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# Unique Requirements for Mono- and Polyubiquitination of the Peroxisomal Targeting Signal Co-receptor, Pex20*[S]

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**Background:** Pex20 recycling and degradation depend on its ubiquitination.
**Results:** Ubiquitination of Pex20 and the roles of the E2 enzyme Pex4, the RING peroxins (Pex2/Pex10/Pex12), and Pex7 in Pex20 ubiquitination were determined *in vivo*.
**Conclusion:** The RING peroxins and Pex4 are required for Pex20 mono/polyubiquitination, whereas Pex7 is only required for its polyubiquitination.
**Significance:** This novel mechanism of ubiquitination is not used by other PTS receptors.

**In *Pichia pastoris*, the peroxisomal targeting signal 2 (PTS2)-dependent peroxisomal matrix protein import pathway requires the receptor, Pex7, and its co-receptor Pex20. A conserved lysine ($Lys^{19}$) near the N terminus of Pex20 is required for its polyubiquitination and proteasomal degradation, whereas a conserved cysteine ($Cys^{8}$) is essential for its recycling. In this study, we found that $Cys^{8}$ is required for the DTT-sensitive mono- and diubiquitination of Pex20. We also show that the PTS2 cargo receptor, Pex7, is required for Pex20 polyubiquitination. Pex4, the E2 ubiquitin-conjugation enzyme, is required for monoubiquitination of Pex20. However, it is also necessary for polyubiquitination of Pex20, making its behavior distinct from the ubiquitination described for other PTS receptors. Unlike the roles of specific RING peroxins in Pex5 ubiquitination, we found that all the RING peroxins (Pex2, Pex10, and Pex12) are required as E3 ubiquitin ligases for Pex20 mono- and polyubiquitination. A model for Pex20 ubiquitination is proposed based on these observations. This is the first description of the complete ubiquitination pathway of Pex20, which provides a better understanding of the recycling and degradation of this PTS2 cargo co-receptor.**

Peroxisomes are single membrane-enclosed organelles that house oxidative enzymes involved in a variety of metabolic pathways (1). Peroxisomal matrix proteins endowed with peroxisomal targeting signals (PTS),[3] PTS1 and/or PTS2, are imported into the organelle via the action of specific co-receptors and/or receptors. This theme is exemplified by the PTS1 receptor, Pex5 (2), and the PTS2 receptor, Pex7, with its co-receptors, such as Pex5L in higher eukaryotes (3, 4), or Pex18/Pex21 in *Saccharomyces cerevisiae* (5, 6), or Pex20 in other fungi (7–10), respectively.

After delivering the cargo into the peroxisomal matrix, the receptors/co-receptors are recycled for the next round of cargo import or degraded by the proteasome via the receptor accumulation and degradation in the absence of recycling (RADAR) pathway (7, 11) when the recycling is impaired. During the recycling and degradation, the receptors are modified via the ubiquitination pathway, in which the ubiquitin-activating enzyme (E1) transfers ubiquitin (Ub) to an ubiquitin-conjugating enzyme (E2), and a protein-Ub ligase (E3) binds both Ub-E2 and substrate and facilitates the direct or indirect transfer of the Ub moiety onto the substrate protein (12). The Ub is covalently linked via an isopeptide bond between its C-terminal glycine and an $\epsilon$-amino group of a lysine on the substrate protein. However, Ub can also be linked to the $\alpha$-amino group of the N-terminal amino acid on the substrate protein or more rarely via a thioester bond to a cysteine or an oxyester bond to a serine or threonine on the substrate protein (13).

It is known that the recycling and degradation of Pex5 and Pex18 depend on mono- and polyubiquitination pathways, respectively. The N-terminal conserved cysteines of Pex5 ($Cys^{11}$ in mammals and $Cys^{6}$ in *S. cerevisiae*) (2, 14) and Pex18 ($Cys^{6}$ in *S. cerevisiae*) (5) are required for monoubiquitination and recycling. The N-terminal conserved lysines of Pex5 ($Lys^{18}$/$Lys^{24}$ in *S. cerevisiae* and $Lys^{21}$ in *Hansenula polymorpha*) (11, 14) and Pex18 ($Lys^{13}$/$Lys^{20}$ in *S. cerevisiae*) (5) are required for polyubiquitination and degradation. A conserved lysine near the N terminus of Pex20 ($Lys^{19}$ in *Pichia pastoris*) is required for polyubiquitination and degradation (7), whereas an N-terminal conserved cysteine of *P. pastoris* Pex20 ($Cys^{8}$) is essential for its recycling (15). However, it is unknown whether $Cys^{8}$ is a site for monoubiquitination.

Pex4/Pex22 in yeast and plants (16) and UbcH5 in mammals (17) function as the E2 ubiquitin-conjugation enzymes in monoubiquitination of Pex5, whereas Ubc4 in *S. cerevisiae* (18) functions as the E2 enzyme in the polyubiquitination of Pex5.

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[S] This article contains supplemental Tables S1–S3.
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[3] The abbreviations used are: PTS, peroxisomal targeting signals; RADAR, receptor accumulation and degradation in the absence of recycling; Ub, ubiquitin.

The E2 enzymes involved in Pex20 mono/polyubiquitination are not known.

The roles of the RING subcomplex in Pex5 ubiquitination were only determined in *S. cerevisiae in vitro*. The RING peroxins Pex2, Pex10, and Pex12 form a heteromeric complex and stabilize each other *in vivo* (19–21). *In vitro* data showed that ScPex12 functions as E3 ligase for monoubiquitination of Pex5 (18). ScPex2 (18) and ScPex10 (22) have been implicated as E3 enzymes for polyubiquitination of Pex5, because mutation or truncation of ScPex10 only reduces Pex5 polyubiquitination (22), whereas this receptor modification is completely absent when ScPex2 is mutated (18). ScPex10 functions as a central component and directly binds to ScPex2 and ScPex12 while bridging the indirect interaction between these two RING peroxins, and the ubiquitination activity of the Pex10/Pex12 RING domains is enhanced in the presence of Pex4 *in vitro* (21). However, the E2 and E3 ligases involved in Pex20 mono- and polyubiquitination have not been characterized.

Pex20 interacts indirectly with PTS2 cargo through Pex7 and functions in the translocation of the Pex7-cargo complexes, although Pex7 alone does not require Pex20 for translocation across the peroxisomal membrane into the matrix (7). However, no role has been described for Pex7 in Pex20 ubiquitination.

Most of these insights on the sites and enzymes involved in these ubiquitination steps and the biological role of mono- or polyubiquitination of PTS receptors have come from studies on the PTS1 receptor, Pex5, and to a far lesser extent from studies on the PTS2 pathway co-receptor Pex18/Pex20. Understanding these processes for the Pex20 family of proteins, the subject of this paper, is essential for a complete understanding of the PTS2 import pathway, as well as for an appreciation of the co-evolution of the PTS1 and PTS2 import pathways. Additionally, this information on the PTS2 pathway is relevant for disease because impairments in this pathway cause rhizomelic chondrodysplasia punctata in humans, polymorphisms in the PTS2 receptor are associated with some autism spectrum disorders (23), and defects in this pathway impair fungal pathogenicity (24).

In this study, we show for the first time that the $Cys^8$ is required for the DTT-sensitive, mono/diubiquitination of Pex20 that relies on the E2 enzyme, Pex4. Pex7 and Pex4 were also found for the first time to affect polyubiquitination of Pex20. Unlike the roles of specific RING peroxins in Pex5 ubiquitination described in other studies, we found that all three RING peroxins (Pex2, Pex10, and Pex12) are required as E3 protein-Ub ligases for Pex20 mono- and polyubiquitination. A model for Pex20 ubiquitination is proposed based on these observations. This is the first description of the complete ubiquitination pathway of Pex20, which provides new roles of Pex4, Pex7, and RING peroxins in Pex20 mono/polyubiquitination, thereby providing a better understanding of the recycling and degradation of this PTS2 cargo co-receptor.

## EXPERIMENTAL PROCEDURES

*Strains, Plasmids, and Culture Conditions*—Strains, plasmids, and oligonucleotides used are listed in supplemental Tables S1–S3, respectively. Growth media include rich medium YPD and oleate medium YNO (7). All of the cultures were grown at 30 °C in YPD to 1 $A_{600}$/ml, washed with distilled $H_2O$, and shifted to YNO for biochemical experiments. Oleate induction was overnight (16 h) unless otherwise indicated in the figures.

*Immunofluorescence Microscopy*—Samples were prepared as described previously (25). Anti-thiolase antibody (1:40,000 dilution), anti-Pex12 antibody (1:20,000 dilution), or anti-HA antibody (1:100 dilution) was used as primary antibody. Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen; 1:2000 dilution) and/or Alexa Fluor 568 goat anti-rat IgG antibody (Invitrogen; 1:2000 dilution) was used as secondary antibody.

*Subcellular Fractionation*—Oleate-grown cells were grown overnight and homogenized as described previously (26), except that the last centrifugation was performed at 200,000 × *g* to ensure pelleting of peroxisome remnants in *pex* mutants (27).

*Protease Protection Assay*—The cells were broken as for subcellular fractionation but without protease inhibitors. Pellets of a 200,000 × *g* centrifugation (see previous section) were resuspended in ice-cold Dounce buffer (26) to a protein concentration of 1 mg/ml. Freshly prepared proteinase K (40 μg) and trypsin (40 μg) were added to 200 μg of pellet fraction in the absence or presence of 0.5% Triton X-100, respectively. Aliquots were taken after incubation at room temperature for the indicated times. Trichloroacetic acid (final concentration, 12.5%) was added to terminate the reactions. Proteins were precipitated overnight on ice, washed three times with ice-cold acetone, and resuspended in lysis buffer. Equal amounts of samples were loaded to SDS-PAGE and subjected to immunoblot analysis. Anti-HA, anti-Pex8, anti-Pex17, anti-catalase, anti-Pex5, anti-thiolase, anti-Pex2, anti-Pex10, anti-Pex12, and anti-G6PDH antibodies were used as primary antibodies.

*In Vivo Ubiquitination Assay for Pex20*—Oleate-grown cells (8 ODs) were collected and resuspended in 200 μl of ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EDTA) containing the following inhibitors (Sigma): yeast protease inhibitor mixture, NaF (10 mM), leupeptin (1 μg/ml), aprotinin (1 μg/ml), PMSF (1 mM), *N*-ethylmaleimide (20 mM), and MG-132 (100 μM). The cells were broken with glass beads for 10 min at 4 °C and pelleted at 4,000 × *g* for 5 min. SDS sample buffer was then added, and samples were boiled for 5 min. 20 μl were loaded to SDS-PAGE and subjected to immunoblot analysis. Anti-HA and anti-F1β antibodies were used as primary antibodies.

*In Vivo Ubiquitination Assay for Pex5*—Oleate-grown cells (8 ODs) were collected, and crude extracts were prepared in the presence of TCA (28). Anti-HA and anti-F1β were used as primary antibodies for immunoblotting.

*Protein Stability Assay*—Oleate-grown cells (5 ODs) were collected and resuspended in 200 μl of ice-cold lysis buffer as described above. Cell lysates were made using glass beads. 10 μl were loaded to SDS-PAGE and subjected to immunoblot analysis. Anti-HA and anti-F1β antibodies were used as primary antibodies.

## RESULTS

*$Cys^8$ Is Required for Pex20 Mono/Diubiquitination and Recycling*—Pex20-HA was expressed in a *pex20*Δ mutant strain from its endogenous promoter (1.5 kb), which was almost fully

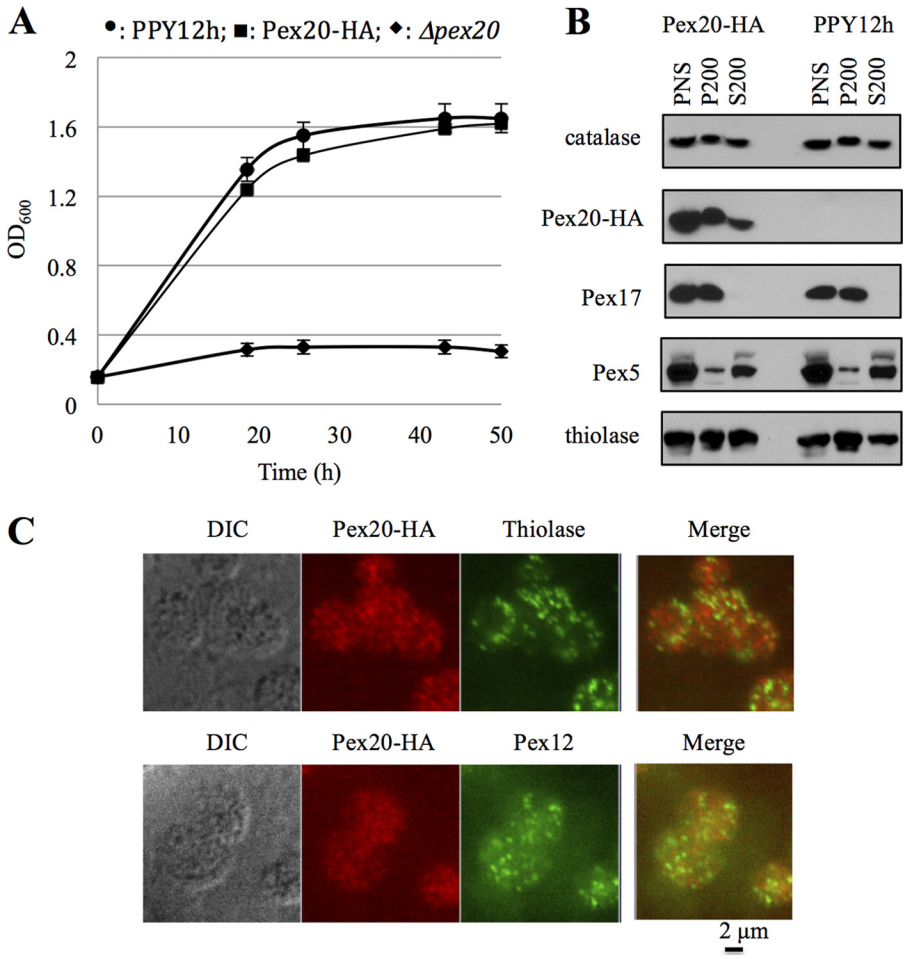


FIGURE 1. **Pex20-HA complements the *pex20*Δ strain.** *A*, growth of Pex20-HA strain compared with wild-type PPY12h and *pex20*Δ in oleate. The strains used were slxq63, PPY12h, and *pex20*Δ. *B*, subcellular fractionation of Pex20-HA. The strains used were slxq63 and PPY12h. *C*, assessment of peroxisomal protein import. Immunofluorescence microscopy of Pex20-HA and peroxisomal matrix (thiolase) and membrane (Pex12) markers in the slxq63 strain. *DIC*, differential interference contrast.

functional as determined by growth curves on oleate (Fig. 1*A*), subcellular fractionation assays (Fig. 1*B*), and import of peroxisomal matrix markers (Fig. 1*C*). Pex20 (C8S)-HA (incapable of recycling), Pex20 (K19R)-HA (incapable of polyubiquitination on Lys[19]), or Pex20 (C8S/K19R)-HA (potentially incapable of mono- or polyubiquitination on Cys[8] or Lys[19]) were expressed from their endogenous promoters in the *pex20*Δ mutant strain. The Pex20 (C8S)-HA strain grew partially, and the Pex20 (C8S/K19R)-HA strain had a strong growth defect, whereas the Pex20 (K19R)-HA strain grew almost as well as the wild-type Pex20-HA strain (Fig. 2*A*). After overnight induction in oleate, Pex20 (C8S)-HA was unstable compared with the Pex20 (K19R)-HA, Pex20 (C8S/K19R)-HA and wild-type Pex20-HA fusion proteins (Fig. 2*B*). These results suggested that the mutation of C8S, which cannot be recycled, might render Pex20 (C8S)-HA unstable by making it available for polyubiquitination on Lys[19] and proteasomal degradation via the RADAR pathway. Consistent with a role for Cys[8] in Pex20 recycling, Pex20 (K19R)-HA was stable and functional because it can be recycled. However, Pex20 (C8S/K19R)-HA, which cannot be recycled or degraded by the RADAR pathway, is stable but nonfunctional. These results, regarding the roles of N-terminal Cys and Lys residues of the PTS receptors in receptor recycling and

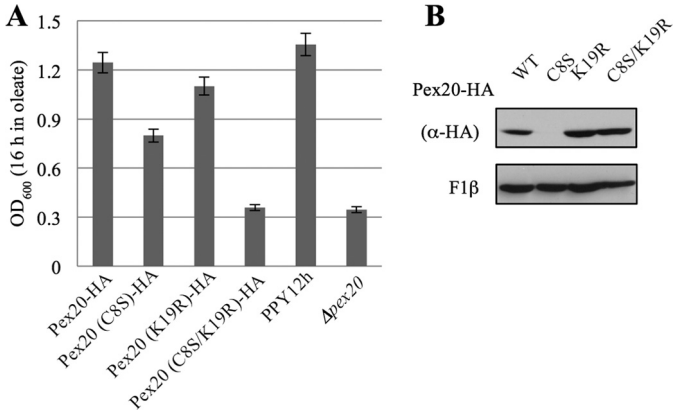


FIGURE 2. **Cys[8] and Lys[19] are required for Pex20 recycling degradation, respectively.** *A*, growth of Pex20-HA and its mutants in oleate. The strains used were slxq63, slxq66, slxq71, slxq96, PPY12h, and *pex20*Δ. *B*, stabilities of Pex20-HA and its mutants. The strains used were slxq63, slxq66, slxq71 and slxq96.

RADAR, are consistent with previous findings for Pex20-GFP (7, 15), Pex5 (2, 14), and Pex18 (5).

The polyubiquitination of Pex20 on Lys[19] was first observed in the Pex20-GFP strain when $His_6$-Myc-tagged Ub (K48R) was constitutively overexpressed to block Lys[48]-branched poly-

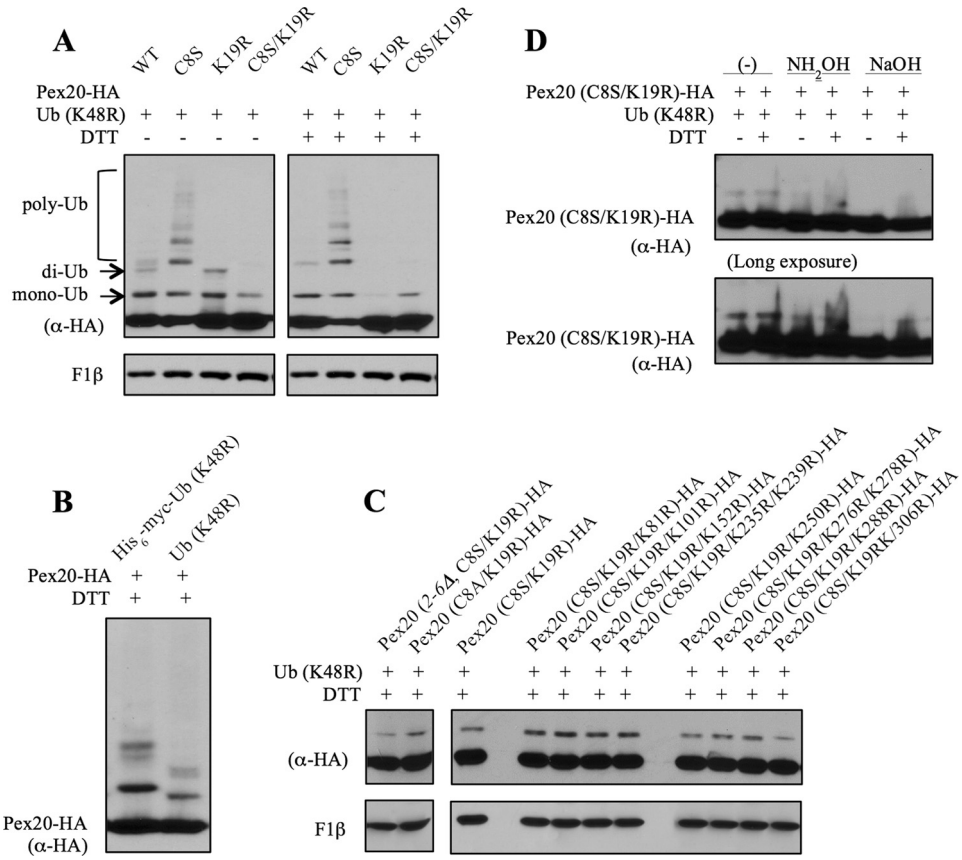


FIGURE 3. **Pex20 is mono/diubiquitinated on Cys[8] and polyubiquitinated on Lys[19].** *A*, ubiquitination of Pex20-HA and its mutants in $His_6$-Myc-Ub (K48R) overexpression strains. The strains used were slxq65, slxq68, slxq73, and slxq97. *B*, ubiquitination of Pex20-HA in $His_6$-Myc-Ub (K48R) and Ub (K48R) overexpression strains. The strains used were slxq65 and slxq174. *C*, the deletion of the first 6 amino acids and mutation of Pex20 (C8A/K19R) or other lysines do not affect the DTT-resistant monoubiquitination of Pex20 (C8S/K19R)-HA. The strains used were slxq160, slxq170, slxq152 to slxq159, and slxq97. *D*, the DTT-resistant monoubiquitination band of Pex20 (C8S/K19R)-HA (strain slxq97) was susceptible to hydroxylamine and NaOH. Cell extracts made for the ubiquitination assay were incubated with hydroxylamine or NaOH at 30 °C for 1 h before SDS-PAGE.

ubiquitination (7). However, the monoubiquitination of Pex20 was not detected (7, 15). To detect the ubiquitination status of Pex20-HA, $His_6$-Myc-tagged Ub (K48R) was overexpressed in Pex20-HA and its mutants. In the *in vivo* ubiquitination assay, we found for the first time the DTT-sensitive mono/diubiquitination of Pex20 (K19R)-HA (Fig. 3*A*). Pex20-HA was found mostly to be mono- and also polyubiquitinated (DTT-resistant) (Fig. 3*A*) and stable (Fig. 2*B*), whereas Pex20 (C8S)-HA was robustly polyubiquitinated (DTT-resistant) (Fig. 3*A*) and unstable, as expected (Fig. 2*A*) (7, 15). The ubiquitinated band shifted to a lower molecular weight (from 10.9 kDa to 8.8 kDa) when the N-terminal $His_6$-Myc tag was removed (Fig. 3*B*), indicating that the modification is ubiquitination. Therefore, these results strongly suggest that Cys[8] acquires the DTT-sensitive, mono/diubiquitination in Pex20 (K19R)-HA.

Notably, Pex20 (C8S/K19R)-HA, which is missing both the normal mono- and polyubiquitination sites on Pex20, was stable (Fig. 2*B*) and gave an aberrant DTT-resistant monoubiquitination band, which could not be on Cys[8] or Lys[19] (Fig. 3*A*). The deletion of the first 6 amino acids or mutation of these ubiquitination sites in Pex20 (C8A/K19R) or each of the lysines in Pex20 (C8S/K19R)-HA singly or doubly, did not affect the DTT-resistant monoubiquitination (Fig. 3*C*), indicating that neither these N-terminal residues nor the serine 8 in C8S or any lysine is involved in the aberrant monoubiquitination. However, the aberrant monoubiquitination band of Pex20 (C8S/K19R)-HA was sensitive to mild alkaline conditions, such as hydroxylamine and NaOH treatment (Fig. 3*D*), indicating that the ubiquitination is by esterification on an unknown serine or threonine (13). Because Pex20 (C8S/K19R)-HA fails to be recycled and degraded and is not functional (Fig. 2), this aberrant monoubiquitination on the Ser/Thr is of minor importance for Pex20 physiological function in peroxisomes.

*RING Subcomplex Components Are Required for Monoubiquitination/Recycling and Polyubiquitination/Degradation of Pex20*—The prototypic RING domain coordinates two zinc atoms in a cross-brace fashion through one histidine and seven cysteine residues and obeys the consensus $^{36}CX_2CX_9CXHX_2$-$CX_2CX_4CX_2C^{50}$ (referred to as the C3HC4 finger) (29, 30). Among the peroxins, Pex2 and Pex10 possess a canonical RING domain in most species (31–34), whereas in Pex12, only five of the eight conserved residues are present (35–37), suggesting that Pex12 can bind only one zinc atom per monomer. The RING peroxins are required for each other's stability (19, 20, 38), whereas the first two Cys residues of the RING domain are known to be essential for their E3 ligase activity (39).

We created double-point mutations at the first two Cys of the RING domains in Pex2, Pex10, and Pex12, to inactivate their E3 ligase without destabilization of the RING subcomplex. The

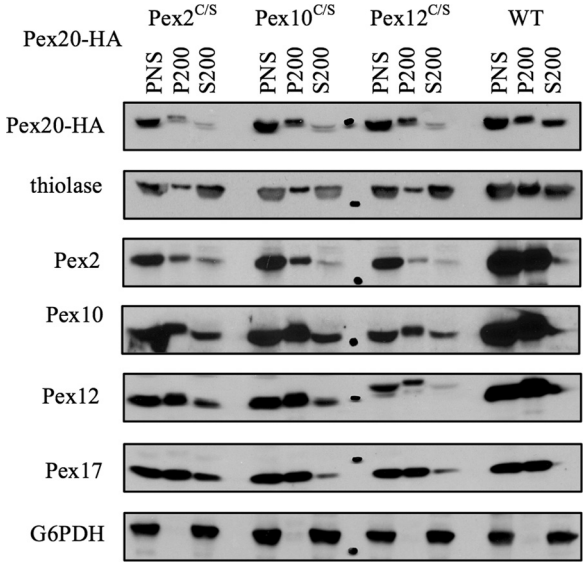


FIGURE 4. **The majority of peroxins (Pex20-HA, Pex2, Pex10, and Pex12), PTS2 cargo thiolase and G6PDH (cytosolic marker) are mostly correctly localized in RING domain point mutants.** The strains used were slxq167, slxq168, slxq169, and slxq63.

subcellular fractionation assay showed that the majority of Pex20-HA, RING peroxins, and peroxisomal membrane protein Pex17 were localized correctly in the RING domain point mutants in comparison to the wild-type strain (Fig. 4), even though less thiolase is imported in the RING mutants, and the recovery of Pex2 (especially in Pex12 C/S) is poor. Therefore, for further studies, Pex2, Pex10, and Pex12 RING domain point mutations were used to maintain their stabilities and that of the RING subcomplex.

Pex2 (C281S/C284S), Pex10 (C313S/C316S), and Pex12 (C339S/C342S) had growth defects in oleate (Fig. 5*A*) (as well as in methanol, data not shown), indicating that Pex2, Pex10, and Pex12 are required for peroxisome biogenesis. After overnight induction in oleate, the Pex2, Pex10, and Pex12 RING domain point mutants were more stable than their expressed counterparts in the *pex2*Δ, *pex10*Δ, and *pex12*Δ deletion strains (Fig. 5*B*).

To check the roles of RING peroxins in the Pex20 ubiquitination, Pex2 (C281S/C284S), Pex10 (C313S/C316S), and Pex12 (C339S/C342S) were expressed in the Pex20-HA, Pex20 (C8S)-HA, and Pex20 (K19R)-HA strains also overexpressing $His_6$-Myc-tagged UB (K48R). In the *in vivo* ubiquitination assay, Pex2, Pex10, and Pex12 RING domain point mutations all affected Pex20-HA ubiquitination (Fig. 5*C*). Note in particular the disappearance of the di-Ub band for Pex20 (K19R)-HA, which is typical of $Cys^8$ ubiquitination (because it is DTT-sensitive) (Fig. 5*D*), as well as the disappearance of Pex20 (C8S)-HA polyubiquitination (Fig. 5*E*). However, the RING peroxin mutants produced the aberrant DTT-resistant monoubiquitination band (Fig. 5*D*, *right panel* with DTT). Therefore, all three RING peroxins are essential for Pex20 monoubiquitination during recycling and for polyubiquitination during degradation.

*Pex4 Is Required for Pex20 Mono- and Diubiquitination on $Cys^8$*—To determine the roles of E2 enzymes (*i.e.*, Pex4 or Ubc4) in Pex20 ubiquitination, the *PEX4* or *UBC4* gene was deleted in the strains expressing Pex20 (C8S)-HA and Pex20 (K19R)-HA, respectively, in which $His_6$-Myc-tagged Ub (K48R) was overexpressed. In the *in vitro* ubiquitination assay, in the *pex4*Δ deletion, Pex20 (K19R)-HA only showed a monoubiquitination band, which was likely to be on $Cys^8$, because most, but not all, of this band was DTT-sensitive (Fig. 6*A*, compare the *second* and *fourth lanes*). The small amount of DTT-resistant monoubiquitination of the Pex20 (K19R)-HA remaining in the *pex4*Δ deletion (Fig. 6*A*, *fourth lane*) corresponds to the aberrant ubiquitination described earlier using Pex20 (C8S/K19R)-HA and is produced only when *both* the receptor recycling and RADAR pathways are nonfunctional. Although the loss of the diubiquitinated Pex20 (K19R)-HA in the *pex4*Δ deletion, relative to wild-type cells (Fig. 6*A*, *first* and *second lanes*), suggested that Pex4 is necessary for the mono- and diubiquitination at $Cys^8$ on Pex20, we were surprised by the appearance of monoubiquitination (Fig. 6*A*, *second lane*) on $Cys^8$ (suggested by DTT sensitivity of most of this species, Fig. 6*A*, *fourth lane*), in the absence of Pex4. This must mean that some other Ubc is capable of monoubiquitination of $Cys^8$ when Pex4 is absent. Our experiments suggest that this monoubiquitination on $Cys^8$ by an alternative Ubc is a peculiar situation that we have uncovered only when the RADAR pathway, which is the normal preferred pathway that is activated in *pex4*Δ cells, is crippled by mutation of the polyubiquitination site ($Lys^{19}$) necessary for the RADAR pathway, and this monoubiquitination is seen only after overnight growth of the cells on oleate (*i.e.*, it is a slow process). This result suggests that in wild-type cells, Pex4 is necessary for the mono- and diubiquitination on $Cys^8$ (Fig. 6*A*); when Pex4 is absent (or when C8S is used), the preferred pathway is that Pex20 is polyubiquitinated on $Lys^{19}$ and degraded rapidly by the RADAR pathway (Fig. 3*A*), but when Pex4 is absent and the RADAR pathway is inactivated, only then is it possible for some other redundant Ubc to substitute for Pex4, to put just a mono-Ub on $Cys^8$, and with much lower efficiency on another DTT-resistant site (Fig. 6*A*, *second* and *fourth lanes*).

Because the overexpression of Ub (K48R) slows down the proteasomal degradation and causes the accumulation of polyubiquitination of Pex20 (C8S)-HA (7), we further checked the effect of Pex4 on Pex20 (C8S)-HA polyubiquitination at shorter time points (from 1 to 3 h) (Fig. 6*B*) and found that Pex20 (C8S)-HA polyubiquitination was delayed at a shorter time point (3 h) in the *pex4*Δ strain and that there was more of the DTT-resistant mono- and diubiquitinated species, relative to the wild-type strain (Fig. 6*B*), indicating that although Pex4 is not essential, the *pex4*Δ deletion does slow the Pex20 polyubiquitination.

To understand the delay of Pex20 (C8S)-HA polyubiquitination in the *pex4*Δ deletion, we checked its stability in the *pex4*Δ deletion strain without overexpressing Ub (K48R). Interestingly, after overnight induction in oleate, Pex20 (C8S)-HA was stabilized in the *pex4*Δ cells and gave the DTT-resistant mono/diubiquitination bands, whereas Pex20 (C8S)-HA was completely degraded by the RADAR pathway in the presence of Pex4 (Fig. 6*C*). This mono/diubiquitination is likely on $Lys^{19}$ because these DTT-resistant bands disappear in Pex20 (K19R)-HA (Fig. 6*A*, *fourth lane*). Thus, Pex4 is required for efficient polyubiquitination of Pex20 (C8S)-HA after some Ubc, other than Pex4, has initiated mono- and diubiquitina-

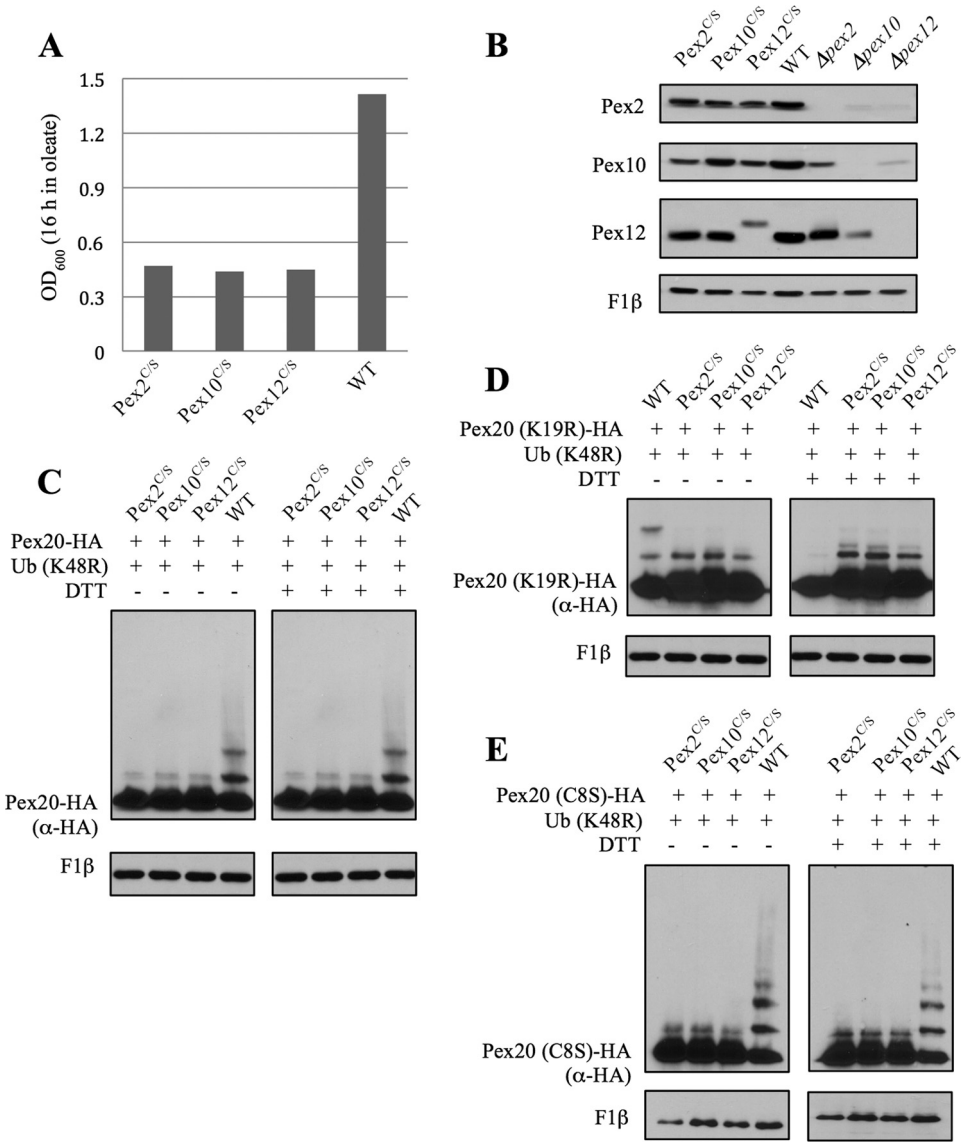


FIGURE 5. **All three RING peroxins are required for both mono- and polyubiquitination of Pex20.** *A*, growth curve in oleate for RING domain point mutants. The strains used were slxq167, slxq168, slxq169, and slxq63. *B*, stabilities of RING peroxins in WT and mutants. The strains used were slxq167, slxq168, slxq169, slxq63, *pex2*Δ, *pex10*Δ, and *pex12*Δ. *C*, RING domain point mutations affect the ubiquitination of Pex20-HA. The strains used were slxq126, slxq127, slxq128, and slxq65. *D*, RING domain point mutations affect the mono/diubiquitination of Pex20 (K19R)-HA. The strains used were slxq73, slxq103, slxq104, and slxq105. *E*, RING domain point mutations affect the polyubiquitination of Pex20 (C8S)-HA. The strains used were slxq99, slxq100, slxq101, and slxq68.

tion at Lys[19]. The action of redundant cellular Ubcs that perform the DTT-resistant mono- and di-Ub additions on Pex20 (C8S)-HA and the nonessential role of Pex4 in polyubiquitination account for the stabilization of the Pex20 (C8S)-HA (Fig. 6*C*) and its slower polyubiquitination in the *pex4*Δ strain (Fig. 6*B*). Additionally, the *ubc4*Δ deletion did not affect Pex20 (K19R)-HA monoubiquitination or Pex20 (C8S)-HA polyubiquitination (data not shown), indicating either that Ubc4 is not required for Pex20 mono/polyubiquitination or that other substitute/redundant E2 enzymes might be involved.

Overall, Pex4 is required not only for Pex20 mono/diubiquitination on its Cys[8] during its recycling, but also for a step in the polyubiquitination of Pex20 at Lys[19]. However, Pex4 is not obligatory for Pex20 polyubiquitination, because other Ubcs can substitute for it, albeit less efficiently. This role of Pex4 in polyubiquitination of other receptors, such as Pex5 and Pex18, may have been missed or alternatively may be peculiar to Pex20.

*Pex7 Is Only Required for Pex20 Polyubiquitination*—To check the requirement of Pex7 in Pex20 mono/polyubiquitination, the *PEX7* gene was deleted in the strains expressing Pex20 (C8S)-HA and Pex20 (K19R)-HA, in which $His_6$-Myc-tagged Ub (K48R) was overexpressed. In the *in vivo* ubiquitination assay, the *pex7*Δ deletion did not affect the DTT-sensitive mono- or diubiquitination of Pex20 (K19R)-HA (Fig. 7*A*) but affected Pex20 (C8S)-HA polyubiquitination (Fig. 7*B*), indicating that Pex7 is only required for Pex20 polyubiquitination. In a previous study, mutation of the conserved Ser residue (S280F) in Pex20 disrupted the interaction with Pex7 (7). We also checked the ubiquitination of Pex20 (C8S/S280F)-HA in which $His_6$-Myc-tagged Ub (K48R) was overexpressed,

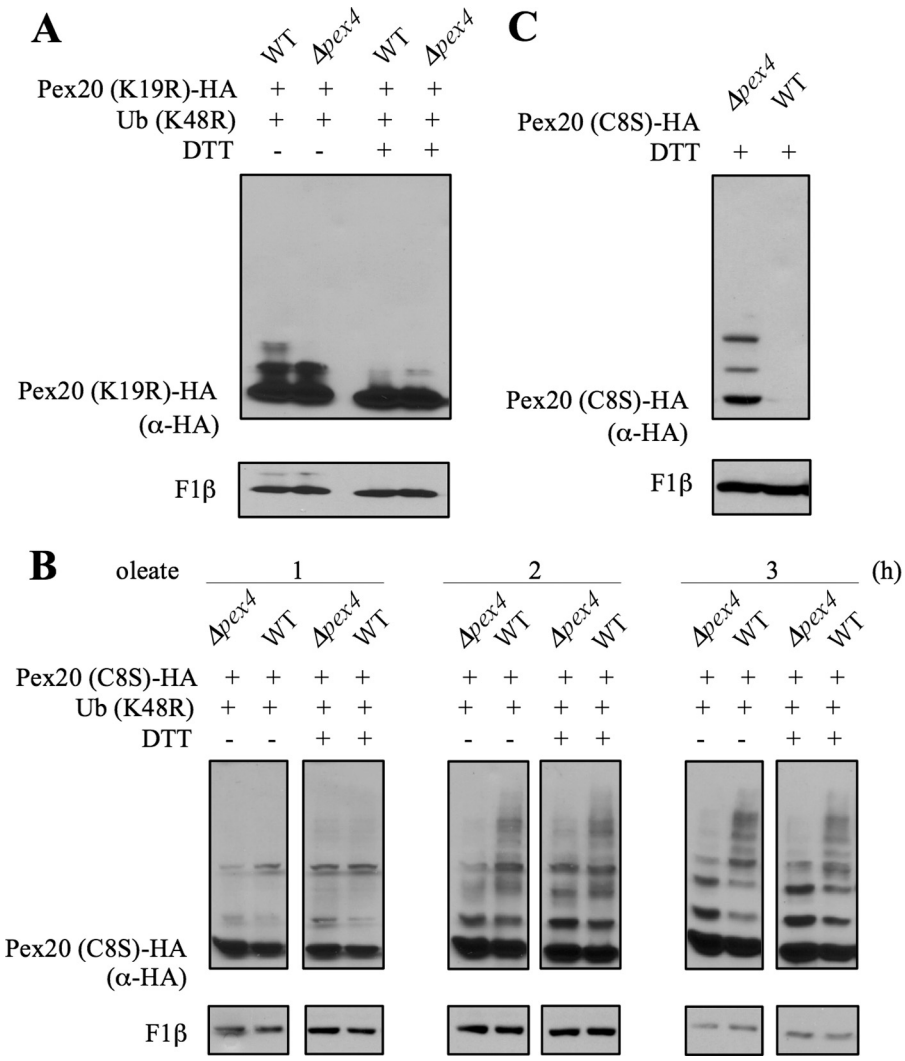


FIGURE 6. **Pex4 is required for Pex20 mono/diubiquitination on Cys[8] and also its polyubiquitination on Lys[19].** *A*, the *pex4*Δ deletion affected Pex20 (K19R)-HA mono/diubiquitination. The strains used were slxq73 and slxq84. *B*, the *pex4*Δ deletion affected Pex20 (C8S)-HA polyubiquitination at shorter time points (1–3 h). The strains used were slxq83, slxq68, and slxq129. *C*, the *pex4*Δ deletion stabilized Pex20 (C8S)-HA. The strains used were slxq175 and slxq66.

which showed the same loss of polyubiquitination (Fig. 7*B*), indicating that the interaction of Pex7 and Pex20 is required for Pex20 polyubiquitination.

After overnight induction in oleate, Pex20 (C8S)-HA was stabilized in the *pex7*Δ strain (Fig. 7*C*), whereas Pex20 (C8S/S280F)-HA was also more stable as compared with Pex20 (C8S)-HA (Fig. 7*C*), which is consistent with the finding that Pex7 is essential for Pex20 polyubiquitination during its degradation. The protease protein assay performed on the P200 fraction from Pex20 (C8S)-HA + *pex7*Δ strain showed that the import of Pex20 (C8S)-HA is not impacted by the *pex7*Δ deletion (Fig. 7*D*).

Overall, we conclude that Pex7 itself and the interaction of Pex7 with Ser[280] on Pex20 are not required for Pex20 import into peroxisomes but are indeed necessary either for Pex20 export or polyubiquitination. However, because Pex20 monoubiquitination, which also requires Pex20 import and export, does not require Pex7, it is most likely that Pex7 is required specifically for Pex20 polyubiquitination and degradation by the RADAR pathway, which is a novel finding.

## DISCUSSION

*Pex20 Mono/Diubiquitination and Recycling Depend on Cys[8]*—Pex20 acts as a co-receptor for Pex7 and is required for the import of PTS2 cargos into the peroxisomal matrix. Ubiquitination of Pex20 was first observed in the *P. pastoris pex4*Δ deletion strain or in cells overexpressing Ub (K48R) (7). At the N terminus of Pex20, the conserved Lys[19] is required for polyubiquitination and degradation via the RADAR pathway, whereas Cys[8] is required for recycling (7). However, in this earlier study, monoubiquitination was not found (15). Pex20-GFP, used in this previous study and driven by a 0.5-kb endogenous promoter, only partially complements the *pex20*Δ deletion strain and did not allow us to find the monoubiquitination site (7). In this study, we expressed Pex20-HA and its mutants under the control of a longer endogenous promoter (1.5 kb), which allowed us for the first time to detect the DTT-sensitive mono/diubiquitination of Pex20 on Cys[8] (Fig. 3*A*). The two types of ubiquitination of Pex20, dependent on the conserved Cys[8] (for DTT-

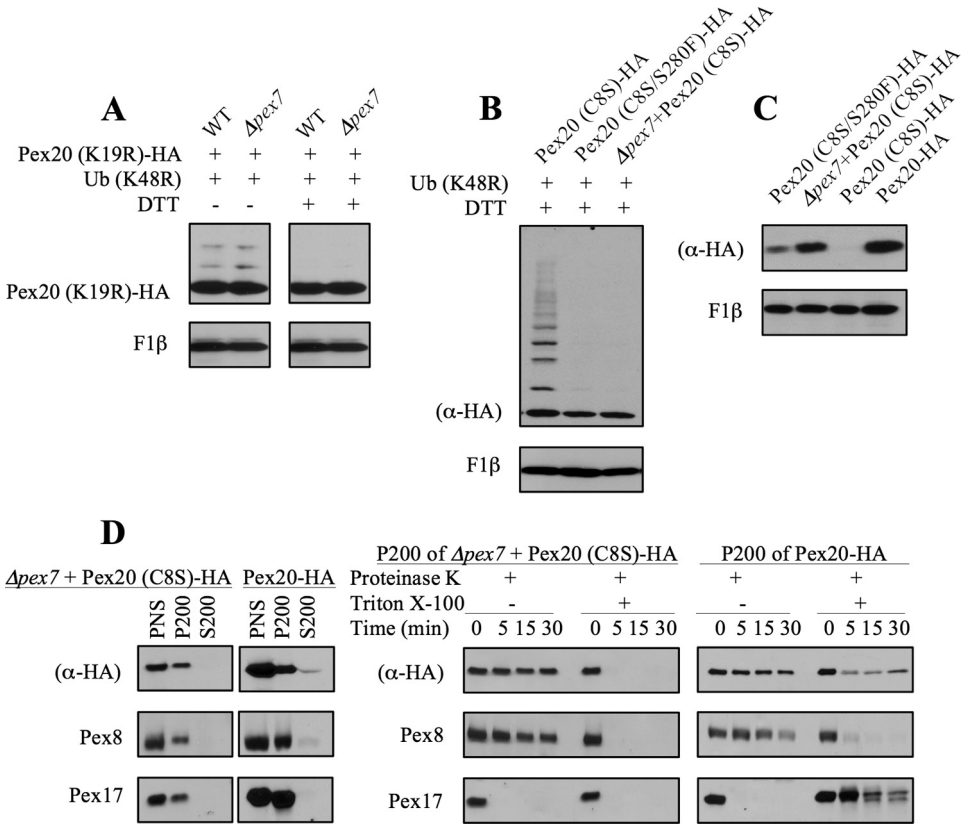


FIGURE 7. **Pex7 is only required for Pex20 polyubiquitination.** *A*, the *pex7*Δ deletion did not affect Pex20 (K19R)-HA mono/diubiquitination. The strains used were slxq73 and slxq140. *B*, the S280F mutation or *pex7*Δ deletion affected Pex20 (C8S)-HA polyubiquitination. The strains used were slxq68, slxq57, and slxq139. *C*, the S280F mutation or the *pex7*Δ deletion stabilized Pex20 (C8S)-HA. The strains used were slxq53, slxq59, slxq66, and slxq63. *D*, subcellular fractionation and protease protection assay of the P200 fraction isolated from a PNS of Pex20 (C8S)-HA + *pex7*Δ and Pex20-HA. The strains used were slxq59 and slxq63.

sensitive mono/diubiquitination) and Lys[19] (for DTT-resistant polyubiquitination) (Fig. 3*A*) are consistent with what was found in Pex5 and Pex18 ubiquitination, suggesting that the N-terminal conserved cysteine and lysine(s) have the same function in Pex20, Pex5, and Pex18 ubiquitination during the recycling and degradation. These receptors/co-receptors therefore appear to have co-evolved similar strategies for the recycling and degradation.

*Pex2, Pex10, and Pex12 Function Nonredundantly as E3 Ub Ligases for Both Mono- and Polyubiquitiantion of Pex20*—The roles of RING peroxins in ubiquitination of Pex5 were first determined in *S. cerevisiae in vitro*. In this study, we analyzed the stabilities of RING peroxins in strains deleted for the corresponding genes or with point mutations in their RING domains, as well as their effect on Pex20 ubiquitination (Fig. 5). Pex2, Pex10, and Pex12 RING domain double point mutants all have strong growth defects on oleate and impair the mono/polyubiquitination of Pex20. We conclude that in *P. pastoris*, Pex2, Pex10, and Pex12 are all required for Pex20 mono/polyubiquitination during its recycling and degradation. The same requirement of RING peroxins in Pex5 ubiquitination was also found *in vivo* (Fig. 8). This is the first *in vivo* finding of the requirements of three RING peroxins in mono/polyubiquitination of the PTS receptors/co-receptors. This is different from the previous *in vitro* studies, which showed that ScPex12 is required for ScPex5 monoubiquitination (18), whereas ScPex10 (22) or ScPex2 (18) is required for ScPex5 polyubiquitination.

*Pex4 Facilitates Both Mono- and Polyubiquitination of Pex20 but Is Not Essential for the Latter*—In *S. cerevisiae*, monoubiquitination of the receptor on a cysteine is facilitated by the E2 protein Pex4, whereas the Pex4-like UbcH5a/b/c does this in humans (11, 14, 18). In *S. cerevisiae*, polyubiquitination of the receptor on lysine(s) is not Pex4-dependent (18). Interestingly, we demonstrated here that Pex4 not only facilitates the Pex20 mono/diubiquitination on Cys[8] but also affects the efficiency of Pex20 polyubiquitination on Lys[19] (Fig. 6). Another E2 enzyme, Ubc4, was required for polyubiquitination of Pex5 in *S. cerevisiae* (14, 18, 40); however, the deletion of the *P. pastoris UBC4* gene did not abolish the mono- or polyubiquitination of Pex20 (data not shown). A role for Ubc4 would be missed in our analysis if a redundant protein fulfilled its function.

*Pex7 Is Only Required for Pex20 Polyubiquitination*—Pex7, the PTS2 cargo receptor, interacting with Pex20 during cargo import, as well as its interaction with Pex20 through Ser[280], are required for Pex20 polyubiquitination (Fig. 7), indicating a new role for Pex7 in Pex20 degradation via the RADAR pathway. Because Pex7 is not necessary for Pex20 mono/diubiquitination on Cys[8], which requires Pex20 import into and export from peroxisomes during the import cycle, we conclude that Pex7 is required for the actual polyubiquitination of Pex20 and its subsequent degradation by the RADAR pathway. Pex7 is believed to shuttle between the cytosol and peroxisomal lumen in an "extended shuttle" model (41), like Pex5. However, no evidence

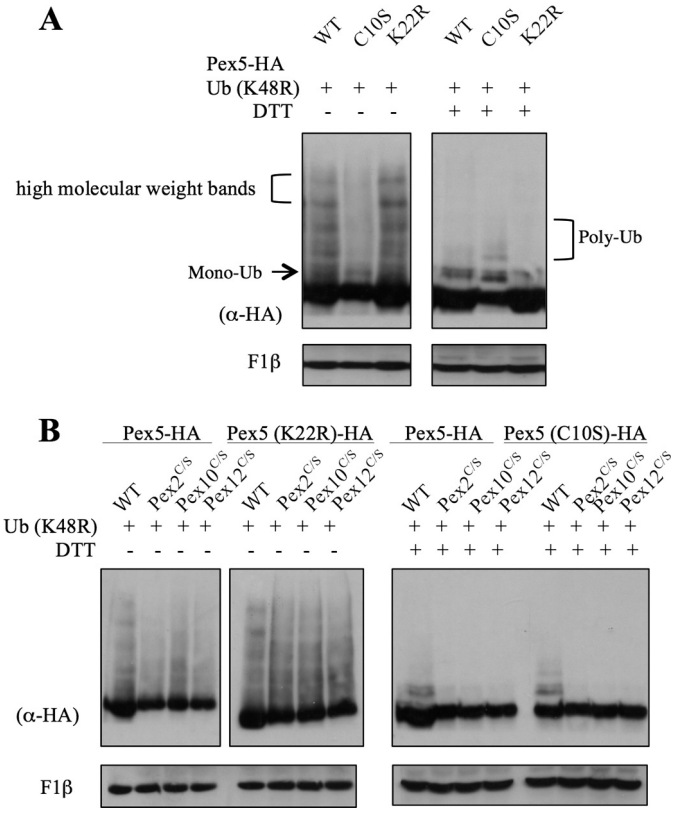


FIGURE 8. **All three RING peroxins are required for both mono- and polyubiquitination of Pex5.** *A*, the ubiquitination of Pex5-HA and its mutants in $His_6$-Myc-Ub (K48R) overexpression strains. The strains used were slxq122, slxq123, slxq124, and slxq125. *B*, RING domain point mutations affect the ubiquitination of Pex5-HA and its mutants. The strains used were slxq122, slxq189, slxq190, slxq191, slxq124, slxq144, slxq145, slxq146, slxq123, slxq141, slxq142, and slxq143.

exists to show that Pex7 recycling and degradation are dependent on ubiquitination.

*A Model for the Pex20 Ubiquitination Pathway*—We propose a schematic model for Pex20 ubiquitination during the peroxisomal matrix protein import cycle based on our findings (Fig. 9). As a PTS2 cargo co-receptor, Pex20, interacts with Pex7 during PTS2 cargo import. After cargo release, Pex20 would be exported back to the cytosol for the next round of import. The mono/diubiquitination of Pex20 occurs on $Cys^8$, and this, without requiring interaction with Pex7, facilitates receptor recycling. We do not know whether the mono/diubiquitination of Pex20 precludes its association with Pex7 or whether recycling is simply the kinetically favored pathway under these circumstances. Alternatively, when the receptor recycling machinery is impaired, Pex20 binding to Pex7 is necessary for its polyubiquitination on $Lys^{19}$ and subsequent degradation by the RADAR pathway.

Pex2, Pex10, and Pex12 function as E3 Ub ligases, which are all required for both mono- and polyubiquitination of Pex20. Pex4 functions as E2 ubiquitin-conjugation enzyme, which facilitates the first (and second) Ub to Pex20 during its mono/diubiquitination, as well as for the initiation or elongation step, during the polyubiquitination process. However, whereas Pex4 is required in an obligatory fashion for Pex20 monoubiquitination, in its absence other Ubcs can assume its role, albeit less efficiently, to allow Pex20 polyubiquitination. The Ub hydrolase, Ubp15 from *S. cerevisiae* and USP9X from mammalian cells, are capable of removing Ub from ubiquitinated Pex5 (42, 43). However, the deletion of the *P. pastoris UBP15* gene did not impair mono- or polyubiquitination of Pex20 (data not

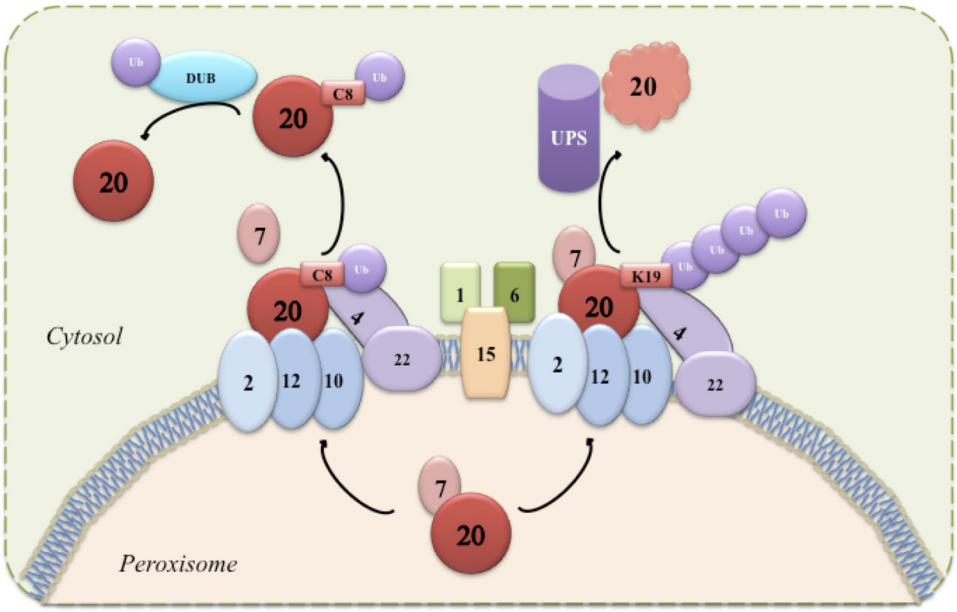


FIGURE 9. **Model for Pex20 ubiquitination during its recycling and RADAR.** The *numbers* denote Pex proteins assigned these designated numbers. *C8* and *K19* represent $Cys^8$ and $Lys^{19}$ on Pex20, respectively. Single or multiple *circles* with *Ub* denote mono- or poly-Ub on $Cys^8$ or $Lys^{19}$ of Pex20, respectively. Pex20 interacts with Pex7 during PTS2 cargo import. After cargo release, Pex20 is recycled back to the cytosol for the next round of import. The mono- and diubiquitination of Pex20 occurs on $Cys^8$, and its recycling does not require Pex7 or interaction between Pex20 and Pex7. When the recycling machinery is impaired by mutation, Pex20 interaction with Pex7 and its subsequent polyubiquitination on $Lys^{19}$ are necessary for Pex20 degradation (Pex20 cloud) by the ubiquitin proteasome system (*UPS*). Pex2, Pex10, and Pex12 function as E3 ubiquitin ligases, which are required for both mono- and polyubiquitination of Pex20. Pex4 functions as the E2 ubiquitin-conjugating enzyme, which is not only required for Pex20 mono- and diubiquitination on $Cys^8$ during its recycling but also as a nonessential kinetic component for a step in the polyubiquitination of Pex20 on $Lys^{19}$.

shown), suggesting that other deubiquitinating enzymes may perform this function in *P. pastoris* cells.

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## REFERENCES


1. Liu, X., Ma, C., and Subramani, S. (2012) Recent advances in peroxisomal matrix protein import. *Curr. Opin. Cell Biol.* **24,** 484–489
2. Okumoto, K., Misono, S., Miyata, N., Matsumoto, Y., Mukai, S., and Fujiki, Y. (2011) Cysteine ubiquitination of PTS1 receptor Pex5p regulates Pex5p recycling. *Traffic* **12,** 1067–1083
3. Honsho, M., Hashiguchi, Y., Ghaedi, K., and Fujiki, Y. (2011) Interaction defect of the medium isoform of PTS1-receptor Pex5p with PTS2-receptor Pex7p abrogates the PTS2 protein import into peroxisomes in mammals. *J. Biochem.* **149,** 203–210
4. Khan, B. R., and Zolman, B. K. (2010) *pex5* mutants that differentially disrupt PTS1 and PTS2 peroxisomal matrix protein import in *Arabidopsis*. *Plant Physiol.* **154,** 1602–1615
5. Hensel, A., Beck, S., El Magraoui, F., Platta, H. W., Girzalsky, W., and Erdmann, R. (2011) Cysteine-dependent ubiquitination of Pex18p is linked to cargo translocation across the peroxisomal membrane. *J. Biol. Chem.* **286,** 43495–43505
6. Stein, K., Schell-Steven, A., Erdmann, R., and Rottensteiner, H. (2002) Interactions of Pex7p and Pex18p/Pex21p with the peroxisomal docking machinery. Implications for the first steps in PTS2 protein import. *Mol. Cell. Biol.* **22,** 6056–6069
7. Léon, S., Zhang, L., McDonald, W. H., Yates, J., 3rd, Cregg, J. M., and Subramani, S. (2006) Dynamics of the peroxisomal import cycle of PpPex20p. Ubiquitin-dependent localization and regulation. *J. Cell Biol.* **172,** 67–78
8. Otzen, M., Wang, D., Lunenborg, M. G., and van der Klei, I. J. (2005) *Hansenula polymorpha* Pex20p is an oligomer that binds the peroxisomal targeting signal 2 (PTS2). *J. Cell Sci.* **118,** 3409–3418
9. Sichting, M., Schell-Steven, A., Prokisch, H., Erdmann, R., and Rottensteiner, H. (2003) Pex7p and Pex20p of *Neurospora crassa* function together in PTS2-dependent protein import into peroxisomes. *Mol. Biol. Cell* **14,** 810–821
10. Einwächter, H., Sowinski, S., Kunau, W. H., and Schliebs, W. (2001) *Yarrowia lipolytica* Pex20p, *Saccharomyces cerevisiae* Pex18p/Pex21p and mammalian Pex5pL fulfil a common function in the early steps of the peroxisomal PTS2 import pathway. *EMBO Rep.* **2,** 1035–1039
11. Platta, H. W., El Magraoui, F., Schlee, D., Grunau, S., Girzalsky, W., and Erdmann, R. (2007) Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling. *J. Cell Biol.* **177,** 197–204
12. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu. Rev. Cell Dev. Biol.* **22,** 159–180
13. Wang, X., Herr, R. A., and Hansen, T. H. (2012) Ubiquitination of substrates by esterification. *Traffic* **13,** 19–24
14. Williams, C., van den Berg, M., Sprenger, R. R., and Distel, B. (2007) A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p. *J. Biol. Chem.* **282,** 22534–22543
15. Léon, S., and Subramani, S. (2007) A conserved cysteine residue of *Pichia pastoris* Pex20p is essential for its recycling from the peroxisome to the cytosol. *J. Biol. Chem.* **282,** 7424–7430
16. Williams, C., van den Berg, M., Panjikar, S., Stanley, W. A., Distel, B., and Wilmanns, M. (2012) Insights into ubiquitin-conjugating enzyme/co-activator interactions from the structure of the Pex4p:Pex22p complex. *EMBO J.* **31,** 391–402
17. Grou, C. P., Carvalho, A. F., Pinto, M. P., Wiese, S., Piechura, H., Meyer, H. E., Warscheid, B., Sá-Miranda, C., and Azevedo, J. E. (2008) Members of the E2D (UbcH5) family mediate the ubiquitination of the conserved cysteine of Pex5p, the peroxisomal import receptor. *J. Biol. Chem.* **283,** 14190–14197
18. Platta, H. W., El Magraoui, F., Bäumer, B. E., Schlee, D., Girzalsky, W., and Erdmann, R. (2009) Pex2 and Pex12 function as protein-ubiquitin ligases in peroxisomal protein import. *Mol. Cell. Biol.* **29,** 5505–5516
19. Agne, B., Meindl, N. M., Niederhoff, K., Einwächter, H., Rehling, P., Sickmann, A., Meyer, H. E., Girzalsky, W., and Kunau, W. H. (2003) Pex8p. An intraperoxisomal organizer of the peroxisomal import machinery. *Mol. Cell* **11,** 635–646
20. Hazra, P. P., Suriapranata, I., Snyder, W. B., and Subramani, S. (2002) Peroxisome remnants in *pex3*Δ cells and the requirement of Pex3p for interactions between the peroxisomal docking and translocation subcomplexes. *Traffic* **3,** 560–574
21. El Magraoui, F., Bäumer, B. E., Platta, H. W., Baumann, J. S., Girzalsky, W., and Erdmann, R. (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
22. Williams, C., van den Berg, M., Geers, E., and Distel, B. (2008) Pex10p functions as an E3 ligase for the Ubc4p-dependent ubiquitination of Pex5p. *Biochem. Biophys. Res. Commun.* **374,** 620–624
23. Ro, M., Park, J., Nam, M., Bang, H. J., Yang, J., Choi, K. S., Kim, S. K., Chung, J. H., and Kwack, K. (2012) Association between peroxisomal biogenesis factor 7 and autism spectrum disorders in a Korean population. *J. Child Neurol.* **27,** 1270–1275
24. Goh, J., Jeon, J., Kim, K. S., Park, J., Park, S. Y., and Lee, Y. H. (2011) The PEX7-mediated peroxisomal import system is required for fungal development and pathogenicity in Magnaporthe oryzae. *PLoS One* **6,** e28220
25. Yan, M., Rachubinski, D. A., Joshi, S., Rachubinski, R. A., and Subramani, S. (2008) Dysferlin domain-containing proteins, Pex30p and Pex31p, localized to two compartments, control the number and size of oleate-induced peroxisomes in *Pichia pastoris*. *Mol. Biol. Cell* **19,** 885–898
26. Faber, K. N., Heyman, J. A., and Subramani, S. (1998) Two AAA family peroxins, PpPex1p and PpPex6p, interact with each other in an ATP-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes. *Mol. Cell. Biol.* **18,** 936–943
27. Harper, C. C., South, S. T., McCaffery, J. M., and Gould, S. J. (2002) Peroxisomal membrane protein import does not require Pex17p. *J. Biol. Chem.* **277,** 16498–16504
28. Baerends, R. J., Faber, K. N., Kram, A. M., Kiel, J. A., van der Klei, I. J., and 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. *J. Biol. Chem.* **275,** 9986–9995
29. Borden, K. L., and Freemont, P. S. (1996) The RING finger domain. A recent example of a sequence-structure family. *Curr. Opin. Struct. Biol.* **6,** 395–401
30. Capili, A. D., Edghill, E. L., Wu, K., and Borden, K. L. (2004) Structure of the C-terminal RING finger from a RING-IBR-RING/TRIAD motif reveals a novel zinc-binding domain distinct from a RING. *J. Mol. Biol.* **340,** 1117–1129
31. Kalish, J. E., Theda, C., Morrell, J. C., Berg, J. M., and Gould, S. J. (1995) Formation of the peroxisome lumen is abolished by loss of *Pichia pastoris* Pas7p, a zinc-binding integral membrane protein of the peroxisome. *Mol. Cell. Biol.* **15,** 6406–6419
32. Okumoto, K., Abe, I., and Fujiki, Y. (2000) Molecular anatomy of the peroxin Pex12p. Ring finger domain is essential for Pex12p function and interacts with the peroxisome-targeting signal type 1-receptor Pex5p and a ring peroxin, Pex10p. *J. Biol. Chem.* **275,** 25700–25710
33. Schumann, U., Wanner, G., Veenhuis, M., Schmid, M., and Gietl, C. (2003) AthPEX10, a nuclear gene essential for peroxisome and storage organelle formation during *Arabidopsis* embryogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 9626–9631
34. Krazy, H., and Michels, P. A. (2006) Identification and characterization of three peroxins–PEX6, PEX10 and PEX12–involved in glycosome biogenesis in *Trypanosoma brucei*. *Biochim. Biophys. Acta* **1763,** 6–17
35. Kalish, J. E., Keller, G. A., Morrell, J. C., Mihalik, S. J., Smith, B., Cregg, J. M., and Gould, S. J. (1996) Characterization of a novel component of the peroxisomal protein import apparatus using fluorescent peroxisomal proteins. *EMBO J.* **15,** 3275–3285

36. Okumoto, K., Shimozawa, N., Kawai, A., Tamura, S., Tsukamoto, T., Osumi, T., Moser, H., Wanders, R. J., Suzuki, Y., Kondo, N., and Fujiki, Y. (1998) PEX12, the pathogenic gene of group III Zellweger syndrome. cDNA cloning by functional complementation on a CHO cell mutant, patient analysis, and characterization of PEX12p. *Mol. Cell. Biol.* **18,** 4324–4336
37. Albertini, M., Girzalsky, W., Veenhuis, M., and Kunau, W. H. (2001) Pex12p of *Saccharomyces cerevisiae* is a component of a multi-protein complex essential for peroxisomal matrix protein import. *Eur. J. Cell Biol.* **80,** 257–270
38. Zhang, L., Léon, S., and Subramani, S. (2006) Two independent pathways traffic the intraperoxisomal peroxin PpPex8p into peroxisomes. Mechanism and evolutionary implications. *Mol. Biol. Cell* **17,** 690–699
39. Amemiya, Y., Azmi, P., and Seth, A. (2008) Autoubiquitination of BCA2 RING E3 ligase regulates its own stability and affects cell migration. *Mol. Cancer Res.* **6,** 1385–1396
40. Platta, H. W., Girzalsky, W., and Erdmann, R. (2004) Ubiquitination of the peroxisomal import receptor Pex5p. *Biochem. J.* **384,** 37–45
41. Nair, D. M., Purdue, P. E., and Lazarow, P. B. (2004) Pex7p translocates in and out of peroxisomes in *Saccharomyces cerevisiae*. *J. Cell Biol.* **167,** 599–604
42. Debelyy, M. O., Platta, H. W., Saffian, D., Hensel, A., Thoms, S., Meyer, H. E., Warscheid, B., Girzalsky, W., and Erdmann, R. (2011) Ubp15p, a ubiquitin hydrolase associated with the peroxisomal export machinery. *J. Biol. Chem.* **286,** 28223–28234
43. Grou, C. P., Francisco, T., Rodrigues, T. A., Freitas, M. O., Pinto, M. P., Carvalho, A. F., Domingues, P., Wood, S. A., Rodríguez-Borges, J. E., Sá-Miranda, C., Fransen, M., and Azevedo, J. E. (2012) Identification of ubiquitin-specific protease 9X (USP9X) as a deubiquitinase acting on ubiquitin-peroxin 5 (PEX5) thioester conjugate. *J. Biol. Chem.* **287,** 12815–12827