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REVIEW ARTICLE

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# Post-translational modifications of proteins associated with yeast peroxisome membrane: An essential mode of regulatory mechanism

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**Abstract**

Peroxisomes are single membrane-bound organelles important for the optimum functioning of eukaryotic cells. Seminal discoveries in the field of peroxisomes are made using yeast as a model. Several proteins required for the biogenesis and function of peroxisomes are identified to date. As with proteins involved in other major cellular pathways, peroxisomal proteins are also subjected to regulatory post-translational modifications. Identification, characterization and mapping of these modifications to specific amino acid residues on proteins are critical toward understanding their functional significance. Several studies have tried to identify post-translational modifications of peroxisomal proteins and determine their impact on peroxisome structure and function. In this manuscript, we provide an overview of the various post-translational modifications that govern the peroxisome dynamics in yeast.

**KEYWORDS**

peroxisomes, phosphorylation, post-translational modification, ubiquitination, yeast

## 1 | INTRODUCTION

Organisms have evolved to act and adapt to the changing environment, and this can be achieved in multiple ways. The most extensively studied mode of adaptation at the cellular level is the transcriptional and translational regulation of proteins (Khraiwesh et al., 2010; Preiss et al., 1998; Schüller, 2003). Post-translational modifications (PTMs) are regulatory mechanisms that allow a quick and reversible modulation to adapt to intracellular or extracellular stimuli (Tripodi et al., 2015). PTMs involve alterations in the polypeptide chain either through the addition or removal of small chemical molecules to amino acid residues, proteolytic cleavage of the protein or covalent cross-linking between protein domains (Walsh et al., 2005). Several PTMs like phosphorylation, ubiquitination, acetylation, glycosylation, methylation, lipidation, flavinylation, thiolation, mannosylation and palmitoylation are identified in eukaryotic cells (Walsh et al., 2005). PTMs tightly regulate the activity and turnover of proteins and modulate their molecular structure and function. Each of these modifications is catalyzed by specialized proteins. Given the important role of PTMs in cellular function, a need and constant effort to develop novel techniques to identify these modifications is underway. Advanced high-throughput MS-based proteomic methods coupled with traditional biochemical methods such as SDS-PAGE and Western blotting

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help in identifying the modifications and understand their biological significance in vivo (Mnatsakanyan et al., 2018). Combinatorial methods such as multidimensional protein identification technology or MudPIT, which utilizes both chromatography and mass spectrometry to discover global PTMs, are also developed (Schirmer et al., 2003). The development of in silico algorithms that reliably predict the modifications in a protein sample enables researchers to identify PTMs (Audagnotto & Dal Peraro, 2017).

Yeast in general and *Saccharomyces cerevisiae*, in particular, is an attractive model organism for studying various cellular processes in higher eukaryotes owing to their similar cellular architecture and ease of performing genetic and molecular manipulations (Karathia et al., 2011). Yeast cells respond dynamically to the changes in the cellular environment by adjusting their metabolism. Several PTMs that regulate yeast metabolism have been identified (Oliveira & Sauer, 2012; http://www.yeastgenome.org). PhosphoPep or UniProt databases predict phosphorylation sites in approximately 52% of enzymes involved in yeast metabolism (Bodenmiller et al., 2008; Farriol-Mathis et al., 2004; Oliveira & Sauer, 2012). PTMs of proteins involved in other cellular pathways such as autophagy have also been reported in yeast (Xie et al., 2015).

Peroxisomes are single membrane-bound organelles that play an important role in cellular metabolism. They are involved in various catabolic processes such as fatty acid oxidation, glyoxylate cycle and methanol metabolism in yeast (Figure 1; Deb & Nagotu, 2017; Sibirny, 2016). Several proteins required for the biogenesis of these organelles and involved in various associated functions have been identified (Akşit & van der Klei, 2018). PTMs of peroxisomal proteins are proposed to be an important regulatory mechanism (Sandalio et al., 2019). Various phospho-proteome studies have reported that multiple peroxisomal proteins undergo phosphorylation (Albuquerque et al., 2008; Holt et al., 2009; Soulard et al., 2010; Swaney et al., 2013). However, concerns have been raised about the quality in identifying these sites, both in terms of technical and biological noise. It has also been suggested that approximately 65% of the identified phosphorylation sites in yeast might have little or no functional importance (Landry et al., 2009). This review provides an overview of all the major validated PTMs of peroxisomal proteins and highlights their importance in peroxisome biogenesis and its functions in yeast (Table 1).

## 2 | PHOSPHORYLATION

Protein phosphorylation is one of the most ubiquitous regulatory mechanisms in both prokaryotes and eukaryotes. Phosphorylation involves the addition of covalently bound phosphate groups to the side chains of amino acids (Ardito et al., 2017). In eukaryotes, phosphorylation occurs majorly at serine (S), threonine (T) and tyrosine (Y) residues, whereas in prokaryotes, it is limited to histidine (H) and aspartic acid (D) (Nishi et al., 2014; Pearlman et al., 2011). Phosphorylation and dephosphorylation of proteins are dynamic processes controlled by a set of kinases and phosphatases, respectively. In *S. cerevisiae*, 120 kinases and 40 phosphatases are reported to be involved in this reversible mechanism (Oliveira & Sauer, 2012). The addition or deletion of a phosphate group at a specific amino acid residue within a protein leads to alteration in the protein's conformation, localization, stability and catalytic activity. These changes enable the protein to form complexes or subject it to degradation (Oeljeklaus et al., 2016; Oliveira et al., 2012). Phosphorylation of proteins holds prime importance in regulating multiple processes with respect to peroxisome biology such as protein import, fission, de novo biogenesis and inheritance (Oeljeklaus et al., 2016; Figure 2). Almost 50% of proteins involved in yeast metabolism are reported to be phosphorylated, showing the high relevance of this regulatory mechanism in the optimum functioning of cells (Vlastaridis et al., 2017).

### 2.1 | Pex11

Pex11 was the first peroxisomal membrane protein (PMP) identified to be involved in the division and proliferation of peroxisomes (Erdmann & Blobel, 1995). Deletion of Pex11 resulted in fewer but enlarged peroxisomes, and overexpression resulted in multiple small peroxisomes in yeast cells (Erdmann & Blobel, 1995; Krikken et al., 2009). Peroxisome division involves tubulation of the membrane, followed by fission. A conserved N-terminal amphipathic helix of Pex11 was reported to play an important role in the tubulation process by inducing membrane curvature (Opaliński et al., 2011). In addition, Pex11 acts as a membrane channel that nonselectively transfers metabolites across the peroxisomal membrane in *S. cerevisiae* and thereby affects the rate of β-oxidation (Mindthoff et al., 2016). Pex11 also aids in the establishment of contact sites between peroxisomes and mitochondria through the ERMES complex by physical interaction with the mitochondrial protein Mdm34 in *S. cerevisiae* (Ušaj et al., 2015). Interestingly, a role for Pex11 in peroxisome-ER contact formation has been recently reported (Wu et al., 2020).

Knoblach and Rachubinski first reported phosphorylation of Pex11 at $Ser^{165}$ and/or $Ser^{167}$ residues in *S. cerevisiae* (Knoblach & Rachubinski, 2010). Pex11 in the methylotrophic yeasts *Komagataella phaffii* and *Ogataea polymorpha* was also reported to be phosphorylated at the conserved $Ser^{173}$ and $Ser^{174}$ residues, respectively (Joshi et al., 2012; Thomas et al., 2015). $Ser^{174}$ was also reported to be conserved in *Yarrowia lipolytica* (Dulermo et al., 2015).

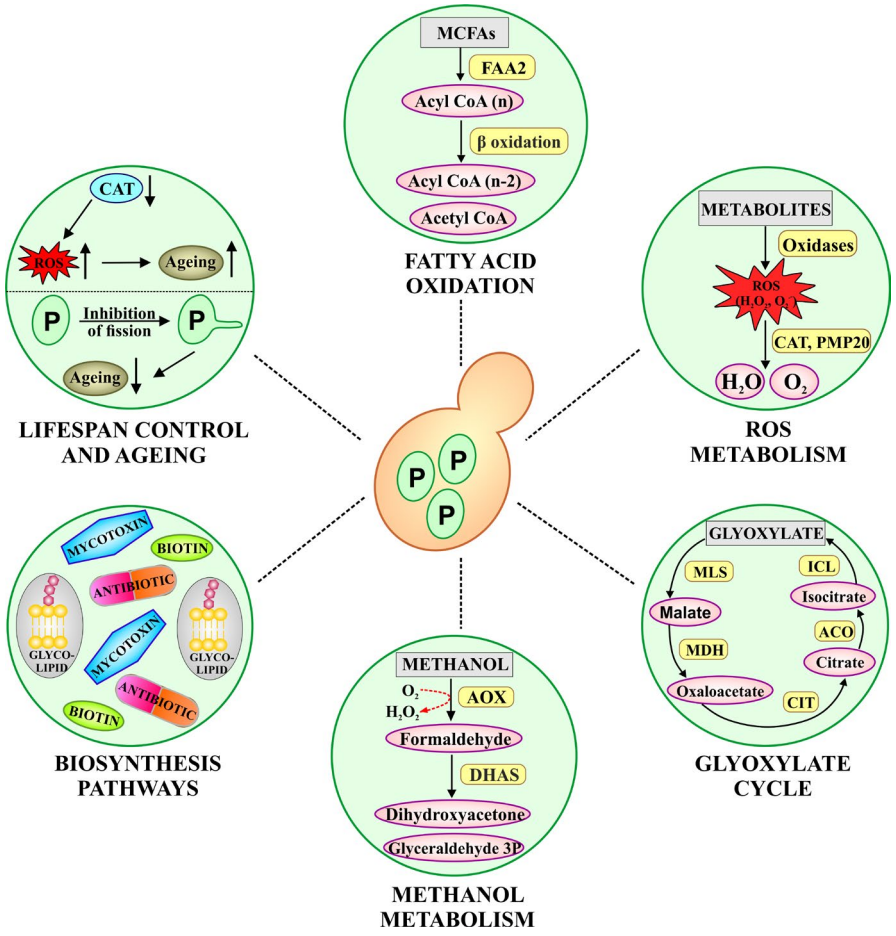


**FIGURE 1** Diverse functions of yeast peroxisomes. Peroxisomes in yeast are associated with a diverse array of functions. They are the sole site of β-oxidation, unlike higher eukaryotes, where it occurs in mitochondria and peroxisomes (Hiltunen et al., 2003; Poirier et al., 2006). Peroxisomes harbor catalase and glutathione peroxidase which scavenge toxic reactive oxygen species (Gómez et al., 2019). A role for catalase-mediated ROS homeostasis and peroxisome fission in modulating yeast lifespan has also been proposed (Kawałek et al., 2013; Lefevre et al., 2015; Mesquita et al., 2010). Peroxisomes are also involved in glyoxylate metabolism and catabolism of unusual carbon and nitrogen sources such as methanol, methylamine and purines and aid the organism to adapt to the prevailing environmental conditions (Kunze et al., 2006; Sibirny, 2016; van Zutphen et al., 2010). Interestingly, peroxisomes are also used as a subcellular site for the synthesis of secondary metabolites, heterologous expression of proteins and for the biosynthesis of valuable chemicals (Dusséaux et al., 2020; Gidijala et al., 2008; Stehlik et al., 2014; Zhou et al., 2016). ACO, aconitase; AOX, alcohol oxidase; CAT, catalase; CIT, citrate synthase; DHAS, dihydroxyacetone synthase; FAA2, medium-chain fatty acyl-CoA synthetase; ICL, isocitrate lyase; MCFAs, medium-chain fatty acids; MDH, malate dehydrogenase; MLS, malate synthase; PMP20, glutathione peroxidase; P, peroxisome

On analyzing the nonphosphorylated (PEX11-A) and phosphomimetic (PEX11-D) mutants in *S. cerevisiae* and *K. phaffii*, PEX11-A cells showed enlarged and clustered peroxisomes similar to *pex11* cells, whereas numerous small and hyper-proliferated peroxisomes that resemble the phenotype of Pex11 overexpression were observed in PEX11-D mutant cells (Joshi et al., 2012; Knoblach & Rachubinski, 2010). This phenotype could be obtained even at a lower concentration of PEX11-D (Knoblach & Rachubinski, 2010). This provides evidence that the control of peroxisome dynamics by Pex11 is regulated by phosphorylation of the protein. However, in *O. polymorpha*, phosphorylation of Pex11 did not affect the number and size of peroxisomes (Thomas et al., 2015).

Phosphorylation of Pex11 also regulates the intracellular distribution and trafficking of the protein. Dual localization of Pex11 to ER and peroxisomes was observed in wild-type (WT) *S. cerevisiae* cells grown in oleic acid media, and upon shifting the cells to a glucose-containing media (which increases Pex11 phosphorylation), only peroxisomal localization of the protein was observed (Knoblach & Rachubinski, 2010). Analysis of localization of Pex11 mutants revealed that Pex11-A was confined to peroxisomes and Pex11-D mostly appeared at the ER-peroxisome interface. However, there was no change in the localization of both the proteins when the cells were shifted from oleic acid to glucose media (Knoblach & Rachubinski, 2010). The kinase Pho85 was identified as a prime candidate that

**TABLE 1** Peroxisomal proteins and their modifications identified in various yeast species

| S. no | Protein | Function in peroxisome homeostasis | *S. cerevisiae* | *O. polymorpha* | *Y. lipolytica* | *K. phaffii* | Reference |
|---|---|---|---|---|---|---|---|
| *1. Phosphorylation* | | | | | | | |
| 1. | Pex11 | Membrane elongation | + ($Ser^{165}$, $Ser^{167}$) | + ($Ser^{174}$) | NR | + ($Ser^{173}$) | Knoblach and Rachubinski (2010)<br>Joshi et al. (2012)<br>Thomas et al. (2015) |
| 2. | Pex14 | Part of the docking complex and required for matrix protein import, role in pexophagy | + ($S^{266}$) | + ($Th^{248}$, $Ser^{258}$) | NR | + | Johnson et al. (2001)<br>Tanaka et al. (2013)<br>Schummer et al. (2020) |
| 3. | Pex15 | Recruitment of Pex6 to the peroxisome membrane | + | NR | NR | NR | Elgersma et al. (1997) |
| 4. | Vps1 | Peroxisome fission | + ($S^{599}$) | NR | NR | NR | Smaczynska-de Rooij et al. (2016) |
| 5. | Myo2 | Peroxisome segregation and movement | + ($Ser^{113}$, $Th^{1132}$, $Ser^{1134}$) | NR | NR | NR | Legesse-Miller et al. (2006) |
| 6. | Inp2 | Peroxisome inheritance | + | NR | NR | NR | Fagarasanu et al. (2009) |
| 7. | Vps34 | Pexophagy | + | NR | NR | NR | Stack and Emr (1994) |
| *2. Ubiquitination* | | | | | | | |
| 8. | Pex5 | Receptor for the import of PTS1 containing matrix proteins | + ($Cys^{6}$, $Lys^{18}$/$Lys^{24}$) | + ($Lys^{21)}$ | NR | ($Cys^{10)}$ | Kragt et al. (2005)<br>Platta et al. (2004)<br>Platta et al. (2007)<br>Kiel, Otzen, et al. (2005)<br>Ma et al. (2013) |
| 9. | Pex7 | Receptor for the import of PTS2 containing matrix proteins | NR | NR | NR | + | Hagstrom et al. (2014) |
| 10. | Pex13 | Part of the docking complex and require for matrix protein import | NR | + | NR | NR | Chen et al. (2018) |
| 11. | Pex18 | Coreceptor for the import of PTS2 containing matrix proteins | + ($Cys^{6}$, $Lys^{13}$and $Lys^{20}$) | NR | NR | NR | El Magraoui et al. (2013)<br>Hensel et al. (2011) |
| 12. | Pex20 | Coreceptor for the import of PTS2 containing matrix proteins | NR | NR | NR | + ($Cy^{8}$, $Lys^{19}$) | Léon et al. (2006)<br>Liu and Subramani (2013) |
| 13. | Pex3 | Essential for peroxisome biogenesis | NR | + | NR | NR | Williams and van der Klei (2013) |
| *3. Glycosylation* | | | | | | | |
| 14. | Pex2 | Member of RING finger complex and required for matrix protein import | NR | NR | + | NR | Titorenko and Rachubinski (1998) |

(Continues)

**TABLE 1** (Continued)

| S. no | Protein | Function in peroxisome homeostasis | *S. cerevisiae* | *O. polymorpha* | *Y. lipolytica* | *K. phaffii* | Reference |
|---|---|---|---|---|---|---|---|
| 15. | Pex16 | Peroxisome division and assembly | NR | NR | + | NR | Titorenko and Rachubinski (1998) |
| *4. Farnesylation* | | | | | | | |
| 16. | Pex19 | Targeting of peroxisome membrane proteins | + ($Cys^{347}$) | + ($Cys^{286}$) | – | – | Götte et al. (1998)<br>Lambkin and Rachubinski (2001)<br>Snyder et al. (1999)<br>Otzen et al. (2004) |

*Note*: +: presence of the PTM in the particular yeast species, –: absence of PTM and NR: Not reported.

phosphorylates Pex11 in *S. cerevisiae* cells. Overexpression of Pho85 led to phosphorylation-induced activation of Pex11, resulting in the exit of the protein from ER and sole localization to peroxisomes (Knoblach & Rachubinski, 2010). In *K. phaffii* and *O. polymorpha*, Pex11 trafficking between ER and peroxisome was reported to be independent of its phosphorylation status (Joshi et al., 2012; Thomas et al., 2015). However, in *K. phaffii*, the interaction of Pex11 with the fission protein Fis1 was phosphorylation-dependent. This most likely accounts for the block in peroxisome fission in PEX11-A mutants (Joshi et al., 2012). Phosphorylation of Pex11 also affects the rate of β-oxidation as the PEX11-D and PEX11-A show differential ion selectivity (Mindthoff et al., 2016). These results indicate that phosphorylation of Pex11 has an intriguing role in controlling the number and function of peroxisomes. However, the extent of the effect varies between different yeast species studied so far.

Mutations in *PEX*11β that resulted in defective peroxisome fission leading to PBD have been reported (Ebberink et al., 2012). Interestingly, phosphorylation-dependent regulation of *PEX*11β was not observed in human cells. Self-interaction of the protein was reported to be essential for the membrane reorganization required for peroxisome fission (Bonekamp et al., 2013).

## 2.2 | Pex14

Pex14 is a member of the docking complex, which mediates the import of peroxisomal matrix proteins (Albertini et al., 1997; van der Klei & Veenhuis, 2013). Peroxisomal matrix protein import depends on the presence of specific targeting signals called peroxisomal targeting signal (PTS) type 1 and type 2 on the cargo proteins. Proteins with PTS1 and PTS2 are recognized and bound by Pex5 and Pex7 receptors, respectively, in the cytosol (Ma & Subramani, 2009). This cargo-receptor complex is then imported into the peroxisomal matrix by the Pex13-Pex14 docking complex that also includes Pex17 in *S. cerevisiae* (Rucktäschel et al., 2011). Mislocalization of peroxisomal matrix proteins such as alcohol oxidase (AOX) and dihydroxyacetone synthase (DHAS) to cytosol was observed in *O. polymorpha pex14* cells. However, upon overexpression of Pex5, the cells showed partial localization of these proteins to peroxisomes, albeit the efficiency was reduced (Salomons et al., 2000). In *K. phaffii pex14* cells, functional peroxisomes were absent (Johnson et al., 2001). A role for Pex14 in the selective degradation of peroxisomes in *O. polymorpha* was also reported (van Zutphen et al., 2008).

Phosphorylation of Pex14 was reported in both *O. polymorpha* and *K. phaffii* (Johnson et al., 2001; Komori & Veenhuis, 2000). In *K. phaffii*, this modification of Pex14 was identified as an additional protein band with a slower

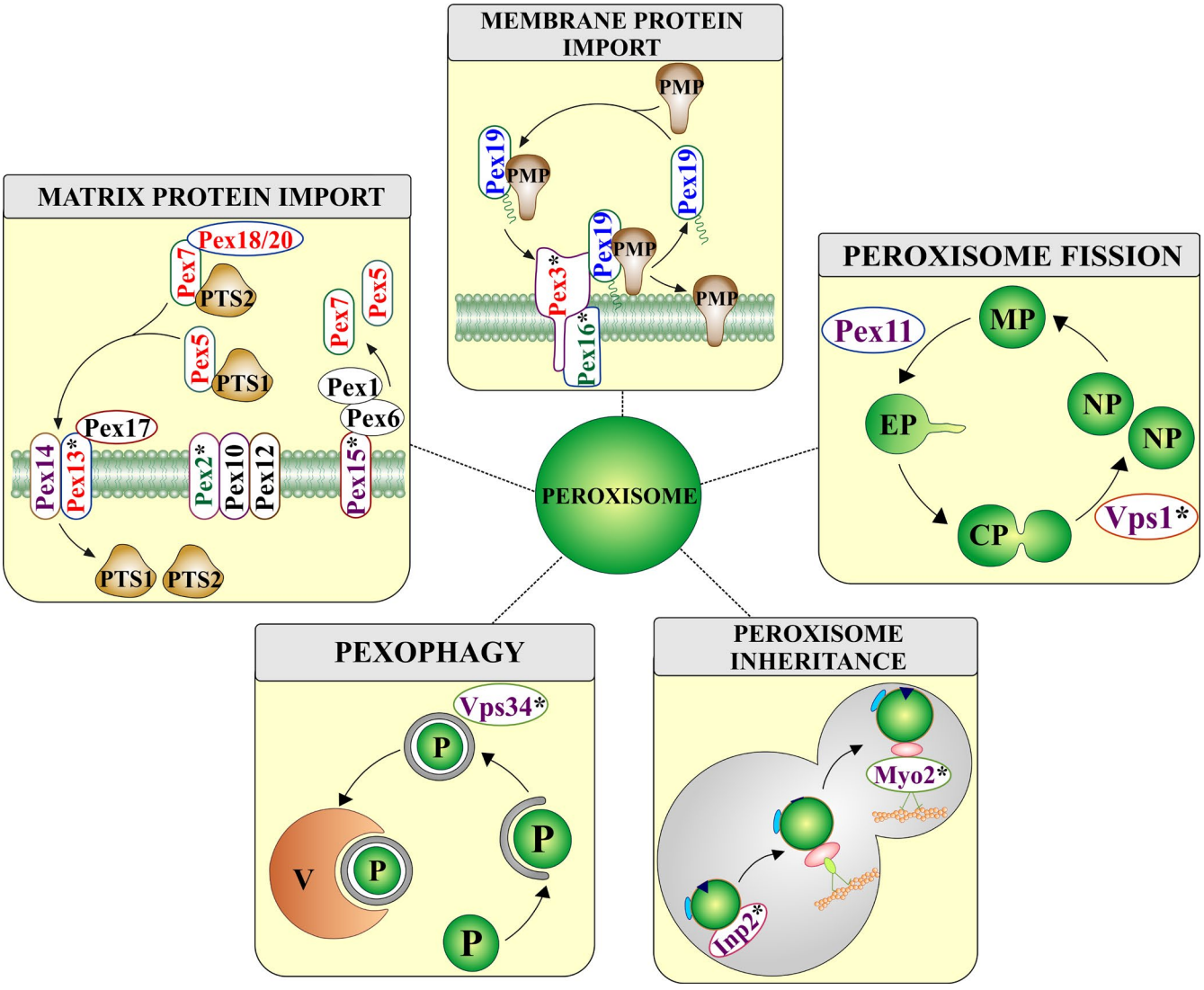


**FIGURE 2** An overview of mechanisms that govern peroxisome homeostasis. The proteins involved and PTMs that regulate them are depicted. Peroxisomal homeostasis is governed by a multitude of processes such as import of peroxisomal membrane and matrix proteins, fission, inheritance and degradation of peroxisomes. Various proteins involved in these processes are found to be post-translationally modified and some of these modifications are found to be crucial for their function. Proteins that are phosphorylated are depicted in purple, ubiquitinated in red, farnesylated in blue and glycosylated in green. CP, constricted peroxisome; EP, elongated peroxisome; MP, mature peroxisome; NP, nascent peroxisome; P, peroxisome; PMP, peroxisome membrane protein; PTS1, peroxisome targeting signal 1; PTS2, peroxisome targeting signal 2; V, vacuole. *: PTMs of proteins are identified but their role in peroxisome homeostasis is either not studied or unidentified

migration speed when run on an SDS-PAGE. The disappearance of the heavier band on treatment with phosphatase confirmed phosphorylation of the protein (Johnson et al., 2001). The interaction of Pex14 with other peroxisomal proteins was not dependent on the phosphorylation status of the protein (Johnson et al., 2001). Phosphorylation of Pex14 in *O. polymorpha* was shown in vivo by [$^{32}$P] orthophosphate incorporation (Komori & Veenhuis, 2000). Thr$^{248}$ and Ser$^{258}$ were identified as phosphorylation sites by mass spectrometry (Tanaka et al., 2013). The Pex14-A mutants of both the sites showed similar growth as WT cells when cultured in peroxisome inducing conditions. Further, no change in the localization of PTS1 and PTS2 containing peroxisomal matrix proteins AOX and amine oxidase (AMO) was observed (Tanaka et al., 2013). These data indicate that phosphorylation of Pex14 does not affect protein import and peroxisome proliferation in *O. polymorpha*. The authors also studied pexophagy in Pex14-A mutants and observed that glucose-induced macropexophagy was not affected by phosphorylation of Pex14. However, rapid loss of the phosphorylated form of Pex14 during pexophagy was observed (Tanaka et al., 2013). A recent study identified 16 phosphorylation sites in the N and C-terminal regions of *S. cerevisiae* Pex14 by mass spectrometry (Schummer et al., 2020). Among

all the identified sites, phosphorylation of Pex14 at $Ser^{266}$ resulted in reduced protein import and significantly impaired growth in oleic acid media compared with WT cells (Schummer et al., 2020). Further, the distribution of different peroxisomal matrix proteins was studied in the Pex14-S266D mutant. Citrate synthase Cit2 (the enzyme required for the growth of yeast cells on nonfermentable carbon sources) exhibited dual localization to peroxisomes and cytosol, highlighting defects in its import in the mutant cells (Schummer et al., 2020). Earlier studies have also identified $Ser^{266}$ and $Ser^{313}$ residues of *S. cerevisiae* Pex14 to be phosphorylated (Albuquerque et al., 2008). $H_2O_2$-induced phosphorylation of Pex14 at $Ser^{232}$ was reported in mammalian cells recently, and this PTM suppressed the peroxisomal import of catalase in mammalian mitotic cells (Okumoto et al., 2020; Yamashita et al., 2020).

## 2.3 | Pex15

Pex15 is an integral membrane protein with a single transmembrane domain at its C-terminus (Elgersma et al., 1997). In *S. cerevisiae*, Pex15 helps in the recruitment of AAA protein Pex6 to the peroxisome membrane (Birschmann et al., 2003). Overexpression of Pex15 led to increased ER proliferation and impaired peroxisome assembly, whereas deletion of Pex15 in *S. cerevisiae* resulted in the accumulation of ubiquitinated Pex5 at the peroxisomes, triggering pexophagy (Elgersma et al., 1997; Nuttall et al., 2014). Phosphorylation of *S. cerevisiae* Pex15 at Ser/Thr residues present in the cytosolic domain of the protein was identified (Elgersma et al., 1997). Immunoblotting showed different migration rates of Pex15 and loss of the higher molecular weight species upon treatment with potato acid phosphatase confirmed the modification (Elgersma et al., 1997). The high-throughput phospho-proteome analysis also revealed that *S. cerevisiae* Pex15 could be phosphorylated at $Ser^{297}$ (Swaney et al., 2013). However, further experiments are needed to elucidate the role of the PTM in the functioning of Pex15.

## 2.4 | Dynamin like proteins: Vps1 and Dnm1

Vps1 is a dynamin-like protein (DLP), initially identified to be involved in vacuolar protein sorting and localized to the Golgi body (Vater et al., 1992). Later studies showed that deletion of Vps1 led to fewer but elongated peroxisomes in *S. cerevisiae*, emphasizing its role in peroxisome biogenesis (Hoepfner et al., 2001; Kuravi et al., 2006). High-throughput phospho-proteome studies in *S. cerevisiae* predicted $Ser^{599}$ to be phosphorylated (Swaney et al., 2013). Further, phosphorylation at $Ser^{599}$ was confirmed in vivo and in vitro using phostag gel, kinase assay and mass spectrometry. To study the effect of phosphorylation on the function of Vps1, nonphosphorylated (Vps1-V) and phosphomimetic (Vps1-D) variants were generated and expressed in cells lacking endogenous *VPS1*. Interestingly, these mutations exhibited defects in endocytosis but did not affect the functions of Vps1 associated with peroxisome membrane fission in *S. cerevisiae* (Smaczynska-de Rooij et al., 2016). In *S. cerevisiae* and *O. polymorpha*, a role for another DLP, Dnm1, in peroxisome fission has also been reported (Kuravi et al., 2006; Nagotu et al., 2008). Interestingly, in higher eukaryotes, the homologue of Dnm1 and DLP1/DRP1 is required for peroxisome division (Li & Gould, 2003; Schrader, 2006). DRP1 undergoes several PTMs, and among them, phosphorylation of DRP1 regulates its activity and subsequently organelle division (Chang & Blackstone, 2010). Several human disorders are associated with the nonfunctioning of DRP1 because of mutations or altered PTM regulation (Assia Batzir et al., 2019; Serasinghe & Chipuk, 2017). Interestingly, when the disease causing mutations are mimicked in yeast Dnm1, variable phenotypes of mitochondrial morphology were observed (Banerjee et al., 2021). It will be of interest to know whether Dnm1 in yeast is also regulated by PTMs similar to its homologue and whether this has a role in peroxisome fission.

## 2.5 | Inp2

Inp2 acts as a specific receptor for Myo2 in *S. cerevisiae* and is involved in the inheritance of peroxisomes from the mother to the daughter cell (Fagarasanu et al., 2006). Lack of Inp2 results in daughter cells devoid of peroxisomes (Fagarasanu et al., 2006). On the contrary, in cells overexpressing Inp2, retention of peroxisomes in the mother cells was hampered, resulting in the inheritance of all peroxisomes to the daughter cell (Fagarasanu et al., 2006). Interestingly, the expression of the protein was cell cycle-dependent (Fagarasanu et al., 2006). Phosphorylation of the protein was identified using immunoblot analysis where multiple bands of Inp2 were observed on SDS-PAGE. Treatment with calf intestine alkaline phosphatase resulted in a faster migrating single band and confirmed the phosphorylation status of the protein (Fagarasanu et al., 2009). The level of phosphorylation was reported to be dependent on the progression of the cell cycle (Fagarasanu et al., 2009).

## 2.6 | Vps34

Phosphatidylinositol 3-phosphate (PtdIns3P) and phosphatidylinositol 4-phosphate (PtdIns4P) play essential roles in

the regulation of peroxisome degradation in vacuolar lumen (Platta & Erdmann, 2007). Vps34 is a class III phosphatidylinositol 3-kinase (PI3K) involved in pexophagy and is the only PI3K reported to localize to peroxisomes under peroxisome inducing growth conditions in *S. cerevisiae* (Grunau et al., 2011; Reidick et al., 2017). Cells lacking Vps34 exhibited impaired pexophagy, indicating the requirement of PtdIns3P signaling in the regulation of peroxisome degradation (Grunau et al., 2011). In vivo and in vitro autophosphorylation of Vps34 was reported (Reidick et al., 2017; Stack & Emr, 1994). Phosphorylation of Vps34 on serine residues in vivo and autophosphorylation on serine, threonine and tyrosine residues in vitro was observed by [$^{32}$P] orthophosphate incorporation and protein kinase assay (Stack & Emr, 1994). However, the effect of this autophosphorylation on pexophagy is unknown.

### 2.7 | Myo2

The segregation of peroxisomes between mother and daughter cells during cell division involves the actin cytoskeleton in yeast (Hoepfner et al., 2001). The movement of organelles across the actin filament is dependent on myosin motor proteins (Sellers, 2000). Myo2 is one such myosin motor protein involved in the movement and segregation of peroxisomes (Hoepfner et al., 2001). In *S. cerevisiae*, Myo2 phosphorylation was first reported in vivo in its tail region. Mass spectrometry analysis showed that approximately 30% of Myo2 was phosphorylated on $T^{1132}$ or $S^{1134}$, 10% was doubly phosphorylated on $T^{1132}/S^{1134}$ or $T^{1132}/S^{113}$, and 60% of the tail region was nonphosphorylated. Although several mutants of Myo2 were generated and studied, the actual phosphoregulatory mechanism of the protein is not yet understood completely (Legesse-Miller et al., 2006).

## 3 | UBIQUITINATION

Ubiquitin is a conserved 76 amino acid peptide that covalently attaches to the target proteins in a process called ubiquitination (Callis, 2014). An important role of ubiquitination in several cellular processes is identified (Metzger et al., 2012). Ubiquitination of a target protein happens via a cascade of steps that requires a ubiquitin-activating E1 enzyme, a ubiquitin-conjugating E2 enzyme and a ubiquitin-ligating E3 enzyme (Deshaies & Joazeiro, 2009). Activation of ubiquitin by the E1 enzyme is ATP-dependent and leads to the formation of a thioester bond with the C-terminal glycine of ubiquitin. The activated ubiquitin is subsequently transferred to the active-site cysteine of the E2 conjugating enzyme and results in the formation of the E2-Ub thioester. Finally, ubiquitin from the E2 is transferred to a lysine residue or N-terminus of the substrate by the E3 ligase (Berndsen & Wolberger, 2014). Specificity of ubiquitination via recognition of the substrates is achieved by the E3 ligases (Berndsen & Wolberger, 2014). Peroxisome biogenesis is an ubiquitination-dependent process and is regulated via the ubiquitination of the PTS receptors (Erdmann, 2016; Honsho et al., 2016; Platta et al., 2016).

### 3.1 | Pex5

Pex5 functions as the receptor for most PTS1 containing matrix proteins and helps in the transport of newly synthesized proteins from the cytosol into the peroxisomal matrix (Kiel et al., 2005; van der Leij et al., 1993). In *S. cerevisiae*, Pex5 together with Pex14 forms a transient translocation pore on the peroxisome membrane (Meinecke et al., 2010). The C-terminal domain of Pex5 consists of six TPRs (tetratricopeptide repeats) and acts as the binding site for the tripeptide PTS1 (Platta et al., 2004). The N-terminal domain interacts with the PMPs of import complex, that is, Pex13 and Pex14 and helps in the docking of the receptor-cargo complex at the peroxisomal membrane followed by the release of cargo into the peroxisomal matrix (Ma et al., 2011; Walter & Erdmann, 2019). Subsequently, Pex5 is recycled back to the cytosol in an ATP-dependent mechanism for the next round of protein import (Wang & Subramani, 2017).

Pex5 undergoes both mono and polyubiquitination. Monoubiquitination of Pex5 at the conserved N-terminal cysteine residue ($Cys^6$) is required for its export back to the cytosol from the peroxisomal membrane. This removal of Pex5 from the membrane also requires AAA proteins, Pex1 and Pex6 (Kiel, Emmrich, et al., 2005; Schwartzkopff et al., 2015). In *S. cerevisiae*, recruitment of the Pex1-Pex6 complex to the peroxisomal membrane requires Pex15 (Birschmann et al., 2003). Monoubiquitination of Pex5 is catalyzed by the E2 enzyme Pex4 (Platta et al., 2007). Pex4 is anchored to the peroxisome membrane by Pex22 (El Magraoui et al., 2014). A complex of three RING E3 ligases (Pex2, Pex10, and Pex12) facilitates the monoubiquitination of Pex5 at the conserved $Cys^6$ (Platta et al., 2009; Schwartzkopff et al., 2015). This ubiquitination at the $Cys^6$ was also proposed to protect Pex5 from proteasomal degradation (Schwartzkopff et al., 2015). Pex5 deubiquitination is mediated by the enzyme Ubp15 and facilitates the next round of import by the receptor (Debelyy et al., 2011). In *K. phaffii*, an interesting role for the monoubiquitination of Pex5 on cysteine residue ($Cys^{10}$) as a mechanism that senses cellular redox status has been reported (Ma et al., 2013).

Polyubiquitination of Pex5 was identified in cells lacking components of the recycling machinery (Platta et al., 2004). This was observed either because of the disruption of dislocase activity of the AAA subcomplex or a disordered monoubiquitination step because of mutations in the Pex4/

Pex22 subcomplex (Kiel, Emmrich, et al., 2005; Kragt et al., 2005; Platta et al., 2004, 2016; Williams et al., 2007). E2 enzymes Ubc4/5 and the RING finger proteins are required for the polyubiquitination of Pex5 (Kiel, Emmrich, et al., 2005; Kragt et al., 2005; Platta et al., 2004). Two lysine residues ($Lys^{18}$, $Lys^{24}$) present at the N-terminus of Pex5 are required for ubiquitin attachment by K48 linkage and act as a signal for the degradation by the proteasome in *S. cerevisiae*. Polyubiquitinated Pex5 is subsequently recognized by the Pex1 and Pex6 and is directed to the proteasome for degradation. Accumulation of polyubiquitinated Pex5 in in *S. cerevisiae* cells with defective 26S proteasomes was reported (Kiel, Emmrich, et al., 2005; Platta et al., 2004). Polyubiquitination of *O. polymorpha* Pex5 at the conserved $Lys^{21}$ has also been reported (Kiel et al., 2005). A role for the quality control RADAR (Receptor Accumulation and Degradation in Absence of Recycling) pathway that removes nonfunctional, membrane-accumulated Pex5 by proteasomal degradation was also proposed (Wang & Subramani, 2017). Recent studies have also identified an oleic acid-inducible paralog of Pex5 in *S. cerevisiae* called Pex9, which also acts as a receptor for some of the PTS1-containing proteins (Effelsberg et al., 2016; Yifrach et al., 2016).

PEX5 stability was also reported to decrease in PBD patient cells deficient in *PEX1*, *PEX6* or *PEX26*. However, no mutations corresponding to the ubiquitination sites are reported to date in any PBD patients (Wang & Subramani, 2017). In human cells, Pex5 was also reported to function as a stress sensor via the conserved Cys11 required for its monoubiquitination (Apanasets et al., 2014; Walton et al., 2017). Under oxidative stress, reduced import of catalase via PEX5 was reported (Walton et al., 2017). Interestingly, phosphorylation-dependent ubiquitination of PEX5 in human cells was also observed (Wang & Subramani, 2017). Peroxisome-localized protein kinase ataxia-telangiectasia mutated (ATM) phosphorylates PEX5 at $Ser^{141}$, which promoted the ubiquitination of the adjacent K209. The ubiquitinated PEX5 was further recognized by the autophagy receptor p62 and is subsequently targeted for degradation via pexophagy (Wang & Subramani, 2017).

## 3.2 | Pex7

Pex7 mediates the delivery of PTS2 containing PMPs into peroxisome lumen with the help of coreceptors Pex18/Pex21 in *S. cerevisiae* and Pex20 in other fungi (Marzioch et al., 1994; Purdue et al., 1998; Yuan et al., 2016). The cysteine and lysine residues present in Pex5 required for ubiquitination are not reported in Pex7. Constitutive proteasome-mediated degradation of Pex7 was observed in methanol-grown *K. phaffii* cells (Hagstrom et al., 2014). Peroxisome proliferation is induced when the *K. phaffii* cells are cultured in media containing either oleic acid or methanol as a carbon source (Gould et al., 1992). Monoubiquitination of Pex7 was reported in oleic acid-grown *K. phaffii* cells, where the protein is indispensable for cell growth. It served as a signal for the shuttling of the protein between the peroxisome and cytosol before it gets degraded by the proteasome (Hagstrom et al., 2014). However, when the cells are shifted to methanol (where Pex7 is not necessary for growth), the protein was observed to be polyubiquitinated and targeted for proteasomal degradation (Hagstrom et al., 2014). In methanol medium, accumulation of Pex7 was observed in mutants defective for matrix protein import (*pex2*, *pex8* and *pex14*), unlike Pex5, suggesting that degradation of Pex7 follows a different pathway from the conventional RADAR pathway (Hagstrom et al., 2014).

## 3.3 | Pex13

Pex13 is an integral membrane protein with its amino and carboxy termini exposed to the cytoplasm and helps in the translocation of peroxisomal matrix proteins (Erdmann & Blobel, 1996; Girzalsky et al., 1999). As mentioned earlier, it forms a docking complex along with Pex14 and Pex17 required for the import of matrix proteins (Erdmann & Schliebs, 2005). A recent study in *O. polymorpha* reported ubiquitin-mediated degradation of Pex13 (Chen et al., 2018). Pex13 is monoubiquitinated by the E2 Pex4 and degraded via the proteasome by the peroxisomal E3 ligase complex (Pex2, Pex10 and Pex12) (Chen et al., 2018). This finding was further supported by the observation that deletion of the components of ubiquitination machinery resulted in increased levels of Pex13 (Chen et al., 2018). A significant increase in Pex13 was observed in cells lacking Pex2 compared to WT cells (Chen et al., 2018). An increased level of Pex13 was also observed in *pex5*, *pex8* and *pex14* cells. Interestingly, in *atg1* cells (where pexophagy was inhibited), increased levels of Pex13 was not observed. This further confirmed that the increased level of Pex13 was not because of the inhibition of pexophagy but rather because of a block in the ubiquitin-proteasome system (UPS)-dependent degradation. Upon treatment of the WT cells with a protein synthesis inhibitor cycloheximide, rapid decrease in Pex13 level was observed suggesting that degradation of Pex13 is dependent on ubiquitination (Chen et al., 2018).

## 3.4 | Pex18

Pex18 is a peroxisomal protein required to import PTS2 containing matrix proteins in *S. cerevisiae* (Brown & Baker, 2003; Purdue & Lazarow, 2001; Purdue et al., 1998). As mentioned above, it is one of the coreceptors of Pex7 (Schliebs

& Kunau, 2006). In *S. cerevisiae*, monoubiquitination of Pex18 on a cysteine residue ($Cys^6$) and polyubiquitination on lysine residues ($Lys^{13}$and $Lys^{20}$) has been reported (Hensel et al., 2011). The E2 enzyme Pex4 catalyzes the monoubiquitination along with the E3 RING ligases Pex10 and Pex12 (El Magraoui et al., 2013). Monoubiquitination of Pex18 facilitates the translocation of cargo-bound Pex7 (Hensel et al., 2011). Mutation of the conserved cysteine ($Cys^6$) arrested Pex18 in a membrane-protected state in a protease protection assay (Hensel et al., 2011). Pex7, on the other hand, was found to be protease-sensitive and remained associated with the peroxisome on the cytosolic face. This showed that mutation of the cysteine residue resulted in the loss of Pex18 export, thereby inhibiting the translocation of Pex7 across the peroxisomal membrane. Pex18 export triggers the peroxisomal import of cargo-loaded Pex7 (Hensel et al., 2011). As Pex18 and Pex21 are paralogs and Pex21 also contains the conserved cysteine residue ($Cys^6$), monoubiquitination of Pex21 was also proposed. However, there is no experimental evidence for the same (Hensel et al., 2011; Purdue et al., 1998).

Polyubiquitination of Pex18 at the lysine residues ($Lys^{13}$ and $Lys^{20}$) occurs similarly to Pex5 (Hensel et al., 2011; Platta et al., 2007). Polyubiquitination and turnover of Pex18 were blocked when these lysines were mutated to arginine (Hensel et al., 2011). However, this residue change did not hamper the PTS2-protein import. El Magraoui and colleagues reported that the polyubiquitination of Pex18 was catalyzed mainly by Pex2 and Pex10, in combination with the E2 enzyme Ubc4 (El Magraoui et al., 2013). A high turnover of Pex18 by proteasomal degradation compared to Pex7 in cells grown on oleic acid was reported (Hensel et al., 2011; Purdue & Lazarow, 2001).

### 3.5 | Pex20

Pex20 is the sole coreceptor for Pex7 in yeast such as *K. phaffii*, *O. polymorpha*, *Y. lipolytica* and is required to import PTS2 containing proteins (Einwächter et al., 2001; Léon & Subramani, 2007; Otzen et al., 2005). In *K. phaffii*, monoubiquitination of Pex20 on the conserved cysteine residue ($Cys^8$) required for its recycling and the PTS2-protein import was reported (Léon & Subramani, 2007; Liu & Subramani, 2013). Pex4 catalyzes this modification of Pex20 in conjugation with the RING–complex proteins (Liu & Subramani, 2013). Unlike Pex18 of *S. cerevisiae*, the cysteine mutant of Pex20 was partially functional and facilitated protein import (Hensel et al., 2011; Léon & Subramani, 2007). Similar to Pex5 and unlike Pex18, the cysteine mutant of Pex20 degraded rapidly (Hensel et al., 2011).

Polyubiquitination of Pex20 occurs via Lys48-linked chains and degradation of the protein was observed in cells lacking AAA complex or Pex4/Pex22 (Léon et al., 2006). A conserved lysine residue ($Lys^{19}$) is the target for polyubiquitination, and the activity of all three RING–peroxisomal proteins is essential for this process (Liu & Subramani, 2013). Pex20 was reported to be nonfunctional when $Lys^{19}$ was mutated to arginine (K19R) (Léon & Subramani, 2007). The polyubiquitination-dependent degradation of Pex20 was observed to be faster than Pex18 in *pex1* and *pex4* cells. This removal of dysfunctional Pex20 via proteasomal degradation is proposed to be dependent on RADAR (Léon & Subramani, 2007; Léon et al., 2006).

### 3.6 | Pex3

Pex3 is a multi-functional PMP that acts as the docking site for Pex19 and subsequently aids the import of other PMPs into the peroxisomal membrane (Hettema et al., 2000; Höhfeld et al., 1991; Jansen & van der Klei, 2019). Apart from a role in peroxisome biogenesis, Pex3 was also reported to be important for pexophagy and the inheritance of peroxisomes in yeast (Bellu et al., 2002; Farre et al., 2008; Krikken et al., 2020; Motley et al., 2012). Pex3 in *O. polymorpha* was identified as a substrate for the peroxisomal ubiquitination machinery (Williams & van der Klei, 2013). This degradation of Pex3 was dependent on Pex2 and Pex10 and independent of Pex1 (Williams & van der Klei, 2013).

## 4 | FARNESYLATION

Farnesylation or prenylation is a type of PTM wherein a farnesyl group is covalently attached to the conserved cysteine residues at the C-terminus CAAX box motif of the protein (Palsuledesai & Distefano, 2015; Zhang & Casey, 1996). This modification is catalyzed by the enzyme farnesyltransferase (FTase) (Zhang & Casey, 1996). Farnesylation of proteins is reported to aid in the localization of the protein to the membrane and is also crucial for protein-protein interactions (Maurer-Stroh et al., 2007).

### 4.1 | Pex19

Pex19 is a soluble cytosolic protein that binds newly synthesized PMPs in the cytosol and aids in their recruitment to the peroxisome membrane with the help of Pex3 (Fujiki et al., 2006). Mislocalization of peroxisomal matrix proteins to cytosol and defects in peroxisome biogenesis were reported in *S. cerevisiae* cells lacking Pex19 (Götte et al., 1998). Deletion of *PEX19* in *S. cerevisiae* and *O. polymorpha* resulted in the absence of functional peroxisomes (Hettema et al., 2000; Otzen et al., 2004). Mislocalization of peroxisomal proteins to the cytosol was also reported. However, small peroxisomal

remnants were observed in *K. phaffii pex19* cells (Snyder et al., 1999). Morphologically identifiable peroxisomes resembling those in WT cells were observed in *pex19* cells of *Y. lipolytica* (Lambkin & Rachubinski, 2001). A recent study adds to the repertoire of Pex19 functions and highlights its role in importing tail-anchored proteins to both mitochondria and peroxisomes (Cichocki et al., 2018). Farnesylation of Pex19 at $Cys^{347}$ was first reported in *S. cerevisiae* (Götte et al., 1998). The interaction of Pex19 with Pex3 and thus peroxisome biogenesis was reported to be dependent on the presence of farnesyl group (Götte et al., 1998). However, this observation was contradicted by later reports, which showed that farnesylation of Pex19 was not essential for peroxisome biogenesis in *S. cerevisiae* (Vastiau et al., 2006). Further studies reported the requirement of farnesylation for the recognition of PMPs by Pex19 both in vivo and in vitro in *S. cerevisiae* (Rucktäschel et al., 2009). Farnesylation induced a conformational change in the secondary structure of Pex19 by increasing its α-helical content (Rucktäschel et al., 2009). A role for Pex19 in peroxisome inheritance in *O. polymorpha* was reported and blocking the farnesylation resulted in partly defective inheritance of peroxisomes (Otzen et al., 2006). This was further confirmed by the observation that interaction of Pex19 with Myo2 was essential for peroxisome inheritance in *S. cerevisiae*. This interaction was affected by mutations that block the farnesylation (Otzen et al., 2012). The CAAX domain essential for farnesylation is also conserved in other yeast species such as *K. phaffii* and *Y. lipolytica*. Mutation of conserved residues did not significantly change peroxisomal phenotype in these yeast models (Lambkin & Rachubinski, 2001; Snyder et al., 1999).

Expression of a truncated version of PEX19 with a 44-amino acid deletion at the C-terminus was observed in a PBD human cell line (Rucktäschel et al., 2009). This might signify the importance of the farnesylation motif in these cells. However, it is unclear whether the truncated Pex19 is expressed stably in the patient cells (Rucktäschel et al., 2009).

## 5 | GLYCOSYLATION

*N*-linked glycosylation is the attachment of glycan residues to the amide nitrogen of asparagine (Asn) residue of the protein with the help of an enzyme oligosaccharyltransferase (OST) (Aebi, 2013). This modification affects protein folding and thereby helps the endoplasmic reticulum (ER) to allow the passage of only properly folded proteins (Aebi et al., 2010).

### 5.1 | Pex2

Pex2 is a member of the RING finger complex required for matrix protein import and peroxisome biogenesis in yeast (Platta et al., 2009). Trafficking of Pex2 to the peroxisome membrane via the ER has been proposed (Van Der Zand et al., 2012). Glycosylation of Pex2 in the ER lumen and subsequent delivery of the glycosylated forms to the peroxisome was reported in *Y. lipolytica* (Titorenko & Rachubinski, 1998). A characteristic canonical sequence Asn-Xaa-Thr, for N-linked glycosylation in the membrane PTS (mPTS) of Pex2, was observed in *Y. lipolytica* (Titorenko & Rachubinski, 1998). Radiolabeled WT cells (grown in oleic acid-containing medium at 32°C) treated with tunicamycin (inhibitor of N-linked glycosylation) showed increased electrophoretic mobility of Pex2 when compared to untreated cells (Titorenko & Rachubinski, 1998). The exact role of glycosylation in the function of Pex2 and if the protein undergoes this PTM in other yeast species is not reported.

### 5.2 | Pex16

Pex16 is a PMP present in *Y. lipolytica* required for peroxisome division and assembly (Eitzen et al., 1997; Guo et al., 2003). Interestingly, a homologue has not been identified in other yeasts such as *S. cerevisiae* and *O. polymorpha* (Kiel et al., 2006). Pex16 also facilitates the import of peroxisomal proteins, such as catalase, thiolase and isocitrate lyase (Eitzen et al., 1997). Similar to Pex2, trafficking of Pex16 to the peroxisome membrane via the ER has also been proposed (Titorenko & Rachubinski, 1998). Glycosylation of Pex16 in the ER lumen and subsequent delivery of the glycosylated forms to the peroxisome was reported in *Y. lipolytica* (Titorenko & Rachubinski, 1998). Glycosylated Pex16 was dually localized to the ER lumen and peroxisomal matrix and was also observed to be associated with the membranes of both organelles. Similar to Pex2, Asn-Xaa-Thr canonical sequence is also found in Pex16 and upon treatment with tunicamycin, increased electrophoretic mobility of Pex16, compared to untreated cells, was observed (Titorenko & Rachubinski, 1998). However, the role of N-glycosylation in the function of Pex16 and peroxisome biogenesis is still not known. Mutations in human PEX16 are identified as one of the genetic causes for PBDs. Glycosylation of human PEX16 has not been reported, but a role for the C-terminal half of the protein in its function has been reported (Honsho et al., 1998).

## 6 | CONCLUSION

Post-translational modifications modulate several aspects of proteins such as their subcellular localization, interactions and functions in eukaryotic cells. Interestingly, a ubiquitous role for these modifications in various cellular functions is

reported. Studying these has become more relevant as several disease-associated functions of a protein are regulated by the modifications it undergoes. A role for PTMs of proteins in optimum functioning of peroxisomes is also under investigation. Although many modifications have been identified using high-throughput studies, few are validated and studied for their significance. One key remaining question is how diverse PTMs on multiple peroxisomal proteins impact their function. Interestingly, modifications in response to conditions such as enhanced oxidative stress have also been reported. This brings us to another interesting avenue of research to identify conditions that induce/alter PTMs that can lead to protein-protein interactions and modify the functions of peroxisomes. Furthermore, it is important to recognize whether these modifications occur in specific cell types or are conserved. Cross-regulation between PTMs can also be envisaged, and future studies can elucidate their impact on peroxisome function.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

TI, RD and SG performed the literature search, wrote the initial draft and prepared the figures. SN conceived the idea, wrote, edited and reviewed the manuscript. All authors read and approved the final manuscript.

## ETHICAL APPROVAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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