Peroxin-dependent targeting of a lipid-droplet-destined membrane protein to ER subdomains

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Lipid droplets (LDs) are endoplasmic reticulum (ER)-derived lipid storage organelles uniquely encapsulated by phospholipid monolayers. LD membrane proteins are embedded into the monolayer in a monotopic hairpin topology and are therefore likely to have requirements for their biogenesis distinct from those inserting as bitopic and polytopic proteins into phospholipid bilayers. UBXD8 belongs to a subfamily of hairpin proteins that localize to both the ER and LDs, and are initially inserted into the cytoplasmic leaflet of the ER bilayer before partitioning to the LD monolayer. The molecular machinery responsible for inserting hairpin proteins into membranes, however, is unknown. Here, we report that newly synthesized UBXD8 is post-translationally inserted into discrete ER subdomains by a mechanism requiring cytosolic PEX19 and membrane-integrated PEX3, proteins hitherto exclusively implicated in peroxisome biogenesis. Farnesylation of PEX19 uncouples ER/LD and peroxisome targeting, expanding the function of this peroxin to an ER-targeting pathway and suggesting a coordinated biogenesis of LDs and peroxisomes.

RESULTS
Post-translational insertion of UBXD8 into ER membranes
To study the mechanisms underlying membrane insertion of HP proteins, we synthesized 35S-labelled, epitope-tagged UBXD8 in rabbit reticulocyte lysate (RRL) in the absence or presence of...
ER-derived rough microsomes (RMs; Fig. 1a). Reactions were fractionated into soluble cytosolic proteins (S), peripheral membrane proteins released by extraction with sodium carbonate (P), and carbonate-resistant, membrane-integrated proteins (M). When translation was conducted in the presence of RMs, most UBXD8 was detected in the membrane fraction (M) similar to the fractionation behaviour of an SRP-dependent signal anchor protein, invariant chain (Ii)15, and a tail-anchored protein, RAMP4op10. UBXD8 and RAMP4op were efficiently integrated into RMs when RMs were present during (co-translational) or after termination (post-translational) of protein synthesis. In contrast, Iiop was only co-translationally integrated and glycosylated as expected for an SRP substrate (Fig. 1a). The opsin-tag contains a consensus N-glycosylation sequon that, on ER membrane translocation, causes a shift in relative molecular mass of ~2,000 (M, ~2K), reflecting addition of a N-linked glycan. Iiop and RAMP4op were efficiently glycosylated when incubated with RMs (Fig. 1a). However, no UBXD8 glycosylation was detected, irrespective of whether the opsin-tag was at the amino or carboxy terminus, consistent with it being inserted into RMs in its native HP topology where both termini face the cytosol. Protease treatment of RMs caused the M, 50K band corresponding to full-length UBXD8 to collapse into a single fragment with the expected size (M, ~5K) of the protected HP domain (Fig. 1b, lanes 1–2), which sedimented with membranes after re-fraccionation and was digested on detergent solubilization of RMs, consistent with it being membrane-integrated (Fig. 1b, lanes 3–5). Thus, in vitro-synthesized UBXD8 can be post-translationally integrated into RMs with the same HP topology as in ER and LD membranes.

The hydrophobic sequence in UBXD8 (amino acids 90–118) serves as a membrane HP anchor3 that is necessary and sufficient for targeting UBXD8 to LDs in vivo16,17. To assess the role of the HP domain in post-translational insertion, we deleted the hydrophobic region (UBXD8ΔHP) or used a minimal UBXD8 version consisting of the HP domain plus flanking residues (UBXD8ΔAC) and monitored membrane insertion in vitro (Fig. 1c). No UBXD8ΔHP was detected in the membrane fraction after in vitro translation/translocation whereas UBXD8ΔAC was efficiently inserted into RMs under co- and post-translational conditions. A minor fraction of UBXD8ΔAC became glycosylated (Fig. 1c), resisted protease treatment, and was efficiently affinity captured following protease treatment irrespective of the position of the tags (Fig. 1d). Therefore, a fraction of this minimal HP construct was fully translocated across the ER membrane in vitro. Most membrane-associated UBXD8ΔAC, however, gave rise to a protease-resistant M, ~5K fragment (Fig. 1d, lanes 2, 8) that failed to bind to N- or C-terminal affinity-capture reagents (Fig. 1d, lanes 4, 6, 10, 12), indicating correct UBXD8ΔAC insertion into RMs in a HP topology. Thus, the HP domain is necessary and sufficient for post-translational UBXD8 insertion into the ER membrane.

**UBXD8 membrane insertion is independent of SRP and TRC40**

Post-translational membrane insertion of UBXD8 could suggest employment of the TRC40-mediated ER-targeting pathway. To test this possibility, we used recombinant WRBcc, a soluble fragment of the TRC40 receptor WRB that binds to substrate-loaded TRC40, to block membrane insertion of tail-anchored proteins by competing with endogenous WRB12. Inclusion of excess WRBcc in our in vitro translocation assays failed to alter UBXD8 insertion efficiency into RMs, despite substantially reducing the insertion of the tail-anchored protein RAMP4op (Fig. 1e). Therefore, ER insertion of UBXD8 is independent of the TRC40–WRB pathway.

To verify this conclusion and to assess the role of the canonical SRP pathway for UBXD8 insertion into RMs, we performed import assays using RMs that had been pre-treated with either N-ethylmaleimide or trypsin (Fig. 1f and Supplementary Fig. 1). Both conditions block SRP-18,19 and TRC40/WRB-dependent protein insertion10,20. While both treatments prevented insertion of the SRP substrate liop, neither interfered with UBXD8 insertion (Fig. 1f), establishing that UBXD8 integration is independent of the SRP and TRC40 pathways and, moreover, might not require ER-integrated proteins. Indeed, in vitro-synthesized UBXD8 was present in buoyant fractions following incubation with protein-free liposomes, similar to the behaviour of cytochrome b5 (Fig. 1g), a protein known to insert into membranes independently of membrane-integrated proteins21. Association of UBXD8 with liposomes required the HP domain as UBXD8ΔHP was retained in dense fractions on sucrose gradient fractionation. Protease treatment of UBXD8-containing liposomes led to accumulation of a M, ~5K protected fragment that bound to neither N- nor C-terminal affinity-capture reagents, indicating a correct HP topology (Fig. 1h). Thus, UBXD8 can insert into membranes post-translationally and independently of canonical SRP- or TRC40-targeting pathways, protein-conducting channels or membrane protein receptors.

**UBXD8 inserts into discrete ER subdomains**

Membrane insertion of UBXD8 independently of membrane-integrated proteins raises the question of how its strict localization in cells to the ER and LDs2 is established and/or maintained. We used immunofluorescence microscopy to determine into which membranes in vitro-synthesized UBXD8 inserts in semi-permeabilized cells (Fig. 2a). Full-length UBXD8 (sUBXD8ΔOP), but not sUBXD8ΔHP-OP, was recruited to discrete subcellular foci (Fig. 2b) that are strikingly different from the characteristic reticular distribution that endogenous UBXD8 exhibits in cells at steady state. This punctate localization was not due to a general inability of proteins to insert into reticular ER in semi-permeabilized cells, because in vitro-translated RAMP4op co-localized precisely with the ER marker calreticulin (Fig. 2c). In contrast, UBXD8 foci did not strictly co-localize with the ER markers calreticulin (Fig. 2c) or calnexin (Fig. 2d) but exhibited a reticular pattern that closely followed the distribution of the ER (Fig. 2d and Supplementary Video 1), suggesting UBXD8 insertion into distinct ER subdomains. Indeed, immuno-electron microscopy of semi-permeabilized cells revealed labelling of sUBXD8ΔOP at ER membranes and on ~150-nm-diameter electron-dense structures continuous with ER membranes (Fig. 2e, arrows). sUBXD8ΔHP-OP failed to co-localize with LDs (Fig. 2f), indicating that newly synthesized UBXD8 preferentially inserts into ER subdomains and not mature LDs. UBXD8 still inserted into foci after treatment with the long-chain fatty acyl CoA synthetase inhibitor, triacsin C (Fig. 2h), suggesting that neutral lipid synthesis is dispensable for recruitment of newly synthesized UBXD8 to ER subdomains. These findings suggest that, while UBXD8 can spontaneously insert into protein-free membranes.
**Figure 1** UBXD8 is post-translationally inserted into membranes by a non-canonical ER-targeting pathway. The indicated mRNAs were translated in RRL and membrane insertion was monitored after incubation with RMs or liposomes by SDS–PAGE/autoradiography. (a) UBXD8 is post-translationally inserted into RMs. Upper panel: membrane topologies of opsin (op)-tagged signal-anchored (SA) invariant chain (Iiop), opsin-tagged tail-anchored (TA) RAMP4op, and opsin- and S-tagged UBXD8 (sUBXD8op). Opsi-tag glycosylation sites are indicated. Lower panel: proteins were synthesized in vitro without RMs (−), or with RMs present during translation (+co) or added after translation (+post). Membrane insertion was assessed by fractionation into soluble proteins (S), carbonate-labile, peripheral membrane proteins (P) and carbonate-resistant, integral membrane proteins (M). g, glycosylated. (b) Verification of UBXD8 topology. Proteinase K (PK) treatment of RMs with post-translationally inserted full-length (FL) sUBXD8op in the absence or presence of Triton X-100 (TX-100). Lanes 4 and 5: refractionated samples. pF, protected fragment; sup., supernatant. (c) The HP domain is necessary and sufficient for post-translational UBXD8 insertion into RMs. Upper panel: constructs used. Lower panel: in vitro translation/translocation of radiolabelled proteins as in a. UBA, ubiquitin-associated domain; UBX, UBX domain; Thl, thredoxin-like; CC, coiled coil. (d) Verification of UBXD8s14 topology as in b. Samples were precipitated with anti-opsin antibodies (IP anti-OP) or S-affinity purified (SAP). (e) TRC40-pathway-independent UBXD8 insertion. Post-translational protein insertion into RMs in the absence or presence of recombinant WRBcc or MBP maltose-binding protein (MBP). sUBXD8op (upper) was fractionated as in a and RAMP4op (lower) was analysed directly. g, glycosylated. (f) UBXD8 insertion persists after covalent modification of RMs. sUBXD8op (upper) and lipop (lower) were co-translationally incubated with RMs pre-treated with trypsin (T-RM) or N-ethylmaleimide (N-RM). sUBXD8op was fractionated as in a and lipop was analysed directly. g, glycosylated. (g) UBXD8 associates with protein-free liposomes. Post-translational liposome addition to in vitro translation (IVT) followed by density gradient fractionation. Upper panel: paradigm. Lower panel: analysis of sUBXD8op and sUBXD8op insertion into liposomes. (h) The UBXD8 HP domain is inserted into liposomes. Post-translational sUBXD8op insertion into liposomes in g followed by protease digestion as in d. Representative autoradiographs from a single experiment are shown. The experiments in a–c,g were repeated twice and those in d–f,h were repeated once with similar results. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
Semi-permeabilization, cytosol release

Addition of in vitro translation reaction

Wash out soluble proteins, detection of membrane-integrated proteins by IF or immuno-EM

**Figure 2** UBXD8 is inserted into ER subdomains in semi-permeabilized cells. (a) Experimental scheme: cells grown on coverslips were semi-permeabilized to release cytosolic contents and incubated with *in vitro* translation (IVT) reactions. Membrane-integrated proteins were detected by immunofluorescence (IF) microscopy (b–d,f,h) or by immuno-electron microscopy (EM) (e,g). (b) The HP region in UBXD8 is required for membrane insertion in semi-permeabilized cells. IF using anti-opsin and anti-calreticulin (ER marker) antibodies as indicated. (c) Full-length sUBXD8op inserts into discrete foci. IF of *in vitro*-translated RAMP4op and sUBXD8op as in b. (d) UBXD8 insertion sites align with the ER marker calnexin. IF of sUBXD8op co-stained with anti-calnexin antibodies. A single z-section is shown. Supplementary Video 1 shows a full z-stack. (e) UBXD8 insertion sites are associated with the ER. Electron micrographs showing immunogold-labelled sUBXD8op using anti-opsin and fluoronanogold-coupled antibodies after insertion into semi-permeabilized cells. Arrows indicate specific gold labelling at ER membranes. Small gold particles attached to all membrane structures represent nonspecific background labelling due to gold enhancement, which is also found in the control specimen to which no *in vitro*-translated protein was added (not shown). N, nucleus; M, mitochondria. (f,g) UBXD8 is not directly inserted into LDs. Before semi-permeabilization and import of *in vitro*-translated sUBXD8op, cells were treated with oleate to induce LD formation and analysed by IF with anti-opsin antibodies (BODIPY labels LDs) (f) or by EM as in e (g). (h) UBXD8 inserts into ER foci in LD-depleted cells. Cells were treated with triacsin C to inhibit neutral lipid synthesis and sUBXD8op was detected by IF using anti-opsin antibodies. Representative images from a single experiment are shown. The experiments in a–c were repeated twice, in d,e were repeated three times, and in f–h were repeated once, all with similar results. Scale bars, 10 μm unless indicated.

In *in vitro*, it is specifically inserted into discrete ER subdomains in semi-permeabilized cells before it distributes within the ER and eventually partitions to LDs. This implicates the existence of proteins specifying correct membrane targeting for nascent UBXD8 molecules.

**BAG6 and PEX19 bind to newly synthesized UBXD8 in the cytosol**

To identify potential targeting factors of newly synthesized UBXD8, we translated UBXD8 in RRL in the absence of RMs,
which should favour prolonged engagement with cytoplasmic proteins maintaining its insertion-competent state, and used affinity capture followed by LC–MS/MS identification of proteins that specifically bound insertion-competent UBXD8FL but not UBXD8SHP. Two proteins, BAG6 and PEX19, were exclusively captured from UBXD8FL pulldowns (Supplementary Table 1). To verify these interactions and to map the interaction sites within UBXD8, we translated UBXD8 deletion mutants in vitro and assessed the amount of PEX19 and BAG6 present in complex with UBXD8 by affinity isolation and immunoblotting (Fig. 3a). The UBXD8 HP region was both necessary and sufficient to engage PEX19 and BAG6. As the deletion of the proline-rich sequence immediately preceding the annotated HP region (UBXD8153–90) reduced binding to PEX19 and BAG6, we extended the UBXD8

Figure 3 UBXD8 pre-insertion complexes contain PEX19 and BAG6. (a) HP-dependent UBXD8 binding to BAG6 and PEX19. The indicated UBXD8 constructs (upper panel) were translated in RRL in the absence of membranes, S-affinity purified (SAP) and analysed by SDS–PAGE and immunoblotting with opsin- (UBXD8), BAG6- or PEX19-specific antibodies (lower panel). Lanes 1–9 show 1/20 of input reaction; lanes 10–18 show corresponding elution fractions. (b) Direct interaction of UBXD8 with PEX19 and BAG6 assessed by chemical crosslinking. sUBXD8op or sUBXD8153–116op was translated in RRL and treated with the crosslinker BMH, or dimethylsulfoxide, immunoprecipitated under denaturing conditions using the indicated antibodies and visualized by autoradiography. The mobilities of monomeric UBXD8 and crosslinked adducts for UBXD8–BAG6 and UBXD8–PEX19 are indicated. (c) Full-length UBXD8 forms distinct complexes with PEX19 and BAG6. sUBXD8op or sUBXD8153–116op translated in RRL was fractionated on sucrose density gradients, and UBXD8 complexes were S-affinity purified from individual fractions and analysed by SDS–PAGE and immunoblotting using anti-opsin (UBXD8), -BAG6 and -PEX19 antibodies. (d,e) UBXD8 insertion competence correlates with PEX19 but not BAG6 association. (d) Sucrose gradient fractionation of radiolabelled sUBXD8op complexes as described in (c) but analysed by autoradiography (upper). RMs were added to individual fractions and UBXD8 insertion competence quantified by densitometry after fractionation into soluble (S) and membrane-integrated (M) proteins as in Fig. 1 (lower). (e) Relative UBXD8 insertion competence into RMs as quantified in (d) was plotted against the relative protein amounts of PEX19 or BAG6 associated with UBXD8 in the individual sucrose gradient fractions. Immunoblot analysis and protein quantification of a parallel sucrose gradient fractionation is shown in Supplementary Fig. 2. Linear least-squares regression analysis of association with PEX19 (R² = 0.89) or BAG6 (R² = 0.67) versus UBXD8 insertion competence. a.u., arbitrary units. Representative autoradiographs from a single experiment are shown. The experiments in a–d were repeated once with similar results. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
HP deletion to include amino acids 53–111 for all subsequent experiments.

To test whether BAG6 and PEX19 bind to UBXD8 directly, we used chemical crosslinking to generate radiolabelled, covalent pre-insertion complex adducts of in vitro-synthesized UBXD8, which were immuno-isolated following protein denaturation (Fig. 3b). The BAG6 antibody precipitated high-molecular-weight ($M_r > 250$K) crosslinked adducts containing $UBXD8_{FL}$ (lane 4), while the PEX19 antibody captured adducts of $M_r \sim 80$K (lane 6). Neither antibody precipitated $UBXD8_{A33-111}$ adducts. These findings confirm HP-domain-dependent interactions of UBXD8 with BAG6 and PEX19 and strongly suggest that they are direct.

To determine whether PEX19 and BAG6 bind UBXD8 in the same or in distinct complexes, we assessed their presence in affinity-isolated UBXD8 pre-insertion complexes fractionated on sucrose gradients (Fig. 3c). $UBXD8_{FL}$ forms higher molecular weight complexes compared with $UBXD8_{A33-111}$ and cofractionated with BAG6 in fractions 6–10, whereas PEX19 was associated with $UBXD8_{FL}$ in fractions 3–6, indicating that $UBXD8_{FL}$ forms distinct complexes with PEX19 and BAG6.

UBXD8 in fractions containing PEX19 was twofold more efficiently integrated into RMs than was UBXD8 in BAG6-containing fractions (Fig. 3d). Insertion competence correlated positively with the ratio of PEX19 to UBXD8 in individual fractions (Fig. 3e and Supplementary Fig. 2), suggesting that PEX19-containing complexes facilitate insertion of newly synthesized UBXD8 into ER membranes.

**PEX19 specifies the subcellular localization of UBXD8**

To test whether UBXD8 is a client for PEX19-mediated protein targeting in living cells, we overexpressed PEX19 appended with a nuclear localization signal, NLS–PEX19, and monitored UBXD8 localization (Fig. 4a). Redirection of both co-expressed sUBXD8op and endogenous UBXD8 to the nucleus demonstrates that PEX19 is sufficient to re-localize UBXD8 in cells.

To determine whether PEX19 is required for ER localization of UBXD8, we generated $PEX19^{+/−}$ cell lines by genome editing (Supplementary Fig. 3a–c). Strikingly, endogenous UBXD8 in $PEX19^{+/−}$ cells was mislocalized to mitochondria and co-localized with HSP60 (Fig. 4bc). UBXD8 localization to ER and LDs was restored when PEX19 was reintroduced into these cells, confirming that its mislocalization was caused by the absence of PEX19 (Supplementary Fig. 3d). Thus, PEX19 specifies steady-state ER localization of endogenous UBXD8.

**PEX19 and PEX3 cooperate in UBXD8 insertion into ER subdomains**

To investigate how PEX19 targets newly synthesized UBXD8 to ER subdomains, we tested the effect of recombinant PEX3ΔN40, a soluble variant of the membrane-resident PEX19 receptor PEX3 (ref. 22), which is required for membrane insertion of peroxisomal membrane proteins21 (PMPs), on the insertion of in vitro-synthesized UBXD8 in semi-permeabilized cells. In the presence of excess PEX3ΔN40, but not WRBcc or MBP, sUBXD8$_{FL}$op was not inserted into ER foci but instead distributed more diffusely consistent with mitochondrial mislocalization (Fig. 5a, upper). In contrast, RAMP4op insertion was unaffected by PEX3ΔN40 addition but sensitive to WRBcc (Fig. 5a, lower). These data establish an essential role for PEX19 in specifying insertion of newly synthesized UBXD8 into ER subdomains, which we propose to be ER entry sites for newly synthesized UBXD8.

PEX3 knockdown in wild-type cells (Fig. 5b) abolished the insertion of in vitro-translated sUBXD8$_{FL}$op into foci after semi-permeabilization (Fig. 5c), indicating that PEX3 is essential for correct UBXD8 insertion. In contrast, PEX19 knockdown did not affect sUBXD8$_{FL}$op insertion (Fig. 5bc), most likely because of the presence of RRL-derived PEX19 bound to in vitro-translated sUBXD8$_{FL}$op (Fig. 3). Semi-permeabilized PEX19$_{WT}$/− cells, however, were not competent for inserting in vitro-translated sUBXD8$_{FL}$op into ER subdomains (Fig. 5d), most likely because these cells are also depleted of PEX3 (Supplementary Fig. 3b), consistent with a PEX19 role in stabilizing PEX3 (ref. 24). Thus, both cytosolic PEX19 and membrane-integrated PEX3 are required for correct targeting and insertion of UBXD8 into ER subdomains.

**UBXD8 ER insertion sites co-localize with endogenous PEX3 but are distinct from mature peroxisomes**

As PEX19 and PEX3 are known to insert PMPs into peroxisomes, we investigated the spatial relationship of UBXD8 insertion sites with peroxisomes in semi-permeabilized cells. Catalase-positive peroxisomes did not co-localize with UBXD8 foci but were found in close apposition (50–250 nm) in about half of the cases (Fig. 6a–d). This relationship between UBXD8 insertion sites and peroxisomes was confirmed with the peroxisomal membrane protein PEX14 (Supplementary Fig. 4).

In contrast, most UBXD8 foci (75%) co-localized with endogenous PEX3 (Fig. 6ef). However, we observed two distinct co-localization phenotypes: while 44% of total UBXD8 foci co-localized with a single PEX3 focus, an additional 31% of UBXD8 foci co-localized with PEX3 and were also adjacent (50–250 nm) to an additional PEX3-positive but UBXD8-negative focus. PEX3 resides in peroxisomes and the ER$^{25–28}$. As UBXD8 insertion sites are positive for PEX3 but negative for catalase, our data suggest that UBXD8 is specifically inserted into PEX3-containing sites that are not peroxisomes, potentially corresponding to pre-peroxisomal ER$^{29}$.

To assess the role of mature peroxisomes in UBXD8 insertion into ER subdomains, we depleted cells of PEX5, an essential peroxin for import of peroxisomal matrix proteins$^{30}$ (Supplementary Fig. 5a). Although these cells lacked mature peroxisomes (Supplementary Fig. 5b), no defect in the import of in vitro-translated sUBXD8$_{op}$ after semi-permeabilization was observed, indicating that mature peroxisomes are dispensable for UBXD8 insertion (Fig. 6g).

**PEX19 farnesylation is essential for UBXD8 localization to the ER and LDs**

PEX19 is known to be farnesylated in cells but this post-translational modification is dispensable for peroxisome biogenesis$^{31}$. To test whether PEX19 farnesylation affects UBXD8 localization, we overexpressed either PEX19$_{WT}$ or the farnesylation-deficient mutant PEX19$_{C296S}$ in wild-type cells and monitored the steady-state localization of endogenous UBXD8 by immunofluorescence (Fig. 7a–c). Overexpression of either PEX19$_{WT}$ (Fig. 7a) or PEX19$_{C296S}$ (Fig. 7b). These data demonstrate that PEX19 is required for correct targeting and insertion of UBXD8 into ER subdomains.
Figure 4 PEX19 specifies the subcellular localization of UBXD8. (a) UBXD8 follows PEX19 redirected to the nucleus. Effect of wild-type (WT) or NLS-tagged PEX19 on co-expressed or endogenous UBXD8 (UBXD8<sub>end</sub>) localization assessed by IF microscopy. PEX19, sUBXD8<sub>FL</sub>op and endogenous UBXD8 were detected using anti-PEX19, anti-opsin and anti-UBXD8 antibodies, respectively. Open arrowheads indicate transfected cells with high nuclear NLS–PEX19 accumulation. White arrowheads indicate non-transfected cells with reticular staining for endogenous UBXD8. Representative images from a single experiment are shown. The experiment was repeated twice with similar results. All scale bars, 10 μm. (b) UBXD8 mislocalizes to mitochondria in the absence of PEX19. Endogenous UBXD8 was detected by IF in WT cells or two different PEX19<sup>−/−</sup> clonal cell lines using anti-UBXD8 antibodies. Results are representative of nine individual PEX19<sup>−/−</sup> clonal cell lines derived from four different guide RNAs. Representative images from a single experiment are shown. The experiment was repeated twice with similar results. Anti-HSP60 and anti-PDI antibodies stain mitochondria and ER, respectively. All scale bars, 10 μm. (c) Fluorescence intensity line profiles as depicted in the UBXD8 insets reveal increased correlation of UBXD8 localization with mitochondria in PEX19<sup>−/−</sup> cells. a.u., arbitrary units. Representative line profiles from a single experiment are shown. The experiment was repeated twice with similar results.
Figure 5  PEX19 and PEX3 are essential for UBXD8 insertion into ER subdomains. (a) UBXD8 insertion into ER foci is inhibited by competition with PEX3ΔN40. sUBXD8FLop or RAMP4op was translated in RRL, incubated with semi-permeabilized wild-type (WT) cells in the presence of the indicated purified proteins and detected by IF as described in Fig. 2. Representative images from a single experiment are shown. The experiment was repeated twice with similar results. All scale bars, 10 μm. (b,c) UBXD8 insertion into ER foci requires PEX3. (b) WT cells were transfected with the indicated siRNAs and analysed by SDS–PAGE and immunoblotting with the indicated antibodies. NT, non-transfected. Unprocessed original scans of blots are shown in Supplementary Fig. 8. Representative immunoblots from a single experiment are shown. The experiment was repeated once with similar results. (c) In vitro-translated sUBXD8FLop was added to semi-permeabilized WT cells transfected with siRNA constructs as in b and analysed by IF. NT, non-transfected. Representative images from a single experiment are shown. The experiment was repeated once with similar results. All scale bars, 10 μm. (d) UBXD8 insertion into ER foci requires PEX19/PEX3. In vitro-translated sUBXD8FLop was incubated with semi-permeabilized WT or two individual PEX19=clonal cell lines and detected by IF as in Fig. 2. Representative images from a single experiment are shown. The experiment was repeated once with similar results. All scale bars, 10 μm.

(Fig. 7b) did not disrupt the ER distribution of endogenous UBXD8, presumably because endogenous wild-type PEX19 is still present in these cells. Interestingly, however, overexpression of PEX19WT led to pronounced accumulation of UBXD8 on LDs as revealed by BODIPY co-staining (Fig. 7a). We previously reported that UBXD8 accumulation on LDs results either from overexpression of UBXD8 or from induction of LD biogenesis by oleate treatment7. The observation that PEX19WT overexpression induces endogenous UBXD8 accumulation on LDs in cells not loaded with oleate suggests that PEX19 is limiting for UBXD8 trafficking to LDs.

In striking contrast, PEX19C296S overexpression in wild-type cells led to enrichment of UBXD8 in punctate structures that did not correlate with LDs (Fig. 7b), but co-localized with the peroxisome marker PMP70 (Fig. 7c). This dominant-negative effect of overexpressed PEX19C296S on UBXD8 distribution in wild-type cells suggests that PEX19 farnesylation is essential to prevent delivery of UBXD8 to peroxisomes and to promote partitioning to ER sites from where it can be mobilized to LDs. Indeed, while stable expression of PEX19C296S rescued peroxisome biogenesis in PEX19=cells (Fig. 7d–f and Supplementary Fig. 6), it failed to complement PEX19 function on the localization of endogenous UBXD8, which remained mislocalized to mitochondria as in PEX19=cells (Fig. 7e,f). Moreover, a fraction of endogenous UBXD8 in PEX19=PEX19C296S cells was also present on peroxisomes (Fig. 7f), reflecting the profound mistargeting of UBXD8.
to peroxisomes after PEX19C296S overexpression in wild-type cells (Fig. 7bc). Additionally, we found that the amount of endogenous UBXD8 on LDs isolated from olate-treated PEX19−/− PEX19C296S cells was strongly (~70%) reduced compared with wild-type cells (Fig. 7gh). Together, these findings demonstrate an essential role for PEX19 farnesylation in directing UBXD8 to ER and LD membranes.

Figure 6 UBXD8 insertion sites co-localize with endogenous PEX3 but not with mature peroxisomes. (a–d) UBXD8 insertion sites are in close proximity to but distinct from peroxisomes. In vitro-translated sUBXD8op was imported into semi-permeabilized wild-type (WT) cells and visualized by IF microscopy as in Fig. 2 (a) or by structured illumination microscopy (SIM) (b,c,e). Anti-catalase antibodies mark mature peroxisomes. Positions of insets are indicated by white squares, a.u., arbitrary units. (a) Representative single z-slice from deconvolution wide-field microscopy. (b) Representative SIM z-stack projection. (c) A representative SIM z-slice inset (left) and fluorescence intensity profile from the indicated line scan (right) illustrates how co-localization of sUBXD8op foci with catalase-positive foci was quantified. Δ, measured distance between the intensity maxima. (d) Quantification of the distances between sUBXD8op foci and catalase-positive foci from SIM micrographs analysed as illustrated in c. Fifty-two sUBXD8op foci were analysed. Co-loc, co-localization. (e,f) Two distinct co-localization phenotypes for sUBXD8op foci with endogenous PEX3. (e) Left, two representative fields of single SIM z-slices after co-staining for sUBXD8op and endogenous PEX3 are shown to illustrate either complete co-localization (upper) or double foci with one co-localized spot adjacent to a PEX3-positive but sUBXD8op-negative spot (lower). Right, fluorescence intensity profiles from the indicated line scans used to quantify co-localization of sUBXD8op foci with PEX3-positive foci in f. (f) Quantification of the distances between sUBXD8op foci and PEX3-positive foci obtained from line profiles of fluorescence intensity as in e. Fifty-seven sUBXD8op foci were analysed. The grey-shaded bar indicates the percentage of sUBXD8op foci that co-localized with PEX3 foci and were also found to be adjacent to sUBXD8op-negative PEX3 foci within a distance of 50–250 nm. (g) Insertion of UBXD8 into ER foci is independent of mature peroxisomes. WT cells were transfected with indicated siRNAs for 120 h, used for semi-permeabilization and import of sUBXD8op, and stained with anti-opsin or anti-catalase antibodies as indicated. Scale bars, 10 μm. Representative images from a single experiment are shown. The experiment in a was repeated four times, in b,d,f, once, and in g twice, with similar results.
Figure 7 PEX19 farnesylation determines UBXD8 localization in cells. (a–c) Dominant-negative effect of the PEX19 farnesylation mutant on ER and LD targeting of endogenous UBXD8. Wild-type (WT) cells were transfected with either WT PEX19 (a) or with the farnesylation mutant PEX19C296S (b,c). PEX19, UBXD8 and PMP70 were detected by IF microscopy using antibodies and LDs were visualized using BODIPY staining. Insets show outlined regions; open and white arrowheads indicate transfected and non-transfected cells, respectively. Scale bars, 10 μm. (a) Overexpression (OE) of PEX19WT in WT cells promotes trafficking of endogenous UBXD8 to LDs. Representative images from a single experiment are shown, the experiment was repeated twice with similar results. (b) Overexpression of PEX19C296S in WT does not promote trafficking of endogenous UBXD8 to LDs but instead causes mislocalization to peroxisomes. Representative images from single experiments are shown, the experiments were repeated once with similar results. Images in (a) are representatives of three individual clonal PEX19−/− PEX19C296S cell lines. (c) Oleate-treated WT and PEX19−/− PEX19C296S cells were fractionated into membranes (M), cytosol (C) and LDs. A representative immunoblot from a single experiment is shown, the experiment was repeated twice with similar results. Membranes were first decorated with UBXD8-specific antibodies (upper) and then redecorated with anti-ATGL antibodies (lower). Unprocessed original scans of blots are shown in Supplementary Fig. 8. (d–f) Stable expression of PEX19C296S in PEX19−/− cells rescues peroxisome biogenesis but not mitochondrial mislocalization of endogenous UBXD8. WT cells, PEX19−/− cells or PEX19−/− cells stably expressing PEX19C296S (PEX19−/− PEX19C296S) as indicated were analysed by IF microscopy using UBXD8- and PMP70-specific antibodies. Insets show outlined regions; scale bars, 10 μm. Representative images from single experiments are shown; the experiments were repeated once with similar results. Images in (d) are representatives of three individual clonal PEX19−/− PEX19C296S cell lines. (g,h) UBXD8 levels on LDs are severely reduced in PEX19−/− PEX19C296S cells. (g) Oleate-treated WT and PEX19−/− PEX19C296S cells were fractionated into membranes (M), cytosol (C) and LDs. A representative immunoblot from a single experiment is shown; the experiment was repeated twice with similar results. Membranes were first decorated with UBXD8-specific antibodies (upper) and then redecorated with anti-ATGL antibodies (lower). Unprocessed original scans of blots are shown in Supplementary Fig. 8. (h) Relative UBXD8 amounts in LD fractions compared with the LD-resident membrane protein ATGL were quantified by densitometry from immunoblots as shown in (g). n = 3 independent experiments normalized to values obtained from WT cells. Mean ± s.e.m. (4.9%).
**DISCUSSION**

Post-translational protein integration into membranes implies that biosynthesis on cytosolic ribosomes is mechanistically uncoupled from membrane insertion and raises the question of how organelle-specific UBXD8 targeting within a cellular context is achieved. Selective targeting requires favoured delivery to the correct target membrane and prevention of promiscuous integration into inappropriate membranes. In this study we identified PEX19 and BAG6 as direct HP-domain-specific binding partners of newly synthesized UBXD8 and provide three lines of evidence supporting the conclusion that PEX19 specifies correct targeting of UBXD8 to ER membranes in cells. First, misdirecting PEX19 to the nucleus leads to nuclear accumulation of UBXD8. Second, in the absence of PEX19, endogenous UBXD8 is mislocalized to mitochondria, consistent with our observation that UBXD8 can insert into protein-free membranes and with studies showing that other post-translationally inserted membrane proteins can accumulate in mitochondrial membranes when their respective organelle-targeting pathways are disrupted. Third, blocking PEX19 function with a soluble fragment of PEX3 in semi-intact cell import-assays prevents insertion of in vitro-synthesized UBXD8 into ER subdomains and also causes mitochondrial mislocalization.

The role for BAG6 in UBXD8 biogenesis is less clear. This multifunctional chaperone has been implicated in a variety of cellular processes including ER-membrane targeting, protein quality control, and ER-associated protein degradation. BAG6 could contribute to UBXD8 targeting independently of or in collaboration with PEX19, or alternatively, could participate in UBXD8 turnover (Supplementary Fig. 7). Further investigation is required to assess these possibilities.

PEX19 and PEX3 are essential for de novo peroxisome biogenesis at the ER and for post-translational insertion of PMPs into peroxisomal membranes. PEX3 can be biosynthetically inserted into the ER, where it concentrates in a discrete subdomain termed the pre-peroxisomal ER (pER). Pre-peroxisomal vesicles bud from the pER in a process dependent on the interaction of PEX3 with PEX19, and a PEX3 function in intra-ER sorting and packaging of PMPs has been suggested. Our finding that newly synthesized UBXD8 inserts into PEX3-containing ER subdomains leads us to speculate that the pER may have a more general role; perhaps as an ER domain specialized for sorting of membrane proteins that are targeted to the ER by non-canonical insertion pathways. Our data establish that PEX19 farnesylation, which is dispensable for peroxisome biogenesis, is essential for UBXD8 insertion into the ER and LD partitioning. It may therefore serve to segregate HP-anchored proteins destined for LDs from bilayer-spanning peroxisomal proteins. Further studies will reveal whether and how this covalent lipid modification influences the association of PEX19 with PEX3-containing ER subdomains.

LDs and peroxisomes both originate from the ER membrane and have complementary roles in lipid metabolism. LDs store neutral lipids and hydrolyse them into fatty acids, which are further metabolized in peroxisomes. Conversely, peroxisomes uniquely synthesize ether lipids, which account for up to 20% of the neutral lipid content of LDs and are absent from LDs in cells lacking peroxisomes. LDs and peroxisomes are spatially associated and their juxtaposition with the ER suggests that all three organelles are intimately coupled to balance lipid storage and consumption. Our finding that peroxisomal proteins and LD-destined HP proteins share targeting machinery raises the hypothesis that LD and peroxisome biogenesis may be mechanistically coordinated in the ER. We previously reported that UBXD8 positively regulates LD abundance by controlling the activity of the major lipase on LDs. Thus, coordinating the biogenesis of such a protein with peroxisome biogenesis could allow mutual control of metabolic functions fulfilled by these organelles that have to act in concert during metabolic change.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

*Note: Supplementary Information is available in the online version of this paper.*

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**AUTHOR CONTRIBUTIONS**

B.S. performed and analysed all experiments, prepared the figures and wrote the first draft of the manuscript. B.S. and R.R.K. jointly conceived the experimental design, interpreted the results and wrote subsequent drafts of the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Methods

Reagents. Canine pancreas rough microsomes were a gift from B. Dobberstein and stored at 2°eC. In RM buffer (250 mM sucrose, 50 mM Hepes/KOH pH 7.6, 50 mM KAc, 2 mM Mg(OAc)₂, 1 mM diethiothreitol (DTT)), purified WRBcc and MBP was a kind gift from F. Vilardi and have been described earlier. ¹⁵

Plasmids and antibodies. Expression constructs for RAMP4op and Cib5op were a gift from B. Dobberstein and are described elsewhere. ¹⁶ UB8X constructs used in this study are derived from previously published UB8X expression plasmids that were used as templates for PCR-cloning using primers encoding either an 5'-tag (MKTEAAAARKHMQDS) or an opn-tag (GPNNPVYFSDKGTO) as well as either an XbaI or a NotI restriction site. PCR products were digested with the indicated enzymes and ligated into an empty pCDNA3.1(-) vector cut with the same enzymes. UB8X constructs lacking internal amino-acid sequences (AHP, Δ53–90, Δ53–111) were generated by primer-extension overlap PCR. Following digestion of the parental plasmid DNA by DpnI, the PCR reaction was transformed into Escherichia coli DH5alpha, and positive clones were identified by restriction digest/sequencing and then subcloned into an empty pCDNA3.1(-) vector using To generate N-terminally hexa-histidine-tagged PEX3 forward primer encoding the amino-acid sequence MAPKKKRKVGDGS was used. C296S clone ID: 2820701) and cloned into pCDNA3.1 by generating PCR products fragment. PEX19 expression constructs are derived from a cDNA clone (Thermo was used for cloning Iiop into pCDNA3.1(-) by PCR and ligation of the XbaI/NotI digestion of the parental plasmid DNA by DpnI, the PCR reaction was transformed into DH5alpha, and positive clones were identified by restriction digest/sequencing and then subcloned into an empty pCDNA3.1(-) vector using XbaI and NotI. Similarly, to introduce a PreScission protease cleavage site preceding the C-terminal Stag in UB8X constructs, primers encoding the amino-acid sequence LEVLFQGP were used for primer-extension overlap PCR. pRK5rsIiop digest/sequencing and then subcloned into an empty pCDNA3.1(-) vector using digestion with m7G cap analogue (Promega), DNase I digested, and purified using Microspin 7 in this study are derived from previously published UBXD8 expression plasmids with ammonium sulfate followed by SDS–PAGE and autoradiography. For protein insertion into semi-permeabilized cells, cells were seeded onto glass coverslips and semi-permeabilized by 0.003% digitonin in S buffer (250 mM sucrose, 20 mM Hepes KOH pH 7.4, 2.5 mM Mg(OAc)₂, 25 mM KCl 2.5 mM EDTA, 1 mM DTT, protease inhibitors) for 5 min. After washing out the cytosol, in vitro translation reactions were added to the cells post-translationally for 30 min, non-inserted proteins removed by washing in S-buffer and cells fixed with 4% formaldehyde in PBS. Proteins of interest were detected by standard immunofluorescence protocols or samples processed for immuno-electron microscopy.

Pre-treatment of RMs. For trypsin treatment, pelleted RMs were resuspended in 20 μg ml⁻¹ trypsin (Promega, sequencing grade) freshly dissolved in PSB (50 mM Hepes/KOH pH 7.4, 100 mM KAc, 2 mM Mg(OAc)₂) and incubated for 1 h on ice. The reaction was stopped by adding 20 μg ml⁻¹ aprotinin (SIGMA) and 2 mM phenylmethyl sulfonyl fluoride (PMSF) and incubation on ice for 15 min. RMs were collected by centrifugation through a 500 mM sucrose cushion in PSB, washed in PSB containing PSB and re-collected by centrifugation through a sucore cushion. For NEM treatment, pelleted RMs were resuspended in PSB containing 2 mM NEM and incubated for 30 min at 25 °C. The reaction was quenched with 20 mM DTT and RMs were collected by centrifugation through a 500 mM sucrose cushion in PSB.

In both cases the final RM pellet was resuspended in RM buffer to a final volume equal to the starting material and stored in aliquots at −80 °C after flash freezing. As controls, RMs were treated as outlined above without adding trypsin or NEM, respectively.

Protease-protection assays. After protein insertion into RMs, membranes were resuspended in 30 mM Hepes/KOH pH 7.6, 50 mM KOAc, 2 mM Mg(OAc)₂, and incubated with 2 μg ml⁻¹ Protease K (Invitrogen) for 45 min at 30 °C. Digestion was stopped by adding 5 mM PMSF and samples were either directly added to boiling SDS-sample buffer, further fractionated by centrifugation through a sucrose cushion containing 5 mM PMSF (130,000g, 10 min), or subjected to affinity purification after protein denaturation in boiling SDS (1% in Tris/HCl pH 8.0) and 1:10 dilution with 1% Triton X-100, 100 mM NaCl, 50 mM Hepes pH 7.4.

For digestion of proteinsinserted into liposomes, protease K was added to the in vitro insertion reaction and liposomes were isolated by anity purification as described. Protein in the partition was precipitated with TCA precipitated in the presence of 0.5% Triton X-100 as a carrier or affinity isolated after solubilization with 1% Triton X-100 in 10 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM EDTA. Proteins were analysed by autoradiography after separation on 12% BisTris NuPAGE precast gels with MES buffer (Invitrogen). Analyses of UB8X pre-insertion complexes by S-affinity isolation, chemical crosslinking and sucrose gradient fractionation. S-taged UB8X8 variants were translated in RRL (40 μl reaction volume) in the absence of membranes, the reaction stopped by addition of 2.5 mM puromycin and diluted with 900 μl PBS. UB8X8 complexes were affinity isolated using S-agarse beads (Novagen). After washing in cleavage buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA), proteins were eluted from the beads by addition of S-sample buffer and heating at 65 °C for 10 min. The presence of UB8X8, PEX19 and RAG6 in the purified complexes was assessed by immunoblotting following SDS–PAGE.

For chemical crosslinking, radiolabelled UB8X8 variants were translated in RRL as described above. After diluting the reactions with 10 volumes of PBS, they were incubated with 250 mM BMH crosslinker (Pierce), or dimethylsulfoxide as a negative control, for 30 min at 25 °C. The reactions were quenched with 20 mM DTT, supplemented with 10 mM Tris/HCl pH 7.5 and proteins denatured by adding 1% SDS and heating for 10 min at 55 °C. After diluting the reaction tenfold with IP buffer A (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.4% Triton X-100) proteins were immunoprecipitated. After washing the beads with IP buffer A, IP buffer B (10 mM Tris/HCl pH 7.5, 500 mM NaCl, 2 mM EDTA, 0.2% NP-40) and 10 mM Tris/HCl pH 7.5 proteins were eluted with SDS-sample buffer and incubation at 65 °C for 10 min followed by SDS–PAGE and visualization by autoradiography.

To separate UB8X8 pre-insertion complexes, 100 μl in vitro translation reactions were layered onto 2 ml 5–20% sucrose gradients (50 mM Hepes/KOH pH 7.4, 100 mM KAc, 2 mM MgCl₂) and centrifuged for 5 h at 55,000 r.p.m. at 4 °C in a
Electron microscopy. After protein insertion into semi-permeabilized cells, cells were fixed with 4% formaldehyde/PBS for 20 min, washed in PBS, and quenched with 50 mM glycine/PBS for 5 min. After washing in PBS, nonspecific binding sites were blocked with 3% BSA/5% FBS/PBS for 20 min followed by incubation with primary antibodies (2% FCS/PBS) and Alexa Fluor 488 or 594 (Life Technologies) for 1 h. After washing in PBS, cells were incubated with goat anti-rabbit and goat anti-mouse secondary antibodies conjugated with Alexa Fluor (Life Technologies). Cells were washed in PBS, mounted on glass slides, and analyzed using a Leica SP5 laser scanning confocal microscope (Leica Microsystems) and Velocity software (PerkinElmer). Images were deconvolved using Huygens (Sofib) software. All channels were superimposed using ImageJ software (National Institutes of Health). Images were further processed using Adobe Photoshop and Illustrator.

Potential aggregated protein species and ribosomes were removed by centrifugation (90,000 × g, 30 min) and opUBXD8-PPs complexes were isolated on S-agarose beads. Beads were extensively washed in a spin column format with cleavage buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) and incubated in 2 bead volumes of cleavage buffer supplemented with 8 units of PreScission protease (GE Healthcare) for 4 h at 8 °C with shaking. Proteins were eluted from the column by centrifugation (2 min at 205g) and incubated with glutathione Sepharose beads to retain GST-tagged PreScission protease during a second elution. Proteins in the final eluates were precipitated by TCA and separated by SDS–PAGE. Whole lanes were excised from the gel, cut into pieces and subjected to in-gel tryptic digestion, peptide extraction and subsequent peptide identification by LC–MS/MS.

Expression and purification of recombinant His-tagged PEX3ΔN40. Expression of N-terminally hexa histidine-tagged PEX3ΔN40 was induced in E. coli Rosetta (DE3) cells (Novagen) with 1 mM IPTG at an attenuation (D600) of 0.5 for 16 h at 18 °C. Cells were pelleted and resuspended in buffer A (20 mM Tris/HCl pH 8.0, 150 mM NaCl, 5% glycerol, 10 mM imidazole) and cells were lysed by adding 0.5 mg/ml lysozyme, 1 mM EDTA, 1 mM PMSF, and Complete EDTA-free protease inhibitors (Roche) for 15 min on ice and samples were cleared by centrifugation (16,000g, 10 min, 4 °C). Protein concentration was determined by BCA assay (Pierce), and equal protein amounts were separated by SDS–PAGE, followed by wet-transfer onto nitrocellulose membranes. Skimmed milk (5%) in TBS-T was used to block nonspecific binding and to dilute antibodies. IRDye secondary antibodies (LiCor) were used for signal detection by Odyssey imaging (LiCor). Band intensities were quantified by densitometry using either ImageJ or Image Studio Lite software (LiCor).

Immunofluorescence. For immunofluorescence, cells were seeded onto glass coverslips (no. 1.5 high precision), fixed with 4% formaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. Nonspecific binding sites were blocked with either 1% BSA or 3% BSA/5% FBS in PBS followed by antibody incubation in the same solution. BODIPY 493/503 (Invitrogen) was used at 5 μg/ml to stain lipids. Samples were mounted on glass slides using Fluoromount G (EMS) or slow fade gold (Invitrogen) and analysed using a Zeiss AxioImager.M1 microscope with Plan-Apochromat oil objectives (63× or 100×/1.4 N.A.) and appropriate filter sets. Usually eight individual z-sections in 300 nm intervals were collected using a CoolSNAP HQ camera (Photometrics). Where indicated, images were deconvolved using the Slidebook software and the nearest-neighbour setting. Individual data sets were normalized for brightness/contrast and merged, and pseudo-coloured pictures were generated using ImageJ software. Micrographs were cropped and assembled using Adobe Photoshop, and Illustrator.

For structured illumination microscopy, specimens were examined with the DeltaVision OMX Blaze 3D-SIM microscope equipped with three electron-multiplying CCDs (charge-coupled devices; Photometrics, Evolve 512) and an U-PLANAPo SIM objective (100×/1.42 N.A.). Individual z-sections of 125 nm were taken, reconstructed and aligned using the SoftWoRx software. Parameters for channel alignment were verified on the same day by calibration with fluorescein isothiocyanate (FITC) beads.

For assessment of co-localization, fluorescence intensity profiles were plotted and intensity peak distances measured using ImageJ. All SIM micrographs have a pixel size of 40.35 nm.

NATURE CELL BIOLOGY

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diluted to select clonal cell lines, which were screened for the absence of PEX19 by immunoblotting using anti-PEX19 antibodies (Abcam) and by immunofluorescence using anti-catalase antibodies to verify the absence of peroxisomes. At least two individual clonal cell lines derived from each gRNA were used to verify PEX19-mediated effects on UBXD8 insertion into semi-permeabilized cells and on endogenous UBXD8 localization.

Cellular fractionation of lipid droplets. Four sub-confluent 10 cm dishes of oleate-loaded cells were collected in ice-cold PBS, resuspended and incubated for 10 min in hypotonic lysis medium (HLM; 20 mM Tris/HCl pH 7.4, 1 mM EDTA) containing 250 mM sucrose and Complete EDTA-free protease inhibitors (Roche), and lysed by passing ten times through a 25G1/2 needle. The post-nuclear supernatant (5 min, 500 g) was adjusted to a final concentration of 20% sucrose, overlaid with HLM and centrifuged for 1 h at 172,000 g and 4 °C in a TLS-55 rotor. The buoyant LD fraction was collected using a tube slicer (Beckman), the cytosolic fraction by pipetting and the membrane-containing pellet washed three times with HLM. Proteins in the LD fraction were solubilized for 20 min in 2% Triton X-100 at 65 °C, precipitated with 10% TCA and washed twice with acetone. Equivalent percentages of cytosolic and membrane fractions were analysed next to TCA-precipitated LD proteins by SDS–PAGE and quantitative immunoblotting.

Statistics and reproducibility. Uncropped scans of all gels are available in Supplementary Fig. 8.

Experiments used for statistical quantification were repeated independently three times (n = 3) and normalized to values obtained from WT cells. The mean and the standard error of the mean are shown as bar graphs with error bars. Micrographs, autoradiographs and immunoblots shown are representative for at least two independent experiments as depicted in the individual figure legends. For assessment of sUBXD8op foci co-localization with endogenous proteins at least 50 foci, as depicted in the individual figure legends, were analysed from a representative single experiment. The experiment itself was repeated at least once. For genome-edited PEX19−/− cells nine independent clonal cell lines were characterized with similar results. For PEX19−/− cell lines stably expressing PEX19C296S three independent clonal cell lines were analysed with similar results.

Data availability. All data supporting the findings of this study are available without undue qualification from the corresponding author on request.

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**Supplementary Figure 1** Characterization of Pre-treated RMs, Related to Figure 1. Pre-treated rough microsomes (RMs) were analysed by SDS-PAGE and immunoblotting using anti-PDI, anti-calreticulin, anti-calnexin or anti-TRAM antibodies as indicated. T-RM: trypsin-treated RMs; NEM-RM: N-ethylmaleimide treated RMs. A representative blot from a single experiment is shown. The immunoblot analysis was repeated once with similar results. RMs were pre-treated in batch once and used for all experiments. Uncropped scans of all gels are available in Supplementary Figure 8.
Supplementary Figure 2 Analyses of UBXD8 Pre-Insertion Complex Components, Related to Figure 3. a) Sucrose density fractionation experiment of *in vitro* translated sUBXD8\_op performed in parallel with experiment in figure 3d. Here, UBXD8 complexes were S-affinity purified from individual fractions and analysed by SDS-PAGE and immunoblotting using anti-opsin (UBXD8), -BAG6 and -PEX19 antibodies. A representative blot from a single experiment is shown, the experiment was repeated once with similar results. b) UBXD8, PEX19 and BAG6 amounts in the individual fractions of (a) were quantified by densitometry and the relative ratios of PEX19/UBXD8 or BAG6/UBXD8 calculated. A.U. arbitrary units. Uncropped scans of all gels are available in Supplementary Figure 8.
**Supplementary Figure 3** Generation of PEX19−/− Cells by CRISPR/Cas9 Genome-Editing, Related to Figure 4. a) Schematic outline of the genome-editing strategy to delete endogenous PEX19 in HeLa cells. Exons 1 to 8 (E1-E8) are indicated. Selected guide RNAs 1, 2 and 3 target a sequence within exon 1, guide RNA 4 targets exon 2. PAM sequences are depicted in grey. b) SDS-PAGE and immunoblot analysis of wild-type (WT) cells and genome-edited PEX19−/− clonal cell lines using indicated antibodies. A representative blot of a single experiment is shown, the experiment was repeated twice with similar results. Clonal cell lines used in the main figures are indicated in bold. c) PEX19−/− cells lack peroxisomes. Immunofluorescence microscopy of WT and PEX19−/− cell lines using antibodies against catalase, a marker for mature peroxisomes. Representative images of a single experiment are shown, The experiment was repeated once with similar results. Scale bars, 10 µm. d) PEX19 overexpression in PEX19−/− cell lines reverts mitochondrial mislocalisation phenotype of endogenous UBXD8. WT or PEX19−/− cell lines were transfected with plasmids encoding WT PEX19 and analysed by immunofluorescence microscopy using anti-PEX19 and anti-UBXD8 antibodies. Representative images of a single experiment are shown, the experiment was repeated twice with similar results. Open and white arrowheads indicate transfected and non-transfected cells, respectively. Scale bars, 10 µm. Uncropped scans of all gels are available in Supplementary Figure 8.
**Supplementary Figure 4** Colocalisation analysis of sUBXD8 FL-op foci with endogenous PEX14, Related to Figure 6. a) *In vitro* translated (IVT) sUBXD8 FL-op was imported into semi-permeabilised WT cells and visualized by IF/SIM microscopy using anti-opsin and anti-PEX14 antibodies. A representative single SIM z-slice inset (*left*) and a fluorescence intensity profile from indicated line scan (*right*) from a single experiment are shown to illustrate how colocalisation of sUBXD8 FL-op foci with PEX14-positive foci was quantified in (b). A.U.: arbitrary units. b) Quantification of the distance between sUBXD8 FL-op foci and PEX14-positive foci using line profiles of fluorescence intensity as shown in (a). 53 sUBXD8 FL-op foci were analysed from one representative cell. The experiment itself was not repeated but independent SIM images were acquired from three individual cells and showed similar results. Grey-shaded area indicates sUBXD8 FL-op foci that colocalised with PEX14 foci and in addition were found adjacent to sUBXD8 FL-op-negative PEX14 foci within a distance of 50-250 nm.
Supplementary Figure 5 siRNA-mediated PEX5 knock-down in WT cells. Related to Figure 6. WT cells were transfected with the indicated siRNAs for 120h and analysed by either immunoblotting using the indicated antibodies to confirm reduction of endogenous PEX5 levels (a) or by IF microscopy using catalase-specific antibodies to indicate lack of mature peroxisomes (b). Representative images and blots from parallel experiments are shown. The experiments were repeated three times with similar results. NT: non-transfected. Scale bars, 10 µm. Asterisk indicates cross-reactive band. Uncropped scans are available in supplementary Figure 8.
Supplementary Figure 6 Stable expression of PEX19\textsubscript{C296S} in PEX19\textsuperscript{-/-} cells restores peroxisome biogenesis, Related to Figure 7. IF microscopy of WT, PEX19\textsuperscript{-/-}, and PEX19\textsuperscript{-/-} PEX19\textsubscript{C296S} cells as indicated using catalase-specific antibodies. Scale bar, 10 µm. Representative images from a single experiment are shown. For WT cells, the experiment was repeated four times with similar results. For PEX19\textsuperscript{-/-} cells nine independent clonal PEX19\textsuperscript{-/-} cell lines were analysed in two independent experiments with similar results. For PEX19\textsuperscript{-/-} PEX19\textsubscript{C296S} cells, three independent cell lines were analysed in two independent experiments with similar results.
Supplementary Figure 7  Model of HP-protein insertion into ER-subdomains as exemplified by UBXD8. Newly synthesized HP-proteins are released from cytosolic ribosomes and engage either BAG6 or PEX19. We can envision three distinct roles for BAG6 binding in HP-protein biogenesis. First, BAG6 could function in a parallel ER-targeting pathway, competing with PEX19 for binding to the newly synthesized protein (a). However, the inability to detect efficient UBXD8 insertion into RMs when incubated with sucrose density gradient fractions containing BAG6 but not PEX19, and the mitochondrial mislocalisation of UBXD8 in Pex19−/− cells argue against a direct and PEX19-independent role for BAG6 in HP-protein membrane insertion. A second possibility (b) is that BAG6 and PEX19 bind to nascent HP-proteins sequentially in a manner reminiscent of the hand-off of TA proteins from BAG6 to TRC401. Finally (c), BAG6 could serve a “triage” role in its capacity of a quality control “holdase”2,3 directing non-inserted HP-proteins for degradation by the ubiquitin-proteasome system. These latter two models are not mutually exclusive and further investigation is required to assess their relative contributions to HP-protein biogenesis. The PEX19-HP-protein pre-insertion complex does not target clients to lipid droplets (LD) or peroxisomes but inserts them into ER-subdomains with the help of PEX3. PEX19 farnesylation ensures correct client protein insertion into ER-subdomains, whereas non-farnesylated PEX19 can promote mistargeting of a HP-protein to peroxisomes. TG: Triglycerides; FA: fatty acids.
Supporting Information

Figure 1a, figure 1c, figure 1f, figure 1g, figure 3a, figure 3c, figure 3d, figure 3g, figure 5b

Supplementary Figure 8 Uncropped immunoblots and autoradiographs. Boxes indicate areas used for cropped panels.
Supplementary Figure 1

Supplementary Figure 2a

Supplementary Figure 3b

Supplementary Figure 5a

Supplementary Figure 8 continued
Supplementary Table 1  Identification of UBXD8 pre-insertion complex partners by mass spectrometry. In vitro translated UBXD8 was identified using a human database. UBXD8-associated proteins from the RRL were identified using a rabbit database and proteins of which at least 2 peptides were identified for opUBXD8FL PPs and which were absent from the negative control (no RNA added to RRL) are listed. BAG6, PEX19 and CCT2 are exclusively associated with opUBXD8FL PPs but not opUBXD8ΔHP PPs. In a replicate experiment for opUBXD8FL PPs, BAG6 and PEX19 were identified with sequence coverage of 34% and 22%, respectively, whereas for CCT2 only 3 total peptides with sequence coverage of 9% were identified. VCP, KPNB1, HSP90AA1, HSP90AB1 and DDB1 were equally associated with both, opUBXD8FL PPs and UBXD8ΔHP PPs, with VCP being the most abundant protein, consistent with its known interaction with the UBX-domain of UBXD8.
Supplementary Video 1  UBXD8 insertion sites align with the ER marker calnexin. A full z-stack of the specimen shown in figure 2d is shown. *In vitro* translated sUBXD8\textsubscript{FL} op was imported into semi-permeabilised cells, which were examined by immunofluorescence microscopy using anti-opsin (green) and anti-calnexin (red) antibodies, respectively.

Supplementary References

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