with SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- B Quantification of Pex13-mGFP levels in WT and *pex2* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were taken from (Figure 1A). Asterisks denote statistically significant increases in protein levels compared to those in WT samples (*P < 0.05, **P < 0.01, ***P < 0.001).
- C Quantification of Pex14p levels in WT and *pex2* cells. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were taken from Figure 1B.
- D Representative western blots of *pex4* cells expressing Pex13-mGFP derived from cells grown and treated as in (A). Samples were probed with SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- E Quantification of Pex13-mGFP levels in WT and *pex4* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B). Asterisks denote statistically significant increases in protein levels compared to those in WT samples (*P < 0.05, **P < 0.01, ***P < 0.001).
- F Quantification of Pex14p levels in WT and *pex4* cells. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B).

Figure 3: Pex13-mGFP accumulates on peroxisomes in *pex2* cells.

- A** WT and *pex2* cells producing Pex13-mGFP and Pex3-mKate2 were grown on oleic acid containing media to an OD₆₀₀ of 1.0 and fluorescence microscopy images were taken. Images of Pex13-mGFP were processed using ImageJ with optimal settings to show signals in WT and *pex2*. Pex3-mKate2 was used as peroxisomal membrane marker. The following settings were used: for WT cells mGFP (290, 650) and mKate2 (225, 600); for *pex2* cells mGFP (290, 2900) and mKate2 (225, 630). Scale bar: 5µm.
- B** Fluorescence images of Pex13-mGFP in WT or *pex2* shown in (A) were processed using ImageJ with the same settings: mGFP (300, 2000), mKate2 (225, 620). Scale bar: 5µm.
- C** Box plot showing quantification of mGFP fluorescence intensity at the peroxisomal membrane in WT and *pex2* cells producing Pex13-mGFP. Fluorescence intensities (arbitrary units) were measured using ImageJ. The box represents values from the 25 percentile to the 75 percentile; the horizontal line through the box represents the median value. Whiskers indicate maximum and minimum values. Pex13-mGFP measurements were taken as described in the Materials and Methods section.
- D** Average ratio ± SD per cell (*n* = 40) of mGFP to mKate intensities in WT and *pex2* cells. ****P* < 0.001.

- E WT and *pex2* cells producing Pex13-mGFP grown on oleic acid media and TCA samples were taken when the cultures reached an OD₆₀₀ of 1.0. Samples were subjected to SDS-PAGE and immunoblotting using antibodies against mGFP, Pex14p and Pyc1p.
- F Quantification of protein levels in WT and *pex2* cells, normalized to the loading control Pyc1p. Protein levels in WT cells were set to 1. Values represent the mean $\pm$ SD of three independent experiments. Asterisks denote statistically significant increases in protein levels compared to those in WT samples (**P < 0.01).

Cdc48p function is not required for Pex13p degradation

Membrane proteins need to be removed from their native membrane environment before they can be degraded by the proteasome and the AAA-ATPase Cdc48p (p97 in humans) is known to extract membrane proteins from both the endoplasmic reticulum (ER) and mitochondrial membranes and deliver them to the proteasome for degradation (Cao et al, 2003; Wolf & Stolz, 2012). Therefore, we considered the possibility that Cdc48p could be involved in extracting Pex13p out of the peroxisomal membrane and delivering it to the proteasome, for degradation. To investigate this, we utilized a temperature sensitive mutant form of Cdc48p (*cdc48-3*), since *CDC48* is an essential gene (Dargemont & Ossareh-Nazari, 2012; Wolf & Stolz, 2012; Yamanaka et al, 2012). The mutant Cdc48 protein is active when cells are grown at the permissive temperature of 23°C but is inactive when cells are grown at the restrictive temperature of 37°C. We used Cdc5p as a control substrate. Cdc5p is a serine/threonine-protein kinase required for the cell cycle and its degradation requires Cdc48p (Cao et al, 2003). We introduced Pex13-mGFP or Cdc5-HA₆ into *cdc48-3* cells and followed the degradation of these proteins in oleic acid-grown cells treated with CHX, at both 23°C and 37°C (Figure 4). Cdc5-HA₆ degradation was observed in cells grown at the permissive temperature while Cdc5-HA₆ turnover was inhibited in cells growing at the restrictive temperature (Figure 4A-C). Significantly, Pex13-mGFP was degraded at a similar rate in cells grown at both the permissive and the restrictive temperatures (Figure 4D-K), suggesting that Cdc48p function is not required for Pex13p degradation.

Figure 4: Cdc48p function is not required for Pex13p degradation.

- A Representative western blots of *cdc48-3* cells expressing Cdc5-HA₆ derived from cells grown and treated as in (A). Samples were probed with SDS-PAGE and immunoblotting with antibodies against HA and Pyc1p.
- B Representative western blots of *cdc48-3* cells expressing Cdc5-HA₆ derived from cells grown and treated as in (E). Samples were probed with SDS-PAGE and immunoblotting with antibodies against HA and Pyc1p.
- C Quantification of Cdc5-HA₆ levels in *cdc48-3* cells at permissive temperature 23°C or restrictive temperature 37°C expressing. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. (*P < 0.05)
- D WT cells expressing Pex13-mGFP were grown on oleic acid media at 23°C for 18 hrs and treated with DMSO (Ctrl) or Cycloheximide (CHX). TCA samples were taken at the indicated time (hrs) after DMSO/CHX addition and probed by SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- E Representative western blots of *cdc48-3* cells expressing Pex13-mGFP derived from cells grown and treated as in (A). Samples were probed with SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- F Quantification of Pex13-mGFP in (A) and (B). Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments.
- G Quantification of Pex14p levels in WT and *cdc48-3* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments.

Figure 4 continued

- H WT cells expressing Pex13-mGFP were grown on oleic acid media at 23°C for 17 hrs, then shifted to 37°C for 1 hr, and treated with DMSO (Ctrl) or Cycloheximide (CHX). TCA samples were taken at the indicated time (hrs) after DMSO/CHX addition and probed by SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- I Representative western blots of WT cells expressing Pex13-mGFP derived from cells grown and treated as in (E). Samples were probed with SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- J Quantification of Pex13-mGFP in (E) and (F). Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments.
- K Quantification of Pex14p levels in WT and *cdc48-3* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments.

Investigating Pex13p degradation with a tandem fluorescent timer

While our data demonstrate a role for the peroxisomal ubiquitination machinery in the degradation of *S. cerevisiae* Pex13p (Figure 2 and 3), we sought to identify which additional

factors may regulate Pex13p degradation. To achieve this we utilized a tandem fluorescent timer (tFT) approach, which has been previously used to study protein stability in *S. cerevisiae* cells (Khmelniskii et al, 2014). The tFT tag consists of mCherry and sfGFP, which have maturation half times of around 45 and 5 minutes, respectively (in *S. cerevisiae* cells grown at 30°C). Measuring the red and green fluorescent intensities directly in cells can provide information on the relative stability of the tFT-tagged protein in an *in vivo* setting. A high mCherry/sfGFP intensity ratio indicates that the tagged protein is stable while a low mCherry/sfGFP ratio indicates an unstable protein (Khmelniskii et al, 2012). Cells expressing Pex13-tFT only (and hence not the WT version of the protein) can grow on oleic acid (Figure 1A), suggesting that the Pex13-tFT protein is functional. Furthermore, Pex13-tFT was rapidly degraded in cells treated with CHX (Figure 5), demonstrating that Pex13-tFT can be used to study Pex13p degradation.

Figure 5: Pex13-tFT is rapidly degraded in *S. cerevisiae* cells treated with CHX.

- A Representative western blots of samples derived from cells expressing Pex13-tFT or Pex11-tFT grown for 12 hrs on oleic acid media. Blots were probed with antibodies directed against mGFP and Pyc1p. * Denotes a hydrolysed product of mature mCherry during SDS-sample preparation due to chemical breakage of the fluorophore. This cuts mCherry into two pieces, at around position 69 of mCherry, thereby generating a fragment containing the C-term mCherry and sfGFP of approximately 45 kDa. # Denotes an incomplete degradation product of sfGFP due to its rigid fold. This 33 kDa band often appears as a doublet. The presence of this band is a strong signature that the tFT tagged protein is degraded by the proteasome (Khmelniskii et al, 2016).
- B Quantification of Pex13-tFT and Pex11-tFT levels of blots in the left. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments.

Next, we created a library of 152 strains made by synthetic genetic array (SGA) expressing Pex13-tFT that either lacked a gene involved in protein degradation or which contained a mutant version of a protein involved in protein degradation (in case the deletion was lethal) (Tong & Boone, 2006). Gene deletion and gene tagging were validated by PCR (data not shown). Mutant cells expressing Pex13-tFT were grown on synthetic complete media plates containing 0.1% oleic acid and 0.1% glucose for seven days at 30°C. Colonies (four technical replicates for each mutant strain plus controls) were imaged every day to determine mCherry and GFP fluorescence intensities and the average ratio of mCherry to sfGFP intensities was determined for each strain on each day. These ratios were normalized to the ratio measured in WT cells expressing Pex13-tFT and the Z-score, the deviation of the ratio for a particular mutant strain compared to the average ratio across all strains, was calculated for each strain (Figure 6). Further details on how the images were taken and the data were processed can be found in the Materials and Methods section.

We considered mutant strains that displayed an increase in Z-score of more than 1.0 on each of the seven days potentially interesting. These strains, which include cells deleted for *PEX4*, *PEX2*, *PEX10*, *PEX12* and *UBI4* (which depletes the amount of ubiquitin available in the cell, but does not result in an absence of ubiquitin completely because of the presence of additional copies of the *UBI* gene), display an increase in Pex13-tFT stability on each of the seven days (Figure 6). A role for these factors in Pex13p degradation was already shown (Figure 1, 2 & 3), validating our tFT approach. In addition, cells deleted for *UBR2*, *UFD4*, *RCY1*, *YUH1*, *UBP6* and *UBC4* all displayed increased Pex13-tFT stability on each of the seven days (Figure 6). Ufd4p is a cytosolic HECT E3 ligase which regulates the degradation of misfolded proteins (Theodoraki et al, 2012). Interestingly, Ufd4p is also involved in the degradation of Pxa1p (Devarajan *et al*, in preparation), a peroxisomal membrane protein involved in the import of activated long-chain fatty acids from the cytosol to the peroxisomal matrix (Shani et al, 1995). Ubc4p is a ubiquitin-conjugating enzyme (E2) involved in the degradation of abnormal or excess proteins while it also mediates the ubiquitination of Pex5p (Seufert & Jentsch, 1990; Williams et al, 2007). Ubc4p is known to work with Ufd4p in the ubiquitination of certain substrates (Bao, 2015). Ubr2p is a cytosolic RING E3 ligase which like Ufd4p, is involved in the degradation of misfolded proteins (Nillegoda et al, 2010). *S. cerevisiae* cells depleted of Ubr2p cannot grow on oleic acid containing media (Lockshon et al, 2007; Saleem et al, 2010), although the underlying peroxisomal defect in these cells is unknown. Ubp6p is a de-ubiquitinating enzyme that associates with the proteasome and can negatively regulate proteasomal activity (Hanna et al, 2006) and it is also involved in Pxa1p degradation (Devarajan *et al*, in preparation). Rcy1p is involved in recycling plasma membrane proteins internalized by endocytosis (Wiederkehr et al, 2000) and is required for recycling of the v-SNARE Snc1p (Galan et al, 2001). Yuh1p is a de-ubiquitinating enzyme that regulates cellular ubiquitin levels (Miller et al, 1989). Taken together, our tFT analysis identifies additional factors potentially involved in Pex13p degradation.

Figure 6: tFT analysis identifies additional factors involved in Pex13p degradation.

Heat-map indicating relative Pex13-tFT stability in different mutant yeast strains. Strains expressing Pex13-tFT were grown for seven days at 30°C on oleic acid plates with 0.1% glucose (w/v). The mCherry and sfGFP fluorescence intensities were measured for colonies from each strain on each day and the mCherry/sfGFP ratio

was calculated and used to determine the Z-score, the deviation of the ratio for a particular strain on a particular day compared to the average ratio across all strains on that day. Increases in Z-score are colour coded, ranging from 1.0 or less (green) to 5.0 (red). The data for each square in the heat map are derived from four technical replicates.

Ufd4p, Ubc4p and Ubr2p facilitate the targeted degradation of Pex13p

Our tFT data identified six additional candidates that could play a role in Pex13p degradation and we chose to investigate the role of three of these candidates in Pex13p turnover. These were the cytosolic E2 Ubc4p and the cytosolic E3 ligases Ufd4p and Ubr2p. As negative control we chose *atg12* cells, since no increase in Pex13-tFT stability was observed in these cells (Figure 6). We introduced Pex13-mGFP into *ufd4*, *ubc4*, *ubr2* and *atg12* cells and investigated Pex13-mGFP steady state levels in cells grown on oleic acid containing media, establishing that Pex13-mGFP levels are significantly elevated *ufd4*, *ubr2* and *ubc4* cells (Figure 7). In addition, degradation of Pex13-mGFP proceeded at a significantly lower rate in CHX treated *ufd4* (Figure 8), *ubc4* (Figure 9) and *ubr2* (Figure 10) cells, supporting our data on the steady state levels of Pex13-mGFP in these mutant strains (Figure 7A,B). Taken together, these data provide further evidence for a role for Ufd4p, Ubc4p and Ubr2p in Pex13-mGFP degradation.

Figure 7: Pex13-mGFP levels are increased in *ufd4*, *ubr2* and *ubc4* cells.

A Representative western blots of samples derived from WT and mutant cells grown for 12 hrs on oleic acid media. The *atg12* strain was used as a negative control. Blots were probed with antibodies directed against mGFP, Pex14p and Pyc1p.

B Quantification of protein levels in WT and mutant cells, normalized to the loading control Pyc1p. Protein levels in WT cells were set to 1. Values represent the mean $\pm$ SD of three independent experiments. Asterisks denote statistically significant increases in protein levels compared to those in WT samples (* $P < 0.05$, ** $P < 0.01$).

Figure 8: Cells lacking the cytosolic E3 Ufd4p display enhanced Pex13-mGFP stability.

- A** The *ufd4* cells expressing Pex13-mGFP were grown on oleic acid media for 12 hrs and treated with DMSO (Ctrl) or Cycloheximide (CHX). TCA samples were taken at the indicated time (hrs) after DMSO/CHX addition and probed by SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- B** Quantification of Pex13-mGFP level in *ufd4* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B). Asterisks denote statistically significant increases in protein levels compared to those in WT samples (* $P < 0.05$).

C Quantification of Pex14p level in *ufd4* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B).

Figure 9: The cytosolic E2 Ubc4 is involved in Pex13-mGFP degradation.

- A The *ubc4* cells expressing Pex13-mGFP were grown on oleic acid media for 12 hrs and treated with DMSO (Ctrl) or Cycloheximide (CHX). TCA samples were taken at the indicated time (hrs) after DMSO/CHX addition and probed by SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- B Quantification of Pex13-mGFP level in *ubc4* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B). Asterisks denote statistically significant increases in protein levels compared to those in WT samples (* $P < 0.05$).

C Quantification of Pex14p level in *ubr2* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B).

Figure 10: Deletion of UBR2, which encodes for a cytosolic RING E3 ligase, impacts on Pex13-mGFP degradation.

- A The *ubr2* cells expressing Pex13-mGFP were grown on oleic acid media for 12 hrs and treated with DMSO (Ctrl) or Cycloheximide (CHX). TCA samples were taken at the indicated time (hrs) after DMSO/CHX addition and probed by SDS-PAGE and immunoblotting with antibodies against mGFP, Pex14p and Pyc1p.
- B Quantification of Pex13-mGFP levels in WT and *ubr2* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B). Asterisks denote statistically significant increases in protein levels compared to those in WT samples (* $P < 0.05$, ** $P < 0.01$).

C Quantification of Pex14p levels in WT and *ubr2* cells expressing Pex13-mGFP. Protein levels were normalized to the loading control (Pyc1p) at the corresponding time point and to the protein levels at T0. Values represent the mean $\pm$ SD of three independent experiments. Values of WT were same as in (Figure 1B).

Discussion

Pex13p is a PMP and member of the peroxisomal docking complex which is required for MAT import, although its actual role in the import process is still unclear. Pex13p has a relatively short half-life in the yeast *H. polymorpha* and it is degraded via the UPS in a Pex2p-dependent manner (Chen et al, 2018). In addition *Arabidopsis* Pex13p can be degraded by the UPS, in a process involving the RING E3 Ligase SP1 (Pan et al, 2016). Together, these reports suggest that UPS-mediated Pex13p degradation is a conserved process, which led us to investigate Pex13p degradation in the yeast *S. cerevisiae*. Our data clearly establish that Pex13p is degraded in *S. cerevisiae* while additionally demonstrating that Pex13p degradation likely proceeds via a similar UPS-mediated mechanism to that in *H. polymorpha*. Although the function of Pex13p degradation remains unclear (see Chapter 3 of this thesis), the fact that it has been shown to occur in three different organisms strongly suggests that Pex13p degradation is fundamental to peroxisomes. This poses the question as to whether Pex13p degradation also occurs in humans and if so, what would be the impact of blocking Pex13p degradation on human health? Mutations in Pex2p, Pex10p or Pex12p have been reported in patients suffering from peroxisome biogenesis disorders (Gootjes et al, 2004a; Gootjes et al, 2004b; Warren et al, 2000). In many cases the RING E3 complex members displayed reduced activity or loss of function, which resulted in defects in peroxisomal MAT import (Krause et al, 2006). It is probable that many of the defects exhibited by these patients are caused by inhibitions to Pex5p recycling. Nevertheless, because the peroxisomal E3 ligases are clearly involved in Pex13p degradation, it is feasible that some of the effects displayed by patients with reduced peroxisomal E3 ligase activity may stem from blocking Pex13p degradation.

Cdc48p is an AAA-ATPase involved in protein degradation and it is able to extract ubiquitinated substrates from the ER and mitochondrial membranes and target them to the proteasome for degradation (Wolf & Stolz, 2012). However, since our data establish that Cdc48p function is not required for Pex13p degradation, it remains unknown how ubiquitinated Pex13p may target to the proteasome. One possibility is that another AAA-ATPase regulates the transport of Pex13p towards the proteasome. Pex1p and Pex6p are two such ATPases that form a complex and can extract ubiquitinated Pex5p from the peroxisomal membrane (Platta et al, 2008), although Pex3p degradation in *H. polymorpha* did not require Pex1p (Williams & van der Klei, 2013). This may argue against a role for the Pex1p/Pex6p complex in Pex13p degradation. Similarly the membrane bound AAA-ATPase Msp1p, which was reported to extract tail anchored proteins out of the peroxisomal membrane for degradation (Weir et al, 2017), is also a potential candidate to fulfil this function. Another possibility is that the cytosolic proteasome approaches ubiquitinated Pex13p while still at the peroxisomal membrane. The

proteasome can associate with the ER membrane (Lipson et al, 2008; Mayer et al, 1998), which led Mayer *et al.* to propose that dislocation and degradation are coupled (Mayer et al, 1998). They further proposed that Cdc48p and Rpt4p (a subunit of 19S regulatory particle of the proteasome) might work in parallel, due to their structural (both are hexameric AAA-ATPases) and functional (both can bind ubiquitin conjugates) similarities (Dai & Li, 2001; Elsasser & Finley, 2005; Lam et al, 2002). In such a model, the proteasome may not require the action of an additional AAA-ATPase to facilitate Pex13p degradation. Clearly further work is needed to investigate how ubiquitinated Pex13p is extracted from the peroxisomal membrane.

Using a tandem fluorescent timer (tFT) and high throughput screening approach, we observed that, in addition to in cells lacking members of the peroxisomal ubiquitination machinery, Pex13-tFT stability was also increased in cells lacking Ufd4p, Ubc4p or Ubr2p. Furthermore the steady state levels of Pex13-mGFP was increased while Pex13-mGFP degradation was reduced in each of these deletion strains. Together these data strongly suggest that Ufd4p, Ubr2p and Ubc4p play a role in Pex13-mGFP degradation. This raises the question what is the relationship between the different E2s and E3s that play a role in Pex13p degradation? One possibility is that several pathways act in parallel on Pex13p. One pathway may rely on the peroxisomal ubiquitination machinery while another may require Ubc4p as E2 and Ufd4p or Ubr2p as E3. Ubc4p is known to act as E2 for Ufd4p (Bao, 2015). However, another option is that all these factors act together in ubiquitinating Pex13p. Ufd4p can associate with the RING E3 ligase Ubr1p, which allows the rapid formation of poly-ubiquitin chains on substrates (Hwang et al, 2010). Perhaps this is also the case for Pex13p, with Ufd4p, Ubr2p and Ubc4p “joining forces” with Pex4p and the peroxisomal E3 ligases to promote the rapid formation of poly-ubiquitinated Pex13p, to facilitate its degradation. Nevertheless, deletion of *PEX2* or *PEX4* has a dramatic effect on Pex13-mGFP degradation (Figure 2) whereas deletion of *UFD4*, *UBR2* or *UBC4* has a smaller impact on Pex13-mGFP degradation (Figure 7-10). These data could suggest that the peroxisomal ubiquitination machinery is the major player in Pex13p degradation, while the cytosolic factors Ufd4p, Ubc4p and Ubr2p play a more minor role (Figure 11). However, further investigations are required, including the potential roles of the other factors identified in our tFT analysis in Pex13p degradation.

In summary, our results demonstrate that Pex13p degradation is a general process conserved across different organisms yet occurring likely via similar mechanisms while they also identify roles for additional, cytosolic E2s and E3s in Pex13p degradation. However, further study is required before the mechanisms that underlie Pex13p degradation become clear.

Figure 11. A schematic model of Pex13p degradation.

The ubiquitination machinery at the peroxisomal membrane, including Pex4p (E2) and RING complex Pex2p/ Pex10p/ Pex12p (E3), plays a major role in Pex13p ubiquitination. The cytosolic factors Ufd4p, Ubc4p and Ubr2p play a more minor role, possibly involved in a later step of poly-ubiquitination chain formation.

Materials and Methods

Molecular techniques and construction of *S. cerevisiae* strains

S. cerevisiae strains used in this study are listed in Table 1. Strains used in tFT analysis were constructed as described previously (Khmelninskii et al, 2014). The plasmids and primers used in this study are listed in Table 2 and 3 respectively. Phusion DNA polymerase (Thermo Scientific) was used to produce gene fragments.

Competent cells of *S. cerevisiae* were prepared as follows: Cells were inoculated in 20 mL YPD liquid media and incubated with shaking of 200 rpm overnight at 30°C. The OD 600 of the overnight culture was measured and cells were diluted to 0.25 in 50 mL YPD. The cells were harvested at 5000 rpm for 5 min when the OD 600 reached 0.8~ 1.0. Cells were washed twice with sterile water, once with 5 mL of 1 M Sorbitol, and suspended in 300 µL of 1 M Sorbitol. The competent cells were aliquoted into 50 µL and frozen at -80°C.

For *S. cerevisiae* transformation, 15 µL of PCR product, 40 µL of denatured salmon sperm DNA (100°C for 10 min, and immediately cooled on ice-water) and 300 µL of PEG/ LiAc/ TE solution (0.8 mL 50% PEG-3350, 0.1 mL 100 mM Tris-HCl pH7.5 with 10 mM EDTA, 0.1 mL 1M Lithium acetate) were mixed with 50 µL of cells and shaken for 30 min at 30°C. Cells were mixed with 40 µL DMSO and heat shocked for 15 min at 42°C. Cells were cooled on ice for 1 min and washed once with 1mL YPD. Cells were finally resuspended in 5 mL YPD, shaken for 2~ 3 hr at 30°C and plated on either YPD plates containing appropriate antibiotics

or, for cells expressing Ub-K48R, on YND plates without Uracil. Plates were grown at 30°C for 2 to 3 days.

The Pex13-mGFP fragment was prepared as follows: The plasmid pHIPZ-Pex13-mGFP containing *H. polymorpha* Pex13-mGFP and Zeocin resistance gene was used as the template. The Zeocin fragment together with its promoter and terminator was obtained by PCR using the primers ScPex13-mGFP-F and Tcyc1-dnScP13-R, resulting in a product of 2240 bp with homologous region of upstream and downstream to PEX13 at both ends. This PCR-based fragment was used for transformation to obtain strains expressing Pex13-mGFP.

To produce the Pex3-mKate fragment, the plasmid pHIPH-Pex14-mKate2 containing *H. polymorpha* Pex14-mKate2 and Hygromycin resistance gene was used as template. The Hygromycin fragment, together with its promoter and terminator, was obtained by PCR using the primers ScPex3-mKate-F_SRI and ScPex3-mKate-R_SRI, resulting in a product of 2858 bp with homologous region of upstream and downstream to PEX3. This fragment was transformed into yeast, to obtain strains expressing Pex3-mKate.

The Cdc5-HA₆ fragment was prepared as follows: A HA₆ fragment and Hygromycin cassette, together with its promoter and terminator sequences, was obtained by PCR using the primers ScCdc5-HA6-F and HYG-dnCdc5-R and the plasmid pHIPH-AID-HA6 as template. The resulting 1724 bp product, containing sequences homologous to the upstream and downstream regions of *S. cerevisiae* CDC5, was used for transformation, to obtain a *cdc48-3* strain expressing Cdc5-HA₆.

The episomal plasmid Yep-Pcup1-myc-Ub-K48R has myc-Ub-K48R cassette under control of Copper promoter (Pcup1-overexpression promoter), with URA3 marker for selection in yeast. Yeast episomal plasmids, unlike genome integration, are high copy plasmids and remain free in the cell. PCR of CUP1 promoter was performed on the plasmid pCGCN-FAA4-mGFP using primers NotI-CUP1-F and CUP1-BamHI-R, resulting a fragment of 347 bp. The fragment was further digested with *NotI* and *BamHI*, and cloned into *NotI*/*BamHI* digested pRDV2 vector, generating pHIPH-Pcup1-myc-Ub-K48R. PCR was performed on the plasmid pHIPH-Pcup1-myc-Ub-K48R with primers NotI-CUP1-F and UbKR-SacI-R, resulting a 632 bp fragment which was digested with *NotI* and *SacI* and cloned into *NotI*/*SacI* digested pRG226 (ADDGENE), producing a plasmid of 5979 bp, termed as Yep-Pcup1-myc-Ub-K48R.

PEX11 and *PEX13* were endogenously and seamlessly tagged by PCR targeting with tFT as previously described (Khmelninskii et al, 2014; Khmelninskii et al, 2011).

Strains and growth conditions

The *S. cerevisiae* strains used in this study are derivatives of BY4742 or CB199 and are listed in Table S1. All integrations were confirmed by colony PCR using Phire Hot Start II (Thermo Scientific). Strains containing Pex13-mGFP were further checked by fluorescence microscopy

and Western blotting and strains containing Pex3-mKate were further checked by fluorescence microscopy.

Yeast transformants were selected on YPD plates containing 2% agar and 100 µg/ml Zeocin (Invitrogen) or 300 µg/ml Hygromycin (Invitrogen) or on YND plates (6.7g/L Yeast Nitrogen base w/o Amino acids (DIFCO), 5g/L Glucose (BOOM B.V.)) containing 2% agar, for the Ub-K48R strain. The *E. coli* strain DH5α was used for cloning purposes. *E. coli* cells were grown in LB supplemented with 100 µg/ml Ampicillin at 37 °C. For selection of auxotrophic transformants, selective minimal medium was supplemented with 2% glucose and the required amino acids mixture. Cycloheximide (CHX) when used, was added to a final concentration of 6 mg/ml.

All *S. cerevisiae* liquid cultures were grown while shaking at 200 rpm. *S. cerevisiae* cells for TCA lysates, CHX assays and fluorescence microscopy were grown on YM2 media (6.7g/L Yeast Nitrogen base w/o Amino acids (DIFCO), 10g/L Casein hydrolysate (Sigma), 0.06g/L Uracil (Sigma) and 0.06g/L L-Tryptophan (Sigma)). Cells were first grown on YM2 supplemented with 2% glucose at 30°C overnight, then transfer to YM2 plus 0.3% glucose at 2pm on day-2 and grown at 30°C for 8 hr. Finally cells were transferred to YM2 plus 0.1% oleic acid (Sigma), 0.1% glucose and 0.05% Tween-80 (YM2O) and grown at 30°C till an OD600 of around 1.0 (~10-12hr), after which cells were either harvested for TCA lysates or CHX/fluorescence microscopy experiments were performed.

For experiments using the Cdc48 temperature sensitive strain *cdc48-3*, cells expressing Pex13-mGFP were grown on YM2 plus 2% glucose at 23°C overnight, then at 10am on day-2 cells were transferred to YM2 plus 0.3% glucose and grown at 23°C for 12hr. Finally, cells were transferred to YM2O media and grown further at 23°C. After 17hrs of growth, cells were split into two groups, those for CHX treatment at the permissive (23°C) temperature and those for CHX treatment at the restrictive (37°C) temperature. For permissive-temperature growth, cells were grown on YM2O media for a further 1hr at 23°C and treated with DMSO or CHX. For restrictive temperature growth, cells were shifted to 37°C for 1 hr, treated with DMSO or CHX and grown further at 37°C.

Preparation of yeasts TCA lysates

Cell extracts of TCA-treated cells were prepared for SDS-PAGE as detailed previously (Baerends et al, 2000). Three OD₆₀₀ units of cells from each culture (at each time point) were taken for TCA lysis so that the amount of cells is constant, and after TCA lysis, equal volume (10µL) of each sample was loaded per lane and blots were probed with rabbit polyclonal antisera raised against the Myc tag (Santa Cruz Biotech, sc-789), Pex14p (Bottger et al, 2000), or pyruvate carboxylase 1 (Pyc1p) (Fahimi et al, 1993) or mouse monoclonal antisera raised against HA tag (Sigma, H3663) or mGFP (Santa Cruz Biotech, sc-9996). Secondary goat anti-

rabbit (31460) or goat anti-mouse (31430) antibodies conjugated to horseradish peroxidase (Thermo Fisher Scientific) were used for detection. Pyc1p was used as a loading control.

Quantification of Western blots

Blots were scanned by using a densitometer (GS-710; Bio-Rad Laboratories) and protein levels were quantified using Image Studio Lite Ver5.2 software (LI-COR Biosciences). In the case of Pex14p blots, both the phosphorylated and unphosphorylated forms were included in the calculation if both forms were visible. The value obtained for each band was normalized by dividing it by the value of the corresponding Pyc1p band (loading control). For comparison of absolute protein levels (Figures 2 and 4), normalized values obtained for Pex13p and Pex14p levels in WT cells were set to 1 and the levels of these proteins in mutant cells are displayed relative to WT. For CHX experiments (Figures 1, 5, 6 and 7), the normalized values of T0 samples were set to 1.0 and values obtained from the T1-T3 samples are displayed as a fraction of T0 values. Standard deviations were calculated using Excel. * represents P-values < 0.05, ** represents P-values < 0.01 and *** represents P-values < 0.001. The data presented are derived from three independent experiments.

Fluorescence Microscopy

All fluorescence microscopy images were acquired using a 100×1.30 NA Plan-Neofluar objective (Carl Zeiss). Wide-field microscopy images were captured by an inverted microscope (Axio Scope A1, Carl Zeiss) using Micro-Manager software and a digital camera (CoolSNAP HQ²; Photometrics). GFP signal was visualized with a 470/440-nm band pass excitation filter, a 495-nm dichromatic mirror, and a 525/550-nm band pass emission filter. mKate signal was visualized with a 587/525-nm band pass excitation filter, a 605-nm dichromatic mirror, and a 647/670-nm band-pass emission filter.

For images taken of Pex13-mGFP in WT grown on oleic acid containing medium, the optimal settings were mGFP (290, 650) and mKate2 (225, 600), and in *pex2*, the optimal settings mGFP (290, 2900) and mKate2 (225, 630) were applied for processing. The general settings used to compare the signal of Pex13-mGFP in WT and *pex2*, mGFP (300, 2000) and mKate2 (225, 620) were applied for processing.

For quantification of the Pex13-mGFP signal in WT or *pex2* cells, a rectangular area was drawn using the “rectangular tool” from ImageJ (Abramoff et al, 2004) to envelope the region containing the Pex13-mGFP spot and pixel intensity inside the area was measured. The measured maximum fluorescence intensity of GFP on peroxisomes was corrected for the background intensity and a box plot was made using Microsoft Excel. The box represents values from the 25 percentile to the 75 percentile; the horizontal line through the box represents the median value. Whiskers indicate maximum and minimum values.

Spot assay

Yeast strains were grown overnight at 30 °C on YM2 medium containing 2% glucose and shifted to YM2 medium plus 0.3% glucose. After 6h of growth in YM2 plus 0.3% glucose, cells were diluted to 0.1 OD and ten-fold serial dilutions were made up to 0.0001 OD. From every dilution, 3 µL yeast cells were spotted onto oleate plates containing 6.7 g/L YNB, 1 g/L SCSM–LEU dropout mix (Hopkins mixture, FOR MEDIUM), 180 mg/L leucine, 0.1% oleate, 0.05% Tween and 2% agar was added. Plates were incubated at 30°C for 7 before imaging.

Tandem fluorescent timer (tFT) analysis

The tFT analysis in this study is mainly based on previous established tFT technique, and the following procedure is adapted from the detailed protocols already published (Khmelinskii et al, 2014; Khmelinskii et al, 2012). Chromosomal gene tagging and gene deletion were performed using standard procedures based on PCR targeting as previously described (Janke et al, 2004). Gene deletion and gene tagging were validated by PCR. Expression of Pex13p fused to tFT tag which consists of two fluorescent proteins mCherry and sfGFP was validated using immunoblotting and confirmed with fluorescence microscopy.

Pex13-tFT was crossed using the synthetic genetic array method (Tong & Boone, 2006) with a yeast strain library which consisted of 152 strains that either lacked a gene involved in protein degradation or which contained a mutant version of a protein involved in protein degradation (in case the deletion was lethal), resulting in a library expressing Pex13-tFT in each mutant. All the colonies used for imaging were prepared fresh every time. WT and mutant strains expressing Pex13-tFT were first grown on YPD plates with 2% agar at 30 °C. From agar plates, they were pinned together (Singer Instruments) on a single plate for mating and diploid selection. Colonies were then plated on sporulation plates (Potassium Acetate (Sigma) 20g/L, agar 2%). After sporulation, yeast strains expressing Pex13-tFT fusions were grown at 30 °C in synthetic complete medium (yeast nitrogen base with amino acid supplements, Sigma) (Khmelinskii et al, 2012) with 0.1% glucose, 0.1% oleic acid, 0.5% tween and 2% agar.

The tFT library used in this study consists of one WT strain without Pex13-tFT (used to correct the background signal), one WT with Pex13-tFT fluorescent fusion protein (used as negative control), and 152 mutants in each of which a gene from UPS system is disrupted either by deletion for non-essential genes or by mutations for essential genes. Each plate had 1536 colonies, including Pex13-tFT crossed with UPS mutants, non-functional fluorescent protein crossed with UPS mutants used for background correction, and Pex13-tFT crossed with WT as control, with four technical replicates for each strain. Strains were grown for 7 days, and images were taken every day. The plates were imaged with an M1000 Pro plate reader equipped with automatic plate loading stackers (Tecan) and custom temperature control chambers. Measurements were taken with 10 flashes each for sfGFP (488/10nm excitation, 510/10 nm emission) and mCherry (587/10 nm excitation, 610/10 nm emission).

The mCherry/ sfGFP ratio of each mutant was the average of four colonies of the same strain. For the further calculation of Z-score (see below), ratios of fluorescent intensities were normalized to the WT expressing Pex13-tFT on the same plate on the same day, and the ratio in WT expressing Pex13-tFT was set to 1.

A Z-score, also known as a standard score, indicates how many standard deviations an element is from the mean. A Z-score can be calculated from the formula: $Z = (X - \mu) / \sigma$. In the formula, Z is the Z-score, X is the value of the element (the average of four mCherry/ sfGFP intensity ratios of one strain on the plate on the same day), μ is the population mean (the average of all ratios on the same plate on the same day), and σ is the standard deviation (the standard deviation of all ratios on the same plate on the same day). The mCherry and sfGFP fluorescence intensities were measured for colonies from each strain on each day and the mCherry/sfGFP ratio was calculated and used to determine the Z-score, the deviation of the ratio for a particular strain on a particular day compared to the average ratio across all strains on that day. Increases in Z-score are colour coded, ranging from 1.0 or less (green) to 5.0 (red). The data for each square in the heat map are derived from four technical replicates. We applied a cut-off of 1 to screen out most of the background. As for the interpretation of Z-scores, a Z-score less than 0 represents an element less than the mean and a Z-score greater than 0 represents an element greater than the mean. A Z-score equal to 1 represents an element that is one standard deviation greater than the mean while -1 represents one standard deviation less than the mean.

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Table 1. *S. cerevisiae* strains used in this study

Strain	Description	Reference
WT	The SGA entry strain Y8205 (MAT α <i>can1</i> Δ :: <i>STE2pr-SpHIS5</i> <i>hyp1</i> Δ :: <i>STE3pr-LEU2</i> <i>his3</i> Δ 1 <i>leu2</i> Δ 0 <i>met15</i> Δ 0 <i>ura3</i> Δ 0) with the genetic elements (nat ^R) required for seamless protein tagging, generating the library background strain yMaM330.	(Khmelinskii et al, 2014) (Knop lab)
Pex11-tFT	<i>yMaM330, PEX11::mCherry-I-SceI</i> site- <i>SpCYC1</i> term- <i>ScURA3-I-SceI</i> site- <i>mCherryAN-sfGFP</i>	Knop lab
Pex13-tFT	<i>yMaM330, PEX13::mCherry-I-SceI</i> site- <i>SpCYC1</i> term- <i>ScURA3-I-SceI</i> site- <i>mCherryAN-sfGFP</i>	Knop lab
WT Pex13-mGFP	<i>Sc</i> WT with Pex13-mGFP (zeo ^R)	This study
WT Pex13-mGFP+ Pex3-mKate	<i>Sc</i> WT with Pex13-mGFP (zeo ^R) and Pex3-mKate (hyg ^R)	This study
WT myc-Ub-K48R+ Pex13-mGFP	<i>Sc</i> WT with myc tagged Ub-K8R (URA) and Pex13-mGFP (zeo ^R)	This study
BY4741	<i>MATa his3</i> Δ 1 <i>leu2</i> Δ 0 <i>met15</i> Δ 0 <i>ura3</i> Δ 0	(Khmelinskii et al, 2014) (Knop lab)
UPS deletion library	BY4741, <i>goi deletions::kanMX</i>	(Khmelinskii et al, 2014) (Knop lab)
<i>pex2</i>	<i>Sc</i> WT cell deleted <i>PEX2</i> (BY4741, <i>pex2::kanMX</i>)	Knop lab
<i>pex2</i> + Pex13-mGFP	<i>pex2</i> with Pex13-mGFP (zeo ^R)	This study
<i>pex2</i> + Pex13-mGFP+ Pex3-mKate	<i>pex2</i> with Pex13-mGFP (zeo ^R) and Pex3-mKate (hyg ^R)	This study
<i>pex4</i>	<i>Sc</i> WT cell deleted <i>PEX4</i> (BY4741, <i>pex4::kanMX</i>)	Knop lab
<i>pex4</i> + Pex13-mGFP	<i>pex4</i> with Pex13-mGFP (zeo ^R)	This study
<i>ufd4</i>	<i>Sc</i> WT cell deleted <i>UFD4</i> (BY4741, <i>ufd4::kanMX</i>)	Knop lab
<i>ufd4</i> + Pex13-mGFP	<i>ufd4</i> with Pex13-mGFP (zeo ^R)	This study
<i>ubc4</i>	<i>Sc</i> WT cell deleted <i>UBC4</i> (BY4741, <i>ubc4::kanMX</i>)	Knop lab
<i>ubc4</i> + Pex13-mGFP	<i>ubc4</i> with Pex13-mGFP (zeo ^R)	This study
<i>atg12</i>	<i>Sc</i> WT cell deleted <i>ATG12</i> (BY4741, <i>atg12::kanMX</i>)	Knop lab
<i>atg12</i> + Pex13-mGFP	<i>atg12</i> with Pex13-mGFP (zeo ^R)	This study
<i>ubr2</i>	<i>Sc</i> WT cell deleted <i>UBR2</i> (BY4741, <i>ubr2::kanMX</i>)	Knop lab

<i>ubr2</i> + Pex13-mGFP	<i>ubr2</i> with Pex13-mGFP (zeo ^R)	This study
<i>cdc48-3</i>	Temperature sensitive (ts) mutant <i>cdc48-3</i> contains a heat-sensitive allele of CDC48, with permissive (23°C) or the restrictive temperature (37°C)	(Cao et al, 2003)
<i>cdc48-3</i> + Pex13-mGFP	<i>cdc48-3</i> with Pex13-mGFP (zeo ^R)	This study
<i>cdc48-3</i> + Cdc5-HA ₆	<i>cdc48-3</i> expressing Cdc5 with HA ₆ tag fused to its C-terminus, hyg ^R	This study

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pHIPZ-Pex13-mGFP	C-terminal part of Pex13 fused with mGFP, zeo ^R ; amp ^R	(Knoops et al, 2014)
pHIPH-Pex14-mKate2	Plasmid containing the C-terminal region of <i>H. polymorpha</i> PEX14 fused to mKate2; hyg ^R ; amp ^R	(Chen et al, 2018)
Yep Pcup1-myc-Ub-K48R	Yeast episomal plasmid (URA) containing Ub-K48R fused to myc tag on its N-terminus, under control of Copper ion inducible promoter	This study
pCGCN-FAA4-mGFP	Plasmid for <i>S. cerevisiae</i> containing Pcup1, FAA4, mGFP, nat ^R , amp ^R	(Saraya et al, 2010)
pRDV2	Myc tagged ubiquitin mutate (Ub-K48R) under control of DHAS promoter, zeo ^R ; amp ^R	(Williams & van der Klei, 2013)
pHIPH-Pcup1-Myc-Ub-K48R	Plasmid containing myc tagged Ub-K48R under control of CUP1 promoter, hyg ^R ; amp ^R	This study
pRG226	The episomal <i>E. coli</i> / <i>S. cerevisiae</i> shuttle vector (empty backbone), URA ; amp ^R	(Gnügge et al, 2016)
pHIPH-AID-HA6	Plasmid containing Auxin Inducible Degron (AID) with 6xHA tag at its C-terminus, hyg ^R ; amp ^R	(Morawska & Ulrich, 2013)

Table 3. Primers used in this study

Primer	Sequence	Description
ScPex3-mKate-F_SRI	AGCGCCAGCGTATACAGC AACTTTGGCGTCTCCAGCT CGTTTTCTTCAAGCCTAT GGTTTCTGAACTCATCAAG GA	To clone a fragment of 2858 bp containing 3' end of <i>S. cerevisiae</i> <i>PEX3</i> fused to mKate with Hygromycin resistance, forward primer, used to make strain Pex3-mKate
ScPex3-mKate-R_SRI	TACGCTATATATATATATA TTCTGGTGTGAGTGTCAAGT ACTTATTCAGAGATTACGT TTTCGACACTGGATGGCG GC	To clone a fragment of 2858 bp containing 3' end of <i>S. cerevisiae</i> <i>PEX3</i> fused to mKate Hygromycin resistance, reverse primer, used to make strain Pex3-mKate
Pex3mKate-cPCR-F1	GGCAGCGTGAACGAATAC	To check the positive colonies containing Pex3-mKate in the colony PCR, forward primer
Pex3mKate-cPCR-R1	CTAGCCACTGCCACTTCG	To check the positive colonies containing Pex3-mKate in the colony PCR, reverse primer
ScPex13-mGFP-F	TAAAAAGACGGAAGAAAA TTGAGCATGTTGATGATGA AACGCGTACACACAGATC TGTGAGCAAGGGC	To clone a fragment of 2240 bp containing 3' end of <i>S. cerevisiae</i> <i>PEX13</i> fused to mGFP with Zeocin resistance, forward primer, used to make strain Pex13-mGFP
Tcyc1-dnScP13-R	TAGATTTTACTATATATAT ATGCGAATATATGTGTGC AAATATTGATGCACTGTAC AGAAAAAAGAAAAATT TG	To clone a fragment of 2240 bp containing 3' end of <i>S. cerevisiae</i> <i>PEX13</i> fused to mGFP with Zeocin resistance, reverse primer, used to make strain Pex13-mGFP
ScPex13-F	TACGGTGCAGGAGCG	To check the positive colonies containing Pex13-mGFP in the colony PCR, forward primer
mGFP-reverse_SRI	AAGTCGTGCTGCTTCATGT G	To check the positive colonies containing Pex13-mGFP in the colony PCR, reverse primer
NotI-CUP1-F	GCATGCGCCGCCCTTTA TTTCAGGCTGAT	To clone the 347 bp of CUP1 promoter, forward primer
CUP1-BamHI-R	GTGCGGATCCTTTATGTGA TGATTGATTGATTGAT	To clone the 347 bp of CUP1 promoter, reverse primer
UbKR-SacI-R	GTGCGAGCTCTCAACCAC CTCTTAGTCTTAAG	To clone a 632 bp fragment containing Pcup1 and myc-Ub- K48R, reverse primer

ScCdc5-HA6-F	CTTTGATAAAGGAAGGTTT GAAGCAGAAGTCCACAAT TGTTACCGTAGATTACCCA TACGATGTTCTGACTATG C	To clone a fragment of 1724 bp containing 3' end of <i>S. cerevisiae CDC5</i> fused to HA ₆ tag with Hygromycin resistance, forward primer, used to make strain Cdc5-HA ₆ , forward primer
HYG-dnCdc5-R	CAATGGACTGGTAATTTTCG TATTCGTATTTCTTTCTACT TTAATATTGGTTCGAGATT ATTCCTTTGCCCTCGG	To clone a fragment of 1724 bp containing 3' end of <i>S. cerevisiae CDC5</i> fused to HA ₆ tag with Hygromycin resistance, forward primer, used to make strain Cdc5-HA ₆ , reverse primer

References

- Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image Processing with ImageJ. *Biophotonics International* **11**: 36-42
- Baerends RJ, Faber KN, Kram AM, Kiel JA, van der Klei IJ, Veenhuis M (2000) A stretch of positively charged amino acids at the N terminus of *Hansenula polymorpha* Pex3p is involved in incorporation of the protein into the peroxisomal membrane. *Journal of Biological Chemistry* **275**: 9986-9995
- Bao X, Johnson, J. L., & Rao, H. (2015) Rad25 is targeted for degradation by the Ubc4-Ufd4 pathway. *Journal of Biological Chemistry*
- Bottger G, Barnett P, Klein AT, Kragt A, Tabak HF, Distel B (2000) *Saccharomyces cerevisiae* PTS1 receptor Pex5p interacts with the SH3 domain of the peroxisomal membrane protein Pex13p in an unconventional, non-PXXP-related manner. *Molecular Biology of the Cell* **11**: 3963-3976
- Braverman N, Steel G, Obie C, Moser A, Moser H, Gould SJ, Valle D (1997) Human *PEX7* encodes the peroxisomal PTS2 receptor and is responsible for rhizomelic chondrodysplasia punctata. *Nature genetics* **15**: 369-376
- Cao K, Nakajima R, Meyer HH, Zheng Y (2003) The AAA-ATPase Cdc48/p97 regulates spindle disassembly at the end of mitosis. *Cell* **115**: 355-367
- Chen X, Devarajan S, Danda N, Williams C (2018) Insights into the role of the peroxisomal ubiquitination machinery in Pex13p degradation in the yeast *Hansenula polymorpha*. *Journal of molecular biology* **430**: 1545-1558
- Dai RM, Li CCH (2001) Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nature cell biology* **3**: 740
- Dargemont C, Ossareh-Nazari B (2012) Cdc48/p97, a key actor in the interplay between autophagy and ubiquitin/proteasome catabolic pathways. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **1823**: 138-144
- El Magraoui F, Brinkmeier R, Schrotter A, Girzalsky W, Muller T, Marcus K, Meyer HE, Erdmann R, Platta HW (2013) Distinct ubiquitination cascades act on the peroxisomal targeting signal type 2 co-receptor Pex18p. *Traffic* **14**: 1290-1301
- Elgersma Y, Kwast L, Klein A, Voorn-Brouwer T, van den Berg M, Metzger B, America T, Tabak HF, Distel B (1996) The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import PTS1-containing proteins. *The Journal of Cell Biology* **135**: 97-109
- Elsasser S, Finley D (2005) Delivery of ubiquitinated substrates to protein-unfolding machines. *Nature cell biology* **7**: 742
- Fahimi HD, Baumgart E, Vökl A (1993) Ultrastructural aspects of the biogenesis of peroxisomes in rat liver. *Biochimie* **75**: 201-208
- Finley D, Ulrich HD, Sommer T, Kaiser P (2012) The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. *Genetics* **192**: 319-360
- Gabalton T (2010) Peroxisome diversity and evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 765-773
- Galan JM, Wiederkehr A, Seol JH, Haguenaue-Tsapis R, Deshaies RJ, Riezman H, Peter M (2001) Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. *Molecular and cellular biology* **21**: 3105-3117
- Girzalsky W, Saffian D, Erdmann R (2010) Peroxisomal protein translocation. *Biochim Biophys Acta*

- Gnügge R, Liphardt T, Rudolf F (2016) A shuttle vector series for precise genetic engineering of *Saccharomyces cerevisiae*. *Yeast* **33**: 83-98
- Gootjes J, Elpeleg O, Eyskens F, Mandel H, Mitanchez D, Shimozawa N, ..., Wanders RJ (2004a) Novel mutations in the PEX2 gene of four unrelated patients with a peroxisome biogenesis disorder. *Pediatric research* **55**: 431
- Gootjes J, Schmohl F, Waterham HR, Wanders RJ (2004b) Novel mutations in the PEX12 gene of patients with a peroxisome biogenesis disorder. *European journal of human genetics* **12**: 115
- Hanna J, Hathaway NA, Tone Y, Crosas B, Elsasser S, Kirkpatrick DS, ..., Finley D (2006) Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. *Cell* **127**: 99-111
- Hershko A (1996) Lessons from the discovery of the ubiquitin system. *Trends in biochemical sciences* **21**: 445-449
- Hershko A, Ciechanover A (1998) The ubiquitin system for protein degradation. *Annual Review of Biochemistry* **67**: 425-479
- Hwang CS, Shemorry A, Auerbach D, Varshavsky A (2010) The N-end rule pathway is mediated by a complex of the RING-type Ubr1 and HECT-type Ufd4 ubiquitin ligases. *Nature cell biology* **12**: 1177
- Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, ..., Knop M (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**: 947-962
- Johnson MA, Snyder WB, Cereghino JL, Veenhuis M, Subramani S, Cregg JM (2001) Pichia pastoris Pex14p, a phosphorylated peroxisomal membrane protein, is part of a PTS-receptor docking complex and interacts with many peroxins. *Yeast* **18**: 621-641.
- Khmelinskii A, Blaszczyk E, Pantazopoulou M, Fischer B, Omnis DJ, Le Dez G, ..., Kirmmaier D (2014) Protein quality control at the inner nuclear membrane. *Nature* **516**: 410
- Khmelinskii A, Keller PJ, Bartosik A, Meurer M, Barry JD, Mardin BR, Kaufmann A, Trautmann S, Wachsmuth M, Pereira G, Huber W, Schiebel E, Knop M (2012) Tandem fluorescent protein timers for in vivo analysis of protein dynamics. *Nature biotechnology* **30**: 708-714
- Khmelinskii A, Meurer M, Duishoev N, Delhomme N, Knop M (2011) Seamless gene tagging by endonuclease-driven homologous recombination. *PLoS One* **6**: e23794
- Khmelinskii A, Meurer M, Ho CT, Besenbeck B, Füller J, Lemberg MK, ..., Knop M (2016) Incomplete proteasomal degradation of green fluorescent proteins in the context of tandem fluorescent protein timers. *Molecular biology of the cell* **27**: 360-370
- Knoops K, Manivannan S, Cepinska MN, Krikken A, Kram AM, Veenhuis M, Van der Klei IJ (2014) Preperoxisomal vesicles can form in the absence of Pex3p. *J Cell Biol*
- Krause C, Rosewich H, Thanos M, Gärtner J (2006) Identification of novel mutations in PEX2, PEX6, PEX10, PEX12, and PEX13 in Zellweger spectrum patients. *Human mutation* **27**: 1157
- Lam YA, Lawson TG, Velayutham M, Zweier JL, Pickart CM (2002) A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature* **416**: 763
- Ling Q, Li N, Jarvis P (2017) Chloroplast ubiquitin E3 ligase SP1: does it really function in peroxisomes? *Plant physiology* **175**: 586-588
- Lipson C, Alalouf G, Bajorek M, Rabinovich E, Atir-Lande A, Glickman M, Bar-Nun S (2008) A proteasomal ATPase contributes to dislocation of endoplasmic reticulum-associated degradation (ERAD) substrates. *Journal of Biological Chemistry* **283**: 7166-7175
- Lockshon D, Surface LE, Kerr EO, Kaerberlein M, Kennedy BK (2007) The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. *Genetics* **175**: 77-91

- Mayer TU, Braun T, Jentsch S (1998) Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein. *The EMBO Journal* **17**: 3251-3257
- Miller HI, Henzel WJ, Ridgway JB, Kuang WJ, Chisholm V, Liu CC (1989) Cloning and expression of a yeast ubiquitin-protein cleaving activity in *Escherichia coli*. *Nature biotechnology* **7**: 698
- Morawska M, Ulrich HD (2013) An expanded tool kit for the auxin-inducible degron system in budding yeast. *Yeast* **30**: 341-351
- Mukai S, Ghaedi K, Fujiki Y (2002) Intracellular localization, function, and dysfunction of the peroxisome-targeting signal type 2 receptor, Pex7p, in mammalian cells. *Journal of Biological Chemistry* **277**: 9548-9561.
- Nillegoda NB, Theodoraki MA, Mandal AK, Mayo KJ, Ren HY, Sultana R, ..., Caplan AJ (2010) Ubr1 and Ubr2 function in a quality control pathway for degradation of unfolded cytosolic proteins. *Molecular biology of the cell* **21**: 2102-2116
- Pan R, Hu J (2018) The Arabidopsis E3 ubiquitin ligase SP1 targets to chloroplasts, peroxisomes, and mitochondria. *Plant physiology* **176**: 480-482
- Pan R, Satkovich J, Hu J (2016) E3 ubiquitin ligase SP1 regulates peroxisome biogenesis in Arabidopsis. *Proceedings of the National Academy of Sciences* **113**: E7307-E7316
- Platta HW, Debelyy MO, El Magraoui F, Erdmann R (2008) The AAA peroxins Pex1p and Pex6p function as dislocases for the ubiquitinated peroxisomal import receptor Pex5p. *Biochemical Society Transactions* **36**: 99-104
- Platta HW, El Magraoui F, Schlee D, Grunau S, Girzalsky W, Erdmann R (2007) Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling. *The Journal of cell biology* **177**: 197-204
- Ravid T, Hochstrasser M (2007) Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. *Nature cell biology* **9**: 422
- Rodriguez MS, Wright, J., Thompson, J., Thomas, D., Baleux, F., Virelizier, J. L., ... & Arenzana-Seisdedos, F. (1996) Identification of lysine residues required for signal-induced ubiquitination and degradation of I kappa B-alpha in vivo. *Oncogene* **12**: 2425-2435
- Saleem RA, Long-O'Donnell R, Dilworth DJ, Armstrong AM, Jamakhandi AP, Wan Y, ..., Shmulevich I (2010) Genome-wide analysis of effectors of peroxisome biogenesis. *PLoS One* **5**: e11953
- Saraya R, Cepinska MN, Kiel JA, Veenhuis M, van der Klei IJ (2010) A conserved function for Inp2 in peroxisome inheritance. *Biochim Biophys Acta* **1803**: 617-622
- Scheffner M, Nuber U, Huibregtse JM (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373**: 81
- Seufert W, Jentsch S (1990) Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *The EMBO journal* **9**: 543-550
- Shani N, Watkins PA, Valle D (1995) PXA1, a possible *Saccharomyces cerevisiae* ortholog of the human adrenoleukodystrophy gene. *Proceedings of the National Academy of Sciences* **92**: 6012-6016
- Sichting M, Schell-Steven A, Prokisch H, Erdmann R, Rottensteiner H (2003) Pex7p and Pex20p of *Neurospora crassa* function together in PTS2-dependent protein import into peroxisomes. *Molecular biology of the cell* **14**: 810-821
- Theodoraki MA, Nillegoda NB, Saini J, Caplan AJ (2012) A network of ubiquitin ligases is important for the dynamics of misfolded protein aggregates in yeast. *Journal of Biological Chemistry* **jbc-M112**
- Thrower JS, Hoffman L, Rechsteiner M, Pickart CM (2000) Recognition of the polyubiquitin proteolytic signal. *The EMBO journal* **19**: 94-102
- Tong AHY, Boone C (2006) Synthetic genetic array analysis in *Saccharomyces cerevisiae*. In *Yeast Protocol*: 171-191

- Walker K, Skelton H, Smith K (2002) Cutaneous lesions showing giant yeast forms of *Blastomyces dermatitidis*. *Journal of cutaneous pathology* **29**: 616-618
- Wang X, Herr RA, Chua WJ, Lybarger L, Wiertz EJ, Hansen TH (2007) Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *The Journal of cell biology* **177**: 613-624
- Warren DS, Wolfe BD, Gould SJ (2000) Phenotype-genotype relationships in PEX10-deficient peroxisome biogenesis disorder patients. *Human mutation* **15**: 509-521
- Weir NR, Kamber RA, Martenson JS, Denic V (2017) The AAA protein Msp1 mediates clearance of excess tail-anchored proteins from the peroxisomal membrane. *eLife* **6**
- Wiederkehr A, Avaro S, Prescianotto-Baschong C, Haguenuer-Tsapis R, Riezman H (2000) The F-box protein Rcy1p is involved in endocytic membrane traffic and recycling out of an early endosome in *Saccharomyces cerevisiae*. *The Journal of cell biology* **149**: 397-410
- Williams C, van den Berg M, Geers E, Distel B (2008) Pex10p functions as an E3 ligase for the Ubc4p-dependent ubiquitination of Pex5p. *Biochemical and biophysical research communications* **374**: 620-624
- Williams C, van den Berg M, Sprenger RR, Distel B (2007) A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p. *Journal of Biological Chemistry* **282**: 22534-22543
- Williams C, van der Klei IJ (2013) Pexophagy-linked degradation of the peroxisomal membrane protein Pex3p involves the ubiquitin–proteasome system. *Biochem Biophys Res Commun* **438**: 395-401
- Wolf DH, Stolz A (2012) The Cdc48 machine in endoplasmic reticulum associated protein degradation. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **1823**: 117-124
- Yamanaka K, Sasagawa Y, Ogura T (2012) Recent advances in p97/VCP/Cdc48 cellular functions. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **1823**: 130-137

Summary and outlook

Peroxisomes are single membrane bound organelles that are present in nearly all eukaryotic cells. Common peroxisome functions include β -oxidation of fatty acids and detoxification of hydrogen peroxide however, they are also involved in a wide variety of metabolic process that vary depending on the organism, cell type and developmental stage. The functions of peroxisomes are largely determined by peroxisomal matrix proteins that are involved in peroxisomal metabolism and peroxisomal membrane proteins (PMPs), which are involved in peroxisome formation, fission and transport of proteins and small molecules into peroxisomes. Thus, peroxisomal proteins need to be tightly controlled through regulation of protein synthesis, targeting as well as protein degradation. Peroxisomal proteins may undergo degradation either because they are damaged/unfolded by various stress factors or because they are no longer needed. Generally, protein degradation needs to be tightly controlled in order to prevent the accumulation of unwanted proteins or occurrence of unwanted protein degradation events. While many efforts have been placed on studying the post-translational targeting of PMPs to peroxisomes, little is known about PMP degradation. Investigating how and why PMPs are degraded will allow us to better understand the role of PMP degradation in a cellular context.

Damaged and redundant membrane proteins from other organelles such as the ER and mitochondria are generally degraded by the Ubiquitin Proteasome System (UPS), which involves poly-ubiquitination of the substrate protein by a cascade of enzymes (E1, E2 and E3) followed by the degradation in the proteasome. Peroxisomes possess an E2 (Pex4p) and several E3 ligases (Pex2p, Pex10p and Pex12p), which are known to ubiquitinate the cycling receptor proteins Pex5p and Pex20p family members. Recently, this peroxisomal ubiquitination machinery was reported to be involved in the degradation of the PMPs Pex3p and Pex13p in *H. polymorpha*. In spite of these recent reports, questions remain, including which other PMPs undergo UPS mediated degradation, what are the functions of PMP degradation and whether all PMP degradation events share a common mechanism?

The research presented in this thesis provides insights into PMP degradation in *S. cerevisiae*. **Chapter 1** summarizes the current knowledge on how membrane proteins from the ER and mitochondria are recognized, extracted from the membrane and targeted for degradation by the ubiquitin proteasome system. Furthermore, we discuss what is currently known about PMP degradation, including possible functions of PMP turnover and how PMP degradation may be facilitated.

In **Chapter 2** we present our investigations into PMP stability in the yeast *S. cerevisiae* using the tandem fluorescent protein timer (tFT) approach. A tFT tag consists of two fluorescent proteins, in this case mCherry and sfGFP, which are fused to the protein of interest. The ratio of the fluorescent intensity of the two fluorescent proteins serves as an indicator for protein stability. We observed that the tFT approach can report on the stability of individual PMP-tFT fusions in cells, which varied from unstable (Pex13-tFT and Pxa1-tFT) to stable (Pex11-tFT).

The tFT tagged versions of Pex13p, Pxa1p and Pex11p were induced on oleate containing media and displayed peroxisomal-like localization. Furthermore, growth assays demonstrated that the functionality of Pex13p was not compromised by fusion to a tFT tag, whereas the functionality of Pxa1p was impaired by the tFT tag. Together, these data demonstrate that the tFT approach is valid to study PMP stability. The fact that the tFT tag impaired Pxa1p function demonstrates that attaching a tFT tag to a PMP may be used to deliberately destabilize PMPs, to investigate quality control events associated with that particular PMP. On the other hand Pex13-tFT, which retained its functionality, is rapidly degraded, indicating that tFT tags can also be effectively used for studying the targeted downregulation of PMPs. Either way, a strong point of the tFT approach is that it can be used to investigate PMP stability under different growth conditions (as was the case in this chapter) as well as to screen against mutant libraries, to identify factors that are involved in PMP degradation, as we have done in Chapters three and four.

Chapter 3 reports on quality control of the PMP Pxa1p in *S. cerevisiae*. Pxa1p is a yeast homolog of human adrenoleukodystrophy protein (ALDP) and mutations in ALDP, which often lead to protein instability, can result in the severe genetic disorder adrenoleukodystrophy (ALD). We selected two ALD causing mutations and generated the corresponding mutations in yeast Pxa1p (Pxa1^{MUT}), tagged to mGFP. We observed that Pxa1^{MUT}-mGFP is rapidly degraded while Pxa1-mGFP remained stable, establishing a role for quality control in Pxa1^{MUT}-mGFP turnover. Significantly, Pxa1^{MUT}-mGFP degradation was reduced upon proteasome inhibitor treatment, demonstrating that the proteasome is involved and further suggesting a role for ubiquitination in Pxa1^{MUT}-mGFP turnover. Our attempts to detect ubiquitinated forms of Pxa1^{MUT}-mGFP were unsuccessful, possibly because ubiquitinated Pxa1^{MUT}-mGFP is present at a low level or that ubiquitination of another protein and not Pxa1^{MUT}-mGFP may be required for Pxa1^{MUT}-mGFP degradation. Using the tFT approach, we observed that the cytosolic E3 ligase Ufd4p plays a major role in Pxa1^{MUT}-mGFP degradation. The observation that Pxa1^{MUT}-mGFP is stabilized on peroxisomes in *ufd4* cells suggest that Ufd4p dependent quality control of Pxa1^{MUT}-mGFP occurs at peroxisomes. Like Ufd4p, loss of the peroxisomal E3 ligase Pex2p also inhibited Pxa1^{MUT}-mGFP degradation. There could be two reasons to explain why multiple E3s are involved in Pxa1^{MUT}-mGFP degradation; 1) two parallel pathways target Pxa1^{MUT}-mGFP for degradation, one requiring Ufd4p and other involving Pex2p or alternatively 2) the loss of peroxisomal ubiquitination machinery factors impacts on peroxisome function, which in turn inhibits Pxa1^{MUT}-mGFP degradation. The absence of the cycling receptor protein Pex5p also inhibits Pxa1^{MUT}-mGFP turnover. Hence, the loss of Pex2p or Pex4p could indirectly inhibit Pxa1^{MUT}-mGFP degradation. Finally, we show that Pxa1^{MUT}-mGFP retained a certain degree of activity and this was increased by inhibiting Pxa1^{MUT}-mGFP degradation through the deletion of UFD4. Details on the mechanisms of how Ufd4p regulates Pxa1^{MUT}-mGFP turnover will enable us to better understand how cytosolic UPS factors can regulate the turnover of PMPs

and can hence impact on peroxisome function. Furthermore, the fact that inhibiting Pxa1^{MUT}-mGFP degradation can enhance Pxa1p cellular activity demonstrates that blocking ALDP mutant degradation may represent an effective way to treat ALD.

In **Chapter 4** we present our research on *S. cerevisiae* Pex13p. Similar to *H. polymorpha*, we observed that Pex13p fused to mGFP (Pex13-mGFP) in *S. cerevisiae* undergoes rapid degradation in cells grown on peroxisome inducing conditions, establishing that Pex13p turnover is a conserved event. By employing the tFT approach, we demonstrate that the peroxisomal ubiquitination machinery plays a major role in Pex13-mGFP degradation, while the additional cytosolic UPS factors Ufd4p, Ubc4p and Ubr2p play minor roles, possibly through the formation of poly-Ub chains on Pex13-mGFP. The fact that multiple E2s and E3s appear to be involved in Pex13-mGFP turnover suggests that either several pathways act in parallel to facilitate Pex13-mGFP degradation or that all these factors act together in ubiquitinating Pex13-mGFP, to target the protein for proteasomal degradation. Our data also demonstrate that the function of Cdc48p is not required for Pex13-mGFP degradation. Since Cdc48p is involved in the degradation of ER and mitochondrial membrane proteins, this observation sets the degradation of PMPs apart from that of other organellar membrane proteins and establishes that different mechanisms exist to facilitate these degradation processes. One possibility is that the AAA-ATPase Msp1p, which was reported to extract tail anchored proteins such as Pex15p from the peroxisomal membrane for degradation could target Pex13p to the proteasome. Alternatively, the AAA-ATPases Pex1p and Pex6p, which are involved in the extracting ubiquitinated Pex5p from the peroxisomal membrane could also be seen as an interesting candidate for further study. However, recent reports indicate that proteasomes can be recruited directly to membranes, to facilitate the degradation of membrane proteins. Perhaps such a mechanism could also control the degradation of Pex13p. Clearly further work is needed to investigate how ubiquitinated Pex13p is extracted from the peroxisomal membrane and why Pex13p is targeted for degradation.

PMPs play a crucial role in regulating many peroxisomal functions and therefore are important for peroxisome homeostasis and cellular metabolism. Investigating the degradation of PMPs would help us understand how PMP homeostasis is controlled, to what extent PMP degradation is involved in regulating peroxisomal functions and how PMP degradation relates to other protein degradation systems in the cell. In this thesis we have investigated the degradation of PMPs in *S. cerevisiae*. Our data clearly show that Pxa1-tFT and Pxa1^{MUT}-mGFP, which are both inhibited in function, undergo rapid degradation. The fact that only faulty versions of Pxa1p, but not the wild-type version, undergo degradation demonstrates the existence of a quality control pathway at the peroxisomal membrane that targets faulty PMPs for degradation. The strong link between ER and mitochondrial membrane protein quality control and disease tells us that future studies should be aimed at investigating this PMP quality control pathway. Furthermore, the involvement of Ufd4p in the degradation of Pxa1^{MUT}-mGFP,

Pxa1-tFT and (potentially) Pex13-mGFP indicates both that common mechanisms may exist that target PMPs for degradation and that cytosolic proteins can regulate PMP degradation and hence the peroxisome function. Future studies that aim to identify and characterise additional cytosolic UPS factors involved in PMP degradation will provide valuable insights into the role of PMP turnover in health and disease.

Samenvatting en vooruitzichten

Peroxisomen zijn organellen met een enkel membraan die in bijna alle eukaryote cellen voorkomen. Veel voorkomende peroxisoomfuncties zijn onder meer β -oxidatie van vetzuren en detoxificatie van waterstofperoxide, maar ze zijn ook betrokken bij een breed scala aan metabolische processen die variëren afhankelijk van het organisme, celtype en ontwikkelingsstadium. De functies van peroxisomen worden grotendeels bepaald door peroxisomale matrixeiwitten die betrokken zijn bij het peroxisomale metabolisme en peroxisomale membraaneiwitten (PMP's), die betrokken zijn bij de peroxisoomvorming, splitsing en transport van eiwitten en kleine moleculen naar peroxisomen. Het is belangrijk dat peroxisomale eiwitten strak worden gecontroleerd door regulering van eiwitsynthese, targeting en eiwitaafbraak. Peroxisomale eiwitten kunnen worden afgebroken omdat ze zijn beschadigd / ontvouwen door verschillende stressfactoren of omdat ze niet langer nodig zijn. Over het algemeen moet de afbraak van eiwitten streng worden gecontroleerd om de ophoping van ongewenste eiwitten of het optreden van ongewenste eiwitaafbraak te voorkomen. Hoewel er veel inspanningen zijn geleverd om de post-translationele targeting van PMP's op peroxisomen te bestuderen, is er weinig bekend over PMP-afbraak. Door te onderzoeken hoe en waarom PMP's worden afgebroken, zullen we de rol van PMP-afbraak in een cellulaire context beter kunnen begrijpen.

Beschadigde en overtollige membraaneiwitten van andere organellen zoals het endoplasmatisch reticulum (ER) en mitochondriën worden in het algemeen afgebroken door het Ubiquitin Proteasoom Systeem (UPS), dat poly-ubiquitineren van het substraateiwit teweeg brengt door een cascade van enzymen (E1, E2 en E3) gevolgd door de afbraak in het proteasoom. Peroxisomen hebben één E2 (Pex4p) en verschillende E3-ligasen (Pex2p, Pex10p en Pex12p), waarvan bekend is dat ze de cyclische receptoreiwitten Pex5p en Pex20p familieleden ubiquitineren. Onlangs werd gemeld dat deze peroxisomale ubiquitinatie machine betrokken is bij de afbraak van de PMP's Pex3p en Pex13p in *H. polymorpha*. Ondanks deze recente rapporten blijven er vragen liggen, waaronder welke andere PMP's UPS-gemedieerde afbraak ondergaan, wat zijn de functies van PMP-afbraak en of alle PMP-afbraakgebeurtenissen een gemeenschappelijk mechanisme delen?

Het onderzoek in dit proefschrift geeft inzicht in PMP-afbraak in *S. cerevisiae*. **Hoofdstuk 1** vat de huidige kennis samen over hoe membraaneiwitten uit het ER en mitochondriën worden herkend, uit het membraan worden gehaald en voor afbraak worden getarget door het ubiquitine-proteasoom systeem. Verder wordt besproken wat er momenteel bekend is over PMP-afbraak, inclusief mogelijke functies van PMP-verloop en hoe PMP-afbraak kan worden gefaciliteerd.

In **Hoofdstuk 2** presenteren we ons onderzoek naar PMP-stabiliteit in de gist *S. cerevisiae* met behulp van de tandem fluorescent proteïne timer (tFT) benadering. Een tFT-tag bestaat uit twee fluorescerende eiwitten, in dit geval mCherry en sfGFP, die zijn gefuseerd met

het betreffende eiwit. De verhouding van de fluorescentie-intensiteit van de twee fluorescerende eiwitten dient als indicator voor eiwitstabiliteit. We hebben waargenomen dat de tFT-benadering de stabiliteit van individuele PMP-tFT-fusies in cellen kan weergeven, dit varieerde van onstabiel (Pex13-tFT en Pxa1-tFT) tot stabiel (Pex11-tFT). De tFT-gelabelde versies van Pex13p, Pxa1p en Pex11p werden geïnduceerd op oleaat bevattende media en vertoonden peroxisomaal-achtige lokalisatie. Bovendien toonden groei-experimenten aan dat de functionaliteit van Pex13p niet werd aangetast door fusie met een tFT-tag, terwijl de functionaliteit van Pxa1p wel werd aangetast door de tFT-tag. Samen tonen deze resultaten aan dat de tFT-benadering een goede manier is om PMP-stabiliteit te bestuderen. Het feit dat de functie tFT-tag Pxa1p verminderde, toont aan dat het bevestigen van een tFT-tag aan een PMP kan worden gebruikt om PMP's opzettelijk te destabiliseren. Dit kan gebruikt worden om kwaliteitscontrolegebeurtenissen te onderzoeken die verband houden met die specifieke PMP. Aan de andere kant wordt Pex13-tFT, dat zijn functionaliteit behield, snel afgebroken, wat aangeeft dat tFT-tags ook effectief kunnen worden gebruikt voor het bestuderen van de gerichte downregulatie van PMP's. Een sterk punt van de tFT-benadering is dat het kan worden gebruikt om PMP-stabiliteit onder verschillende groeiomstandigheden te onderzoeken (zoals het geval was in dit hoofdstuk) en om te screenen tegen mutante bibliotheken, om factoren te identificeren die betrokken zijn bij PMP degradatie, zoals we hebben gedaan in de hoofdstukken drie en vier.

Hoofdstuk 3 beschrijft de kwaliteitscontrole van de PMP Pxa1p in *S. cerevisiae*. Pxa1p is een gisthomoloog van humaan adrenoleukodystrofie-eiwit (ALDP) en mutaties in ALDP, die vaak tot eiwitinstabiliteit leiden, kunnen leiden tot de ernstige genetische aandoening adrenoleukodystrofie (ALD). We selecteerden twee ALD-veroorzakende mutaties en genereerden de overeenkomstige mutaties in gist Pxa1p (Pxa1^{MUT}), getagd aan mGFP. We hebben waargenomen dat Pxa1^{MUT}-mGFP snel wordt afgebroken, terwijl Pxa1-mGFP stabiel blijft, wat aangeeft dat kwaliteitscontrole een rol speelt in het Pxa1^{MUT}-mGFP -verloop. De afbraak van Pxa1^{MUT}-mGFP werd significant verminderd na behandeling met proteasoomremmers, wat aantoont dat het proteasoom hierbij betrokken is en verder een rol suggereert voor ubiquitinatie in het Pxa1^{MUT}-mGFP -verloop. Onze pogingen om ubiquitinevormen van Pxa1^{MUT}-mGFP te detecteren, waren niet succesvol, mogelijk omdat geubiquitineerde Pxa1^{MUT}-mGFP weinig aanwezig is of omdat ubiquitineren van een ander eiwit en niet Pxa1^{MUT}-mGFP nodig is voor Pxa1^{MUT}-mGFP -afbraak. Met behulp van de tFT-benadering hebben we waargenomen dat het cytosolische E3-ligase Ufd4p een belangrijke rol speelt bij de afbraak van Pxa1^{MUT}-mGFP. De waarneming dat Pxa1^{MUT}-mGFP is gestabiliseerd op peroxisomen in *ufd4*-cellen suggereert dat Ufd4p-afhankelijke kwaliteitscontrole van Pxa1^{MUT}-mGFP plaatsvindt bij peroxisomen. Net als Ufd4p remt het verlies van de peroxisomale E3-ligase Pex2p ook de afbraak van Pxa1^{MUT}-mGFP. Er kunnen twee redenen zijn om uit te leggen waarom meerdere E3's betrokken zijn bij Pxa1^{MUT}-mGFP -afbraak; 1) twee parallelle routes richten zich op Pxa1^{MUT}-mGFP voor afbraak, één vereist Ufd4p waarbij

de andere Pex2p betrokken is, of alternatief 2) het verlies van factoren van het peroxisomale ubiquitineringsysteem beïnvloedt de peroxisoomfunctie, wat op zijn beurt de afbraak van Pxa1^{MUT}-mGFP remt. De afwezigheid van het cyclische receptoreiwit Pex5p kan ook het Pxa1^{MUT}-mGFP -verloop remmen. Dit wijst erop dat het verlies van Pex2p of Pex4p indirect de afbraak van Pxa1^{MUT}-mGFP kan remmen. Ten slotte laten we zien dat Pxa1^{MUT}-mGFP een bepaalde mate van activiteit behoudt en dat dit wordt verhoogd door Pxa1^{MUT}-mGFP -afbraak te remmen door de verwijdering van UFD4. Details over de mechanismen van hoe Ufd4p het Pxa1^{MUT}-mGFP -verloop reguleert, zullen ons in staat stellen om beter te begrijpen hoe cytosolische UPS-factoren het verloop van PMP's kunnen reguleren en dus van invloed kunnen zijn op de peroxisoomfunctie. Bovendien toont het feit dat het remmen van de afbraak van Pxa1^{MUT}-mGFP de cellulaire activiteit van Pxa1p kan versterken aan dat het blokkeren van ALDP-mutantaafbraak een effectieve manier kan zijn om ALD te behandelen.

In **hoofdstuk 4** presenteren we ons onderzoek naar *S. cerevisiae* Pex13p. Net als bij *H. polymorpha*, hebben we waargenomen dat Pex13p gefuseerd met mGFP (Pex13-mGFP) in *S. cerevisiae* een snelle afbraak ondergaat in cellen die zijn gegroeid onder peroxisoom-inducerende omstandigheden, wat aantoont dat het Pex13p-verloop een geconserveerde gebeurtenis is. Door de tFT-benadering toe te passen, laten we zien dat de peroxisomale ubiquitinatiemachines een belangrijke rol spelen bij de afbraak van Pex13-mGFP, terwijl de aanvullende cytosolische UPS-factoren Ufd4p, Ubc4p en Ubr2p een ondergeschikte rol spelen, mogelijk door de vorming van poly-Ub-ketens op Pex13-mGFP. Het feit dat meerdere E2's en E3's betrokken lijken te zijn bij het Pex13-mGFP-verloop, suggereert dat verschillende routes parallel werken om de afbraak van Pex13-mGFP te faciliteren of dat al deze factoren samenwerken bij het ubiquitineren van Pex13-mGFP, om het eiwit te targeten voor proteasomale afbraak. Onze resultaten tonen ook aan dat de functie van Cdc48p niet vereist is voor Pex13-mGFP-afbraak. Aangezien Cdc48p betrokken is bij de afbraak van ER- en mitochondriale membraaneiwwitten, onderscheidt deze waarneming de afbraak van PMP's van die van andere organellaire membraaneiwwitten en bewijst dat er verschillende mechanismen bestaan om deze afbraakprocessen te faciliteren. Eén mogelijkheid is dat het AAA-ATPase Msp1p, waarvan werd gerapporteerd dat het staartverankerde eiwwitten zoals Pex15p uit het peroxisomale membraan haalt voor afbraak, Pex13p zou kunnen targeten om naar het proteasoom getransporteerd te worden. Als alternatief zouden de AAA-ATPases Pex1p en Pex6p, die betrokken zijn bij het extraheren van geubiquitineerd Pex5p uit het peroxisomale membraan, ook kunnen worden beschouwd als een interessante kandidaat voor verder onderzoek. Recente rapporten geven echter aan dat proteasomen rechtstreeks op membranen kunnen worden gerekruteerd om de afbraak van membraaneiwwitten te faciliteren. Misschien zou een dergelijk mechanisme ook de afbraak van Pex13p kunnen reguleren. Er is nog meer onderzoek nodig om duidelijk te krijgen hoe geubiquitineerd Pex13p wordt geëxtraheerd uit het peroxisomale membraan en waarom Pex13p het doelwit is voor afbraak.

PMP's spelen een cruciale rol bij het reguleren van veel peroxisomale functies en zijn daarom belangrijk voor de peroxisoomhomeostase en het celmetabolisme. Onderzoek naar de afbraak van PMP's zou ons helpen te begrijpen hoe PMP-homeostase wordt gereguleerd, in welke mate PMP-afbraak betrokken is bij het reguleren van peroxisomale functies en hoe PMP-afbraak zich verhoudt tot andere eiwitafbraaksystemen in de cel. In dit proefschrift is de afbraak van PMP's in *S. cerevisiae* onderzocht. De resultaten laten duidelijk zien dat Pxa1-tFT en Pxa1^{MUT}-mGFP, welke beide minder goed functioneren, snel worden afgebroken. Het feit dat alleen defecte versies van Pxa1p, maar niet de wildtype-versie, degradatie ondergaan, toont het bestaan aan van een kwaliteitscontrole route op het peroxisomale membraan dat gericht is op defecte PMP's voor degradatie. De sterke link tussen ER en mitochondriale membraaneiwitkwaliteitscontrole en ziekte geeft aan dat toekomstige studies gericht moeten zijn op het onderzoeken van deze PMP-kwaliteitscontrole route. Bovendien geeft de betrokkenheid van Ufd4p bij de afbraak van Pxa1^{MUT}-mGFP, Pxa1-tFT en (mogelijk) Pex13-mGFP aan dat er gemeenschappelijke mechanismen kunnen bestaan die PMP's voor afbraak targeten, en dat cytosolische eiwitten de PMP-afbraak en daarmee de peroxisoomfunctie kunnen reguleren. Toekomstige studies die gericht zijn op het identificeren en karakteriseren van aanvullende cytosolische UPS-factoren die betrokken zijn bij PMP-afbraak, zullen waardevolle inzichten verschaffen in de rol van PMP-verloop bij gezondheid en ziekte.