

Molecular mechanisms underlying topological remodeling of the Pex14 N-terminal domain in peroxisomal matrix protein transport

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<https://hdl.handle.net/2324/7443269>

出版情報 : Kyushu University, 2025, 博士 (理学), 課程博士
バージョン :
権利関係 :



Doctoral Thesis

**Molecular mechanisms underlying topological
remodeling of the Pex14 N-terminal domain in
peroxisomal matrix protein transport**

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CONTENT

INDEX

ABSTRACT	<u>4-5</u>
INTRODUCTION	<u>6-11</u>
RESULTS	<u>12-18</u>
<i>Pex6 and Pex26 Are Specifically Required for Maintaining the Membrane Topology of the Pex14 N-terminal Domain</i>	13-14
<i>Protease Protection Assay Confirms Topological Alterations in the Pex14 N-terminal Domain</i>	14-15
<i>Functional Analysis of Pex6 Walker Motif Mutants Reveals ATPase-Dependent Topological Regulation of Pex14</i>	15-16
<i>ATP Depletion Promotes Cytosolic Exposure of the Pex14 N-terminal Domain</i>	16
<i>Inhibition of AAA+ ATPases Alters the Membrane Topology of Pex14</i>	17
<i>Ubiquitination of Pex5 Promotes Cytosolic Orientation of the Pex14 N-terminal Domain and Pex5</i>	18-20
DISCUSSION	<u>21-27</u>
EXPERIMENTAL PROCEDURES	<u>28-30</u>
ACKNOWLEDGEMENTS	<u>31-32</u>
REFERENCES	<u>33-38</u>
FIGURE LEGENDS	<u>39-46</u>
FIGURES	<u>47-65</u>

ABSTRACT

ABSTRACT

Pex14 is a membrane peroxin that plays a central role in matrix protein import by mediating the docking of the cytosolic receptor Pex5, which delivers cargo harboring a peroxisome targeting signal 1 (PTS1). My laboratory previously reported the crystal structure of the conserved N-terminal domain of Pex14, which harbors the primary binding site for Pex5. However, the mechanistic contribution of this domain to the import process, particularly regarding its membrane orientation, remains unclear. In this study, I investigated the role of the AAA+ ATPase peroxin Pex6 in regulating the membrane topology of the Pex14 N-terminal domain. By a combination of immunofluorescence microscopy and protease protection assays, I show that the orientation of the Pex14 N-terminus is dynamically modulated in an ATP- and ubiquitination-dependent manner. Under normal culture conditions, the N-terminal domain of Pex14 is oriented toward the peroxisomal lumen. Deficiency of Pex6 or its membrane-recruiting partner Pex26, as well as pharmacological inhibition of AAA+ ATPases, resulted in a marked topological shift, exposing the Pex14 N-terminus to the cytosol. Conversely, inhibition of ubiquitin activation using MLN-7243 prevented this reorientation, likely by blocking Pex5 ubiquitination and its subsequent extraction from the membrane. These findings support a model in which Pex14 undergoes reversible, ATP-dependent topological remodeling during Pex5 recycling, functioning as a molecular reset mechanism for the docking/translocation complex. Our study reveals an additional mechanism of regulation in peroxisomal protein import and highlights the coordinated roles of Pex6 and Pex5 ubiquitination in maintaining the structural organization of the translocation machinery.

INTRODUCTION

INTRODUCTION

Peroxisomes are ubiquitous and metabolically versatile organelles that contribute to lipid and redox metabolism across eukaryotes. In mammalian cells, they play central roles in the β -oxidation of very-long-chain fatty acids and other substrates, the synthesis of ether phospholipids such as plasmalogens, and the detoxification of reactive oxygen species, thereby supporting cellular redox homeostasis and metabolic flexibility (1, 2). These functions depend on the correct enzymatic composition of the peroxisomal lumen, which is established and maintained by continuous import of soluble matrix proteins. Accordingly, disruption of peroxisomal matrix protein import compromises multiple metabolic pathways simultaneously and underlies severe inherited disorders, most prominently the Zellweger spectrum disorders, which are characterized by multisystem pathology and often early lethality (3–6). Understanding how peroxisomes achieve selective and efficient matrix protein import is therefore essential not only for organelle cell biology but also for clarifying the molecular basis of peroxisome biogenesis disorders.

A defining feature that distinguishes peroxisomes from other membrane-bound organelles is their ability to import fully folded and even oligomeric proteins. Whereas mitochondrial and ER import typically require cargo unfolding and specialized translocases powered directly or indirectly by nucleotide hydrolysis, the peroxisomal import pathway supports the translocation of folded substrates through a mechanism that is largely ATP-independent at the import step itself (5). This unusual property implies that the peroxisomal translocation machinery must accommodate large and structurally diverse cargoes while preserving membrane integrity, and it has fueled long-standing questions about the physical nature and regulation of the translocation conduit. Despite major progress in identifying the core peroxins and their interactions, the dynamic architecture of the import machinery and how it is reset during successive rounds of import remain incompletely understood (5).

Most soluble peroxisomal matrix proteins are targeted by a C-terminal peroxisomal targeting signal type 1 (PTS1), classically exemplified by the tripeptide Ser-Lys-Leu (SKL), although many functional variants exist (7). The cytosolic receptor Pex5 recognizes the PTS1 motif, binds cargo in the cytosol, and delivers the receptor–cargo complex to the peroxisomal membrane, where it engages the docking/translocation machinery, in which Pex14 and Pex13 constitute key components that mediate receptor docking and cargo translocation (8, 9). A prominent example is catalase, which carries a

C-terminal PTS1-like signal and is imported into the peroxisomal matrix, where it plays a central role in hydrogen peroxide detoxification. Notably, catalase is typically a homotetramer, and peroxisomes can import catalase in an oligomeric state, underscoring the unique ability of this organelle to translocate folded and multimeric proteins. In addition to PTS1, a subset of peroxisomal matrix proteins are targeted by an N-terminal peroxisomal targeting signal type 2 (PTS2), a conserved N-terminal motif that is recognized by the cytosolic receptor Pex7. In many organisms, Pex7 functions together with a co-receptor (for example, distinct co-receptors in yeast or a longer Pex5 isoform in mammals) to deliver PTS2 cargos to the same peroxisomal docking/translocation machinery. Following import, the N-terminal targeting sequence of many PTS2 proteins is often removed in the lumen, consistent with the idea that PTS2 can behave as a cleavable presequence. Despite this framework, it remains unclear to what extent catalase import, canonical PTS1-mediated import, and PTS2-mediated import converge on an identical translocation route and whether they share the same rate-limiting or regulatory steps at the docking/translocation complex. However, the molecular cue by which Pex5 initially recognizes and targets the peroxisomal surface remains incompletely defined. It is unclear whether this early recognition is primarily mediated by the cytosol-facing N-terminal region of Pex14, by the phase-separated condensate formed by the YG domain of Pex13, or potentially by a distinct phase or interaction environment contributed by the cytosolic C-terminal region of Pex14. Among these components, Pex14 in particular functions as a principal receptor-binding platform: its N-terminal region binds multiple WxxxF/Y motifs present in the intrinsically disordered N-terminus of Pex5, allowing multivalent interactions that can stabilize receptor docking and potentially promote assembly of higher-order import complexes (10). This multivalency has been proposed to be critical for the efficiency and robustness of import, consistent with a model in which transient, dynamic assemblies form at the membrane to accommodate cargoes of different sizes and oligomeric states.

Structural studies have provided important mechanistic insight into how Pex14 recognizes Pex5. My laboratory previously determined the crystal structure of the conserved N-terminal domain of Pex14 (residues 25–70), revealing a three-helix bundle that specifically binds the WxxxF/Y motifs of Pex5 (11). This structure established the molecular basis of the Pex14–Pex5 interaction and suggested how multiple motifs in Pex5 could engage Pex14 in a modular, potentially cooperative manner, thereby contributing to organization of the docking/translocation complex. However, while the receptor-binding interface is well defined, how this N-terminal receptor-binding domain is

positioned relative to the membrane and how its membrane orientation relates to the import cycle have remained less clear. Early models did not firmly establish the membrane topology of the Pex14 N-terminus, and the functional implications of its orientation were therefore difficult to assess. More recently, protease protection experiments have reported that the Pex14 N-terminal domain can be protected from externally added protease, consistent with a topology in which the N-terminus faces the peroxisomal matrix (Nin-Cout) (12, 13). These findings raise a fundamental question: is this orientation constitutive, or can it change during the import cycle?

The possibility of dynamic topology is particularly compelling in light of evidence that Pex5 itself exhibits striking topological plasticity. During cargo translocation, Pex5 has been shown to transiently insert into the peroxisomal membrane and adopt a membrane-associated state in which different regions of the receptor can become exposed to distinct compartments, implying that the receptor can span or traverse the membrane in a regulated and reversible manner (14, 15). If receptor docking and translocation involve such transient membrane integration of Pex5, then the interaction partner Pex14—especially its receptor-binding N-terminal domain—may also undergo coordinated rearrangements. In this scenario, Pex14 would not merely serve as a static docking site, but could be an actively remodeled component of an import apparatus that cycles between “receptive” and “reset” conformations. Such remodeling could provide a means to couple cargo release, receptor ubiquitination, and receptor extraction to restoration of the docking/translocation complex for subsequent rounds of import. At the same time, Pex14 contains additional structural features—including predicted coiled-coil regions and conserved GxxxG/AxxxA motifs—that may promote oligomerization or helix–helix packing, further supporting the idea that Pex14 participates in higher-order assemblies whose architecture could be regulated (16). In contrast to a potentially dynamic N-terminus, the C-terminal region of Pex14 is generally described as cytosol-facing and subject to phosphorylation-dependent regulation, indicating that Pex14 integrates multiple regulatory inputs at distinct domains (16–18). Together, these observations motivate a model in which domain-specific regulation and conformational remodeling of Pex14 contribute to the organization and cycling of the import machinery.

After cargo delivery to the lumen, Pex5 must be efficiently recycled to sustain continuous import. This recycling step is tightly controlled and, unlike cargo translocation itself, is strictly ATP-dependent. A key initiating event is monoubiquitination of Pex5 at a conserved N-terminal cysteine residue, which licenses the receptor for extraction from the membrane (19–22). In yeast, this modification is catalyzed by the E2 enzyme Pex4

together with the RING-type E3 ligases Pex2, Pex10, and Pex12 (23). In mammals, where Pex4 is absent, UbcH5 family enzymes serve as E2s, and Pex10 functions as a major E3 ligase for Pex5 monoubiquitination (19–22). Beyond their enzymatic roles, the RING peroxins have also been implicated in the architecture of the translocation machinery and may contribute to formation or stabilization of a conduit through which the unstructured N-terminus of Pex5 is returned to the cytosol (24). Notably, Pex5 can transiently reach the peroxisomal lumen and, during its passage through the RING peroxins in the course of translocation/recycling, is thought to adopt a largely unstructured state, consistent with the idea that the receptor itself forms a dynamic, deformable intermediate within the import apparatus. Following monoubiquitination, Pex5 is removed from the membrane by the heterohexameric type II AAA+ ATPase complex Pex1–Pex6, which is recruited to peroxisomes by the membrane anchor Pex26 in mammalian cells (19, 20, 25–27). ATP hydrolysis at the D2 domains of Pex1/Pex6 is thought to power mechanical extraction of ubiquitinated substrates through the central channel, with ubiquitinated Pex5 representing a principal physiological substrate (28). Thus, even though peroxisomal cargo import is ATP-independent, the overall import cycle is energetically coupled to ATP-driven receptor recycling, emphasizing a mechanistic coordination between ubiquitin signaling and AAA+ ATPase action (17).

Consistent with this coupling, impaired Pex1 or Pex6 function causes accumulation of ubiquitinated Pex5 on the peroxisomal membrane and can trigger pexophagy, reflecting a cellular quality-control response to dysfunctional import machineries (29–31). Under these conditions, both mono- and polyubiquitinated species of Pex5 have been observed, indicating that ubiquitin chain topology and extent can shift depending on the functional state of the recycling machinery (32, 33). Monoubiquitination is generally associated with productive recycling and is thought to suppress competing polyubiquitination events that would target the receptor for degradation (34). Importantly, the conserved cysteine monoubiquitination site also acts as a redox-sensitive regulatory switch: oxidative stress can inhibit this modification and thereby delay matrix protein import, linking peroxisomal import capacity to the cellular redox environment (35, 36). These findings establish Pex5 ubiquitination as both a core mechanistic step in receptor recycling and a regulatory node through which the import pathway responds to cellular physiology.

Despite these advances, an important gap remains in understanding how receptor ubiquitination and AAA+ ATPase-driven extraction feed back onto the structural organization of the docking/translocation complex. In particular, it is unknown whether the docking factor Pex14—especially its receptor-binding N-terminal domain—

undergoes topological or conformational rearrangements as a consequence of Pex5 ubiquitination and Pex6 activity. Although protease protection studies suggest a matrix-facing N-terminus (12, 13), these experiments do not resolve whether Pex14 topology is fixed or dynamically remodeled during ongoing import and receptor recycling. Moreover, while Pex5 topology is known to be dynamic (14, 15), whether this dynamic state is coordinated with a corresponding rearrangement in Pex14 has not been directly investigated. Addressing this question is crucial for developing a unified model of the peroxisomal import cycle in which docking, translocation, cargo release, receptor ubiquitination, and receptor extraction are mechanistically integrated rather than treated as separable modules.

In this study, I investigated whether the N-terminal domain of Pex14 undergoes ATP- and ubiquitination-dependent topological rearrangement during matrix protein import. Using immunofluorescence microscopy and protease protection assays in a PEX6-deficient cellular model, I demonstrate that the membrane orientation of Pex14 is dynamically remodeled during the import cycle. These results reveal a previously unrecognized regulatory layer in peroxisomal protein import in which Pex6 activity and ubiquitinated Pex5 cooperatively modulate the membrane topology of Pex14, thereby coordinating receptor recycling with structural resetting of the import apparatus.

RESULTS

RESULTS

Pex6 and Pex26 Are Specifically Required for Maintaining the Membrane Topology of the Pex14 N-terminal Domain

To explore the possibility that the membrane topology of the Pex14 N-terminal domain may undergo dynamic changes in coordination with matrix protein import, I examined its orientation in control and in peroxin-deficient human fibroblasts under semi- and full-permeabilization conditions. Cells were fixed under standard culture conditions and permeabilized with either digitonin, which selectively solubilizes the plasma membrane while preserving peroxisomal membrane integrity, or Triton X-100, which disrupts all membranes. Immunofluorescence staining was performed using antibodies specific to the N-terminal (α -Pex14N) or C-terminal (α -Pex14C) domains of Pex14.

Under full-permeabilization (Triton X-100), both α -Pex14N and α -Pex14C signals were consistently detected as punctate structures across all cell lines tested, confirming epitope accessibility (Fig. 1A, panels c, d, g, h, k, l, o, and p). In contrast, digitonin-permeabilized control fibroblasts showed punctate staining only with α -Pex14C, while α -Pex14N signals were *undetectable* (Fig. 1A, panels a and b). This staining pattern, consistent with previous reports, indicates that under normal conditions, the C-terminus of Pex14 is exposed to the cytosol and the N-terminus is oriented toward the peroxisomal lumen or embedded within the membrane. A similar staining pattern was observed in *PEX1*-, *PEX2*-, *PEX10*-, and *PEX12*-deficient fibroblasts (Fig. 1A, panels e and f; Fig. S1), suggesting that these peroxins are dispensable for maintaining the membrane topology of Pex14. In contrast, fibroblasts deficient in *PEX6* or *PEX26* exhibited distinct punctate staining for both α -Pex14N and α -Pex14C antibodies following digitonin treatment (Fig. 1A, panels i, j, m, and n), indicating aberrant cytosolic exposure of the Pex14 N-terminus. This staining pattern indicates that the N-terminal domain of Pex14 is abnormally exposed to the cytosol in these mutant cells. Such topological alteration was not observed in fibroblasts lacking other peroxins, including Pex1, Pex2, Pex10, and Pex12, thereby highlighting the specific requirement for Pex6 and Pex26 in maintaining the correct membrane orientation of Pex14. CHO cells also yielded the same results, indicating that this finding is not cell type-dependent (Fig. S2).

Together, these results show that the proper lumen-facing orientation of the Pex14 N-terminal membrane-embedded domain depends specifically on Pex6 and Pex26. Notably,

PEX1-deficient cells retained normal topology, even though Pex1 forms a complex with Pex6 as part of the AAA+ ATPase machinery, indicating that Pex1 is not required for controlling Pex14 membrane topology. The *PEX1*-deficient cells used in this study have been reported to carry a nonsense mutation introducing a stop codon at Gln261, together with additional splicing abnormalities (45). Notably, one of these splicing variants lacks exon 6, resulting in an in-frame partial deletion (Ile414–Leu453), and this truncated Pex1 protein may still retain the ability to associate with Pex6 without completely abolishing Pex6 function. In addition, Pex6 localizes exclusively to peroxisomes, whereas Pex1 is present both in the cytosol and at peroxisomes (37). Thus, although Pex1 and Pex6 function as a complex, it is possible that not all cellular pools of Pex1 and Pex6 exist as a fully functional complex, which may account for the distinct effects observed in *PEX1*- and *PEX6*-deficient cells. Instead, Pex6 and its membrane anchor Pex26 appear to play a more direct role. The cytosolic exposure of the Pex14 N-terminus in the absence of Pex6 or Pex26 implicates a Pex6- and ATP-dependent mechanism that couples Pex14 topological remodeling to dynamic reorganization of the docking/translocation complex during the matrix protein translocation.

Protease Protection Assay Confirms Topological Alterations in the Pex14 N-terminal Domain

To biochemically validate the topological changes observed by immunofluorescence microscopy, protease protection assays were performed on post-nuclear supernatants (PNS) from control and mutant fibroblasts, followed by immunoblotting with an α -Pex14N antibody. In control cells, an ~18-kDa protease-resistant fragment of Pex14 was detected following proteinase K treatment (Fig. 1B, WT). This fragment was completely digested in the presence of Triton X-100, suggesting that the N-terminal domain of Pex14 was partially protected from digestion due to its insertion into the lipid bilayer or translocation across the peroxisomal membrane. Upon addition of Triton X-100, the protected fragment was completely digested, confirming that the observed protection results from membrane embedding rather than its intrinsic resistance to proteolysis. A similar digestion pattern was observed in *PEX1*-deficient cells (Δ *PEX1*), consistent with the immunofluorescence findings, suggesting that Pex1 is not required for maintaining the native membrane topology of Pex14. In contrast, no protease-protected fragment was detected in *PEX6*-deficient cells, indicating that the N-terminal domain of Pex14 is fully exposed to the cytosol and therefore entirely susceptible to proteolytic digestion. In

PEX26-deficient cells, the ~18 kDa protected fragment was faintly detected, suggesting a reduction in membrane-associated protection (Fig. 1B, Δ *PEX6* and Δ *PEX26*). To exclude the possibility that the altered protease accessibility in *PEX6*-deficient cells arises from dissociation of Pex14 from the membrane, sodium carbonate extraction was performed to assess whether Pex14 becomes peripherally associated with the peroxisomal membrane in the absence of Pex6. Membrane fractions prepared from *PEX6*-deficient cells were treated with alkaline sodium carbonate (pH ~11) and separated into supernatant and pellet fractions by ultracentrifugation. Immunoblot analysis revealed that Pex14 remained predominantly in the pellet fraction in *PEX6*-deficient cells (Fig. S4). These results indicate that loss of Pex6 does not convert Pex14 into a carbonate-extractable, peripherally attached protein, but instead that Pex14 remains membrane-embedded. Thus, the aberrant cytosolic exposure of the Pex14 N-terminus observed in *PEX6*-deficient cells reflects altered membrane topology rather than dissociation of Pex14 from the membrane. These biochemical results corroborate the immunofluorescence data and strengthen the conclusion that Pex6, and to a lesser extent Pex26, is essential for maintaining the lumen-facing orientation of the Pex14 N-terminal domain. The protease accessibility model shown in Fig. 1C illustrates that under normal conditions, the N-terminal domain of Pex14 is embedded within the peroxisomal membrane and oriented toward the matrix, rendering it inaccessible to externally added proteases. In the absence of Pex6, this topology is disrupted, resulting in cytosolic exposure of the N-terminal domain and its susceptibility to proteolytic cleavage, whereas loss of Pex26 causes only a mild effect on this topology. Together, these findings indicate that Pex6, which is recruited to the peroxisomal membrane by Pex26, is essential for maintaining the membrane-protected topology of the Pex14 N-terminal domain.

Functional Analysis of Pex6 Walker Motif Mutants Reveals ATPase-Dependent Topological Regulation of Pex14

To dissect the roles of ATP binding and hydrolysis in Pex6-dependent regulation of Pex14 membrane topology, I introduced point mutations in the conserved Walker motifs of Pex6. These included K476E in the Walker A motif of the D1 domain (A1), K750E in the Walker A motif of the D2 domain (A2), and D803N in the Walker B motif of the D2 domain (B2). The Walker B motif of the D1 domain was not targeted for mutagenesis, as it lacks the canonical aspartate and glutamate residues required for ATP hydrolysis. These mutant constructs were separately expressed in *PEX6*-deficient human fibroblasts, and

the membrane orientation of the Pex14 N-terminal domain was assessed by immunofluorescence microscopy and protease protection assays. Expression of wild-type Pex6 restored the membrane-protected orientation of the Pex14 N-terminal domain, as shown by the absence of punctate α -Pex14N signals after digitonin treatment (Fig. 2A, panel b). In sharp contrast, cells expressing any of the Walker motif mutants retained punctate α -Pex14N staining, indicating a failure to promote topological reorientation of the Pex14 N-terminal domain (Fig. 2A, panels c–e). These results suggest that both ATP binding and hydrolysis are essential for Pex6-mediated translocation of the Pex14 N-terminus across the peroxisomal membrane.

To further confirm these observations, I also performed protease protection assays (Fig. 2B). In cells expressing wild-type Pex6, an ~18-kDa protease-protected fragment of Pex14 was detected following proteinase K treatment, indicating successful translocation of the N-terminal domain into a membrane-protected compartment. In contrast, no protected fragment was detected in cells separately expressing the K476E, K750E, and D803N mutants, consistent with cytosolic exposure of the N-terminal domain and a failure to restore the membrane-protected topology of the Pex14 N-terminal domain. Together, these results suggest that both membrane translocation and reorientation of the Pex14 N-terminal domain require ATP binding to the Walker A motifs and ATP hydrolysis by the Walker B motif of the Pex6 D2 domain. These findings support a model in which Pex6 acts as an ATP-dependent regulator of Pex14 topology and are consistent with previous reports highlighting the essential role of the Walker motifs in peroxisomal matrix protein import (37).

ATP Depletion Promotes Cytosolic Exposure of the Pex14 N-terminal Domain

Pex5 is known to accumulate on the peroxisomal membrane under ATP-depleted conditions (29), and our previous work demonstrated that Pex26 acts as a membrane anchor that spatially coordinates the ATPase activity of Pex6 toward the Pex14–Pex5 complex within the functional docking/translocation complex (38). To determine whether ATP availability affects the membrane orientation of the Pex14 N-terminal domain, I assessed its accessibility by immunofluorescence staining and protease protection assays following cellular ATP depletion. Digitonin-permeabilized cells showed no α -Pex14N signal, consistent with luminal orientation or membrane embedding of the Pex14 N-terminal domain under normal conditions. In contrast, ATP depletion induced a distinct

punctate α -Pex14N staining pattern, suggesting that the N-terminal domain became exposed to the cytosolic face (Fig. 3A, panels a and b). Notably, α -Pex14N signals were clearly detected under Triton X-100 permeabilization regardless of ATP availability, indicating that once membrane barriers are removed, the N-terminal epitope is equally accessible to antibody detection (Fig. 3A, panels c and d). Importantly, cycloheximide treatment did not prevent the Pex14 topology change, indicating that the aberrant cytosolic exposure of the Pex14 N-terminus is unlikely to arise from dual-topology insertion during de novo membrane integration (Fig. S5). Instead, these data support a post-insertional topological remodeling model in which pre-existing, membrane-embedded Pex14 undergoes reorientation in the membrane. Consistently, protease protection assays showed that the ~18-kDa protease-protected fragment of Pex14 was lost upon ATP depletion (Fig. 3B). Together, these findings indicate that ATP hydrolysis is required to maintain the membrane-protected, lumen-facing topology of the Pex14 N-terminal domain, and that ATP depletion triggers a post-insertional topological remodeling event rather than reflecting misorientation established during de novo insertion.

Inhibition of AAA+ ATPases Alters the Membrane Topology of Pex14

To assess the dependence of Pex14 membrane topology on AAA+ ATPase activity, I employed two small-molecule inhibitors targeting this enzyme family. CB-5083 and NMS-873 are known to target valosin-containing protein (VCP/p97), a AAA+ ATPase that shares structural homology with Pex6. CB-5083 acts as a competitive inhibitor at the ATP-binding site ($IC_{50} = 11$ nM), whereas NMS-873 functions allosterically ($IC_{50} = 30$ nM) (39-41). Although these compounds have not been defined for direct inhibition of Pex6, they serve as useful chemical probes to evaluate the involvement of AAA+ ATPases, including Pex6, in the regulation of peroxisomal protein import. To evaluate the effect of these compounds on Pex6-mediated matrix protein import, I expressed wild-type Pex6 in the *PEX6*-deficient CHO cell line ZP164 and examined the peroxisomal localization of matrix proteins in the presence or absence of each inhibitor. As shown in Fig. 4A, expression of Pex6 restored peroxisomal targeting of both endogenous PTS1-harboring proteins and catalase in untreated cells (Fig. 4A, panels b and f). Treatment with CB-5083 moderately impaired the import of both the PTS1-targeted protein and catalase (Fig. 4A, c and g), whereas treatment with NMS-873 markedly inhibited their peroxisomal localization (Fig. 4A, d and h). Quantitative analysis confirmed a significant reduction in import efficiency by NMS-873 treatment (Fig. 4B).

To determine whether these effects were associated with alterations in Pex14 membrane topology, I next performed immunofluorescence analysis in CHO-K1 cells and human fibroblasts treated with respective compounds. In control cells, digitonin-permeabilization did not reveal α -Pex14N staining, indicating a membrane-protected or lumen-facing orientation (Fig. 4C, D, panels a). In contrast, NMS-873 treatment resulted in punctate α -Pex14N staining (Fig. 4C, D, panels e), suggesting cytosolic exposure of the N-terminal domain. Treatment with CB-5083 gave rise to a barely detectable increase in signal intensity (Fig. 4C, D, panels c). Under full permeabilization conditions with Triton X-100, a punctate α -Pex14N staining pattern was observed in all samples (Fig. 4C, panels b, d, f), indicating that the epitope remained detectable even after inhibitor treatment. Taken together, these results indicate that pharmacological inhibition of AAA+ ATPases, particularly with NMS-873, disrupts the membrane-protected topology of the Pex14 N-terminal domain. These findings support a model in which ATP hydrolysis by Pex6 is essential for maintaining the correct membrane orientation of Pex14 during peroxisomal matrix protein import.

Ubiquitination of Pex5 Promotes Cytosolic Orientation of the Pex14 N-terminal Domain and Pex5

Previous studies have shown that ubiquitinated Pex5 accumulates on peroxisomal membranes in cells lacking AAA-type peroxins, suggesting a role for ubiquitination in facilitating the recycling of Pex5 from the peroxisome to the cytosol (31). I earlier identified AWP1, a ubiquitin-binding regulator of NF- κ B signaling, as a factor that selectively recognizes cysteine-monoubiquitinated Pex5 and promotes its extraction through interaction with Pex6 (20). Supporting these findings, Skowyra et al. recently reported that MLN-7243, an inhibitor of the E1 ubiquitin-activating enzyme UBA1, significantly impairs the import of PTS1-containing proteins (13). To investigate whether Pex5 ubiquitination contributes to the regulation of Pex14 membrane topology, I initially evaluated the effect of MLN-7243 on Pex6-mediated restoration of matrix protein import in the *PEX6*-deficient CHO cell line ZP164. As shown in Fig. 5A, expression of wild-type Pex6 restored peroxisomal targeting of PTS1-containing proteins, which was markedly suppressed by MLN-7243 treatment (Fig. 5A, panels b and d). In contrast, MLN-4924, an inhibitor of NEDD8-activating enzyme, had only a modest effect (Fig. 5A, panel c). Quantitative analysis confirmed a significant reduction in import efficiency

following MLN-7243 treatment as compared to either MLN-4924 or vehicle control (Fig. 5B), highlighting the critical role of ubiquitination in peroxisomal matrix protein import.

I next assessed the effect of MLN-7243 on the membrane orientation of the Pex14 N-terminal domain in control fibroblasts, *PEX6*- or *PEX26*-deficient fibroblasts, and control fibroblasts depleted of ATP. In control fibroblasts, α -Pex14N staining was not detected following digitonin permeabilization under either untreated or MLN-7243-treated conditions, indicating that the N-terminal domain remained embedded in the membrane or oriented toward the lumen (Fig. 5C, panels a and e). By contrast, *PEX6*- and *PEX26*-deficient cells, as well as ATP-depleted cells, exhibited punctate α -Pex14N staining under untreated conditions (Fig. 5C, panels b–d), consistent with cytosolic exposure of the N-terminal domain. Notably, punctate α -Pex14N staining was no longer detectable following MLN-7243 treatment (Fig. 5C, panels f–h). Quantitative analysis revealed that MLN-7243 induced reorientation of the Pex14 N-terminal domain toward the lumen in more than 70% of cells across all experimental conditions (Fig. 5D). To corroborate these observations biochemically, I performed protease protection assays in control and *PEX6*-deficient fibroblasts. In control cells, the ~18 kDa protease-resistant fragment corresponding to the Pex14 N-terminal domain was consistently detected under both untreated and MLN-7243-treated conditions (Fig. 5E, lanes 2 and 3), indicating stable membrane protection. In contrast, this protected fragment was absent in untreated *PEX6*-deficient cells but was faintly recovered following MLN-7243 treatment (Fig. 5E, lanes 6 and 7), suggesting partial restoration of the lumen-facing topology of the Pex14 N-terminus.

I also examined the membrane association of Pex5 by performing protease protection assays. In control cells, partial digestion products of Pex5 were detected, consistent with partial membrane insertion and protection from proteolysis (Fig. 5F). Treatment with MLN-7243 increased the abundance of both full-length and partially digested forms of Pex5, suggesting enhanced membrane association. In untreated *PEX6*-deficient cells, full-length and partially digested Pex5 bands were barely detectable (Fig. 5F, lane 6), indicating complete cytosolic exposure. Remarkably, MLN-7243 treatment restored both the full-length form and partial digestion products of Pex5 in these cells (Fig. 5F, lane 7). Notably, this restoration coincided with the reappearance of the ~18-kDa Pex14 fragment, implying a coordinated topological shift of both Pex14 and Pex5. To directly confirm that MLN-7243 inhibits Pex5 monoubiquitination under the same conditions that promote reorientation of the Pex14 N-terminal domain and restore membrane association of Pex5, I performed immunoblot analysis of PNS fraction from *PEX6*-deficient

fibroblasts. Samples were fractionated into the cytosolic and organelle fractions and analyzed under both reducing and non-reducing conditions using an anti-Pex5 antibody. In untreated *PEX6*-deficient cells, a slower-migrating band consistent with monoubiquitinated Pex5 (Ub-Pex5) (19, 20) was detected in the organelle fraction (Fig. 5G, lane 3), along with unmodified Pex5. The Ub-Pex5 band was sensitive to DTT and disappeared under reducing conditions, confirming that it represents a cysteine-linked ubiquitinated form. Treatment with MLN-7243 abolished the Ub-Pex5 band without affecting unmodified Pex5 (Fig. 5G, lane 9), indicating that the inhibitor effectively blocked Pex5 monoubiquitination in vivo.

Collectively, these findings suggest that monoubiquitination of Pex5 acts as a regulatory signal that facilitates the cytosolic orientation of both the Pex14 N-terminal domain and Pex5 during peroxisomal matrix protein import. However, the potential involvement of polyubiquitination or other modifications cannot be excluded and should be further investigated. In the absence of ubiquitination, the N-terminal domain of Pex14 remains oriented toward the lumen, implying that reorientation toward the cytosolic face is tightly coupled to the presence of ubiquitinated Pex5. These results further support a model in which ATP-dependent disassembly of the Pex14-Pex5 complex by Pex6 is followed by topological remodeling of the Pex14 N-terminal domain, thereby resetting the import apparatus for subsequent rounds of matrix protein translocation.

DISCUSSION

DISCUSSION

This study reports that the membrane topology of the N-terminal domain of Pex14 is dynamically regulated during peroxisomal matrix protein import, and that this regulation is tightly coordinated with both the ATPase activity of Pex6 and the ubiquitination status of Pex5. I propose that after cargo translocation, the peroxisomal import machinery undergoes a resetting phase, during which the Pex14 N-terminal domain reorients from the cytosol back to the lumen to prepare for the next import cycle. Specifically, I show that Pex6 is required for maintaining the canonical lumen-facing orientation of the Pex14 N-terminal domain, while its membrane-anchoring partner Pex26 contributes to this process. In the absence of Pex6, this domain becomes reoriented toward the cytosol, indicating a failure in this proposed resetting phase of the docking/translocation complex, whereas loss of Pex26 results in a more modest effect. Notably, the membrane orientation of Pex14 in *PEX26*-deficient cells is highly intriguing, as subpopulations of molecules oriented toward the cytosol and those oriented toward the peroxisomal lumen are observed to coexist. This heterogeneity suggests that loss of Pex26 does not merely reduce peroxisomal membrane recruitment of Pex6, but may also affect the stability of Pex14 within the membrane or the post-insertional processes that maintain its topology. Specifically, whereas Pex26 may act as an anchor for the Pex6 complex and thereby increase the likelihood that a cytosol-exposed state of the Pex14 N-terminal domain is rapidly resolved, *PEX26* deficiency could decrease the efficiency of this reset step (lumen reorientation), resulting in a temporal and molecular mixture of lumen- and cytosol-oriented Pex14. Therefore, it will be necessary to systematically test whether Pex26 contributes primarily to post-insertional stabilization and/or the efficiency of topological reorientation. This can be addressed by assessing the strength of Pex14 membrane association using sodium carbonate extraction and combining this with analyses of the appearance patterns and quantitative profiles of protease-protected fragments. The essential role of ATPase activity in this topological regulation is supported by our mutational analysis of Pex6. Expression of Walker motif mutants defective in either ATP binding or hydrolysis failed to restore the lumen-facing orientation of the Pex14 N-terminal domain in *PEX6*-deficient cells, indicating that both functions are required for the topological reorientation of Pex14. Consistent with this ATP-dependent regulatory model, my laboratory previously demonstrated that wild-type FLAG–Pex26/Pex6/Pex1 complexes do not co-immunoprecipitate with Pex14, whereas ATPase-defective Pex6 mutants (K750E and D803N) selectively co-precipitate with Pex14 (38). These findings

support the idea that ATP hydrolysis by Pex6 regulates its interaction with Pex14 and further underscore the essential role of ATPase activity in controlling Pex14 membrane topology. Pharmacological inhibition of AAA-type ATPases with NMS-873 induced a phenotype resembling that observed in *PEX6*-deficient cells, further supporting the conclusion that ATP hydrolysis is essential for topological rearrangement of Pex14. Consistently, ATP depletion alone induced cytosolic exposure of the Pex14 N-terminal domain, highlighting the energy dependence of this membrane configuration.

The second major finding is that Pex5 ubiquitination appears to contribute to cytosolic repositioning of Pex5 and cytosolic exposure of the Pex14 N-terminal domain. Inhibition of ubiquitin activation by MLN-7243 prevented the topological conversion typically observed in *PEX6*- and *PEX26*-deficient cells. This interpretation was further supported by the appearance of protease-protected fragments of Pex14 under the same experimental conditions, suggesting that monoubiquitinated Pex5 may act as a regulatory signal that promotes reorientation of the Pex14 N-terminal domain toward the cytosolic face. Additionally, using a monoubiquitination-defective Pex5 mutant (Pex5-C11A), I confirmed that the Pex14 N-terminal domain remains protected on the luminal side under these conditions (Fig. S3), supporting a specific role of monoubiquitination in this process. However, this study did not directly examine polyubiquitination or systematically test additional non-ubiquitinatable Pex5 variants beyond Pex5-C11A. Therefore, the possible involvement of other ubiquitin linkages or additional regulatory mechanisms cannot be ruled out. Collectively, these results suggest that Pex5 ubiquitination influences the membrane topology of the Pex14 N-terminal domain. They also raise the possibility that Pex6 facilitates topological remodeling of Pex14 by dissociating the interaction between ubiquitinated Pex5 and the N-terminal domain of Pex14 in an ATP-dependent manner, thereby allowing its reorientation. Importantly, our data do not support a model in which Pex6 itself directly provides the driving force for reorientation of the Pex14 N-terminal domain toward the lumen. Rather, I propose that the primary role of Pex6 is to dissociate ubiquitinated Pex5 from Pex14 in an ATP-dependent manner, thereby permitting the N-terminal domain of Pex14 to disengage from its cytosol-oriented state. The subsequent reorientation of the Pex14 N-terminal domain toward the luminal side may instead be driven by additional components of the import machinery, potentially including Pex13, which may form the core of the translocation channel together with Pex14. Although not shown in the paper, our preliminary observations indicate that, in *PEX13*-deficient cells, the N-terminal domain of Pex14 is biased toward a cytosol-facing orientation. This finding suggests that Pex13 may participate in the process that establishes the lumen-

facing orientation of the Pex14 N-terminal domain. In other words, Pex13 could function as a structural scaffold required for Pex14 membrane insertion and/or for topological maturation after insertion, thereby increasing the probability that Pex14 transitions into and is stabilized in the canonical lumen-oriented state. An alternative interpretation, however, is that loss of Pex13 compromises the architecture of the docking/translocation complex, destabilizing Pex14 within the membrane and increasing the fraction of partially inserted and/or aberrantly oriented Pex14 species. Thus, whether Pex13 directly acts as a translocator for Pex14 remains an open question. Addressing this will require a combination of phenotypic rescue by Pex13 re-expression, assays of Pex14 intramembrane stability (e.g., carbonate extraction, limited proteolysis, and crosslinking), and tests of the presence and dependency of Pex13–Pex14 interactions, in order to discriminate whether Pex13 primarily serves as an insertion factor, a stabilization factor, or instead operates on the reorientation/reset-driving side of the import machinery. Elucidating the molecular mechanism underlying this resetting step of Pex14 topology remains an important subject for future investigation.

To further integrate these findings into the established framework of peroxisomal matrix protein import, I present a working model in Supplementary Fig. S6. In this scheme, Pex14 is envisioned to exist in at least two functional states: a basal membrane-embedded, lumen-oriented state and a transient “importomer-engaged” state within the Pex13-containing docking/translocation machinery. The model places the reversible Pex14 topological switch within the import cycle and links it to ubiquitin signaling on Pex5 and AAA-driven receptor extraction by the Pex26-anchored Pex1/Pex6 machinery. Importantly, several steps in this model represent explicit, testable hypotheses rather than directly demonstrated transitions. For example, the proposed Pex26-dependent stabilization of Pex14 via a heterooligomeric membrane subcomplex and the transition of Pex14 into the Pex13-containing import machinery remain to be validated biochemically. These ideas generate clear predictions: (i) Pex26 loss should alter the native assembly state and/or membrane stability of Pex14 (testable by carbonate extraction, native electrophoresis, and crosslinking), (ii) ubiquitination-defective Pex5 variants should suppress formation of the cytosol-exposed Pex14 state (consistent with the MLN-7243 and C11A-based phenotypes), and (iii) AAA-inactive Pex6 should trap a remodeling intermediate in which Pex14 is cytosol-exposed while remaining membrane-embedded. Together, Supplementary Fig. S6 provides a conceptual framework linking ubiquitin signaling and AAA-driven receptor extraction to a reversible topological ‘reset’ of Pex14 after receptor extraction within the import cycle.

On the basis of our observations, I propose the model illustrated in Fig. 6. In this model, Pex14 is embedded in the peroxisomal membrane with its N-terminal domain facing the lumen, where it mediates cargo translocation by interacting with Pex5. During this process, Pex5 partially inserts into the membrane, likely through lipid interactions (14), positioning its N-terminal domain near the luminal face of Pex14, and facilitating interaction with the Pex14 N-terminal domain. Upon cargo release, cysteine-linked monoubiquitination of Pex5 appears to drive its cytosolic repositioning, which may subsequently induce a topological shift of the Pex14 N-terminal domain toward the cytosol. At this stage, it is also conceivable that the trigger for the Pex14 N-terminal domain adopting a cytosol-facing orientation is the episode in which Pex5 is ubiquitinated by the RING finger domain of cytosol-exposed RING peroxins. Specifically, when Pex5 undergoes monoubiquitination on the cytosolic side after translocation, the mode of interaction and/or the spatial arrangement of the Pex5–Pex14 N-terminal interface within the complex may change, and such reorganization could in turn drive cytosolic exposure of the Pex14 N-terminus. Testing this hypothesis requires correlating the spatial context in which ubiquitination occurs with the exposure state of the Pex14 N-terminus under the same experimental conditions. Thus, it would be informative to use site-specific crosslinkers, photo-crosslinking approaches, or compartment-restricted labeling strategies to assess the simultaneity and causality between Pex5 modification in the proximity of the RING complex and cytosolic exposure of the Pex14 N-terminus. Moreover, combining catalytically inactive RING-peroxin mutants, non-ubiquitinatable Pex5 variants like C11A/C11K, and mutants targeting the Pex14 N-terminal interaction surface should help more clearly discriminate whether ubiquitination acts as a trigger for, or instead a consequence of, the topological remodeling event. This cytosol-oriented conformation likely facilitates interaction with the Pex6–Pex26 complex, enabling ATP-dependent extraction of ubiquitinated Pex5. The extraction step is followed by reorientation of the Pex14 N-terminal domain back toward the lumen, thereby resetting the import machinery for subsequent rounds of matrix protein import. Pharmacological inhibition using MLN-7243 or NMS-873 highlights the sequential requirement of Pex5 ubiquitination and ATP hydrolysis by Pex6 in orchestrating this membrane topology remodeling process. However, the mechanism that drives the reorientation of the Pex14 N-terminal domain back toward the luminal side after the dissociation of monoubiquitinated Pex5 by Pex6 has yet to be determined.

These findings collectively suggest that Pex14 is not a static structural scaffold, but rather a dynamic component of the docking/translocation complex whose membrane topology

is subject to regulated remodeling. Such topological plasticity may contribute to the coordination of cargo delivery, receptor recycling, and structural resetting of the import machinery. While this study expands our understanding of the dynamic behavior of Pex14, several mechanistic aspects remain to be clarified. It remains unclear whether the observed reorientation of Pex14 reflects complete membrane translocation of its N-terminal domain across the membrane, or instead involves more subtle rearrangements, such as partial reinsertion or local conformational rearrangements. These potential mechanisms may involve regulatory events that are not readily detectable under the experimental conditions used in this study. Moreover, the contributions of additional components of the docking/translocation complex, including Pex1, Pex13 and the RING peroxins, to the regulation of Pex14 topology, either through direct interaction or through post-translational modifications, remain to be fully elucidated. In addition, whether Pex6 directly promotes the dissociation of ubiquitinated Pex5 from the N-terminal domain of Pex14 to induce topological remodeling has not been clearly established and may require further investigation. Notably, deficiency of Pex1 does not result in cytosolic exposure of the Pex14 N-terminal domain, indicating that Pex1 is not essential for maintaining its proper membrane topology. Although Pex1 assembles into a heterohexameric complex with Pex6, the specific contribution of Pex1 itself to the regulation of Pex14 topology, either independently or in coordination with Pex6, remains to be clarified (28). In PEX1-deficient cells, the architecture of the Pex1/Pex6 complex may be partially disrupted. However, the D2 domain of Pex6, which is a core component of the AAA functional module, could remain and operate in a limited manner. As a consequence, although the extraction efficiency of ubiquitinated Pex5 would be reduced, the Pex5–Pex14 interaction might still shift, to some extent, toward dissociation. In contrast, in PEX6-deficient cells, because Pex6 itself, which is the substrate that Pex26 normally tethers, is absent, Pex1 is not properly recruited to the peroxisomal membrane and local enrichment of the AAA machinery fails to occur. This would be expected to promote retention of ubiquitinated Pex5 near Pex14 and to markedly impair dissociation of the Pex5–Pex14 complex. Thus, the phenotypic differences between PEX1- and PEX6-deficient cells may be explained not only by the presence or absence of AAA activity per se, but also by a combination of parameters such as membrane localization and local concentration of the machinery, residual partial activity, and the extent of substrate retention. To test this idea, it will be necessary to combine quantitative measurements of membrane recruitment of Pex1/Pex6 or Pex6 alone, assessments of the dissociation kinetics of the Pex5–Pex14 interaction (e.g., time-resolved immunoprecipitation and/or live-cell dynamics), and rescue experiments using Pex26 re-expression or membrane-targeting variants, in order to evaluate the

plausibility of this mechanistic model. In parallel, while our findings suggest that monoubiquitination of Pex5 promotes topological remodeling of the Pex14 N-terminal domain, additional studies using non-ubiquitinatable Pex5 mutants or ubiquitin chain-specific probes will be necessary to determine whether monoubiquitination alone is both necessary and sufficient for this process, or whether polyubiquitination and other post-translational modifications may also contribute to this process. With respect to ubiquitination, it is difficult to rigorously disentangle Pex5 mono-ubiquitination from poly-ubiquitination using only cell-based inhibitors and mutant analyses. Therefore, where feasible, it will be important to employ an *in vitro* reconstitution system to directly test the causal relationship between the mode of Pex5 ubiquitination and the topological remodeling of Pex14. Specifically, the use of methylated ubiquitin, lysine-deficient ubiquitin and linkage-restricted ubiquitin variants would allow systematic evaluation of whether single ubiquitin conjugation is sufficient, whether chain formation is required, and which linkage types contribute. On the Pex5 side, extending analyses beyond C11A to substitutions such as Pex5 C11K, in combination with identification and mutagenesis of candidate lysine acceptor sites, could more convincingly define the specific role of cysteine-linked mono-ubiquitination. In addition, integrating ubiquitin linkage-specific probes, DUB treatments, and mass-spectrometry-based mapping of modification sites should enable a quantitative determination of which Pex5 modification state most strongly drives the observed changes in cytosolic exposure of the Pex14 N-terminus.

In conclusion, this study identifies a previously unrecognized mechanism by which ATP hydrolysis by Pex6 and ubiquitination of Pex5 cooperatively regulate the membrane orientation of the Pex14 N-terminal domain. This mechanism coordinates energy-dependent receptor recycling with structural resetting of the import machinery, providing new insight into the dynamic regulation of peroxisomal matrix protein import.

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES

Antibodies

I used rabbit polyclonal antibodies against the N-terminal (Pex14N) and C-terminal (Pex14C) domains of rat Pex14, as previously described (17) (42) (43). Additional rabbit antisera were employed against the PTS1 peptide (10), human catalase (44), and Pex5N (19).

Cell Culture, Plasmids, and DNA Transfection

Fibroblasts derived from Japanese Zellweger syndrome (ZS) patients *pex1* CG1 (PBDE-14), *pex2* CG10 (PBDF-01), *pex10* CG7 (PBDB-01), and *pex12* CG3 (PBD3-02) were provided by Gifu University (45-48). Additional fibroblast lines from ZS patients *pex26* CG8 (GM07371) and *pex6* CG4 (GM13267) were obtained from the Coriell Institute for Medical Research (44). Human fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂, as described previously (19). CHO cells were cultured in Ham's F-12 medium with 10% FCS under the same conditions (49). Plasmids encoding Pex6 variants (37) and Pex5 C11A mutant (19) were described previously. DNA transfection was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

Drug Treatments

AAA+ ATPase inhibitors (NMS-873 and CB-5083) and ubiquitination pathway inhibitors, including a NEDD8-activating enzyme inhibitor (MLN-4924) and a ubiquitin-activating enzyme inhibitor (MLN-7243), were obtained from SelleckChem. Human fibroblasts and CHO-K1 cells were incubated with 3 μM MLN-4924 (13), 10 μM NMS-873 (40) (41), or 50 nM CB-5083 (39) for 72 h at 37°C. MLN-7243 (1 μM) (13) and cycloheximide (25 μg /ml) were added to human fibroblast cultures for 6 h at 37°C.

ATP Depletion

ATP depletion was induced by incubating human fibroblasts in glucose-free DMEM supplemented with 10 mM sodium azide, 10 mM sodium fluoride, and 50 mM 2-deoxy-D-glucose for 6 h at 37°C (38).

Morphological analysis

Cells were fixed with 4% paraformaldehyde, permeabilized with either 0.1% Triton X-100 or 60 μg/mL digitonin in 1% bovine serum albumin, and incubated with primary

antibodies for 1 h at room temperature (10). Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) were used for indirect immunofluorescence (38). Images were acquired using a Zeiss Axioscope with a Plan Apochromat 63×/1.4 NA oil-immersion objective lens. Image processing was performed using Zen software (Zeiss). Peroxisome-restoring activity of Pex6 was verified by counting PTS1- or catalase-positive cells among 100 cells in *pex6* ZP164 cells. The import efficiency of wild-type Pex6 was taken as 100%.

Protease Protection Assay

Post-nuclear supernatant (PNS) fractions were prepared from human fibroblasts and CHO cells. Protease protection was assessed by incubating 0.5 mg/mL PNS with 1 mg/mL proteinase K on ice for 30 min, as described (13) (17). The digestion was terminated by the addition of 1 mM PMSF. The digests were analyzed by SDS-PAGE followed by immunoblotting using rabbit anti-Pex14N and anti-Pex5N antibodies.

Immunoblotting

Proteins were transferred to polyvinylidene membranes (Bio-Rad) and probed with primary antibodies and second antibodies conjugated to horseradish peroxidase (GE Healthsciences) (10). Antigen-antibody complexes were visualized with an ECL Western blotting detection reagent (GE Healthsciences).

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I would like to thank Dr. Tamura, Dr. Yagita, and Dr. Fujiki for their supervision and encouragement. I would also like to thank T. Umemoto for technical assistance and the other members of our laboratories for helpful discussions.

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FIGURE LEGENDS

FIGURE LEGENDS

Figure 1.

Pex6 and Pex26 are required to maintain the proper membrane topology of the Pex14 N-terminal domain.

A, immunofluorescence analysis of the membrane orientation of the N-terminal (N) and C-terminal (C) domains of Pex14 in control, $\Delta PEX1$ (*pex1*), $\Delta PEX6$ (*pex6*), and $\Delta PEX26$ (*pex26*) human fibroblasts. Cells were selectively permeabilized with 60 μ g/mL digitonin (left panels), which preserves peroxisomal membrane integrity while allowing access to cytosolically exposed epitopes, or fully permeabilized with 1% Triton X-100 (right panels). Samples were stained with antibodies directed against the N-terminal domain (α -Pex14N; panels a, c, e, g, i, k, m, and o) or the C-terminal domain (α -Pex14C; panels b, d, f, h, j, l, n, and p) of Pex14. Scale bar, 20 μ m. B, protease protection assay to assess the membrane topology of the Pex14 N-terminal domain. Post-nuclear supernatants (PNS) from control and mutant fibroblasts were treated with 1 mg/mL proteinase K for 30 min on ice in the absence or presence of 1% Triton X-100. Samples were analyzed by SDS-PAGE and immunoblotting using α -Pex14N antibody. A solid arrowhead indicates full-length Pex14; an open arrowhead denotes an ~18-kDa protease-resistant fragment corresponding to the membrane-protected N-terminal domain. C, schematic diagram of the protease accessibility assay. Under normal conditions, the Pex14 N-terminal domain, which includes the Pex5-binding domain, is inserted into the peroxisomal membrane and oriented toward the matrix, rendering it inaccessible to cytosolic protease (Pro. K). In $\Delta PEX6$ (*pex6*) or $\Delta PEX26$ (*pex26*) cells, this domain becomes misoriented and exposed to the cytosol, resulting in proteolytic degradation. CC, coiled-coil domain; TM, transmembrane domain. All experiments shown in A and B were independently repeated at least three times with similar results, and representative images and immunoblots are shown.

Figure 2.

ATPase activity of Pex6 is required for topological reorientation of the Pex14 N-terminal domain.

A, immunofluorescence analysis of the Pex14 N-terminal domain in $\Delta PEX6$ (*pex6*) human fibroblasts transiently transfected with wild-type or Walker motif mutant Pex6 constructs. Cells were permeabilized with 60 μ g/mL digitonin and stained with the antibody against the N-terminal domain of Pex14 (α -Pex14N; panels a–e). Expression of wild-type Pex6 (+*PEX6*) restored the membrane-protected localization of the N-terminal

domain, as indicated by the absence of detectable α -Pex14N signal (panel b). In contrast, cells expressing Walker A mutants K476E (D1) or K750E (D2), or the Walker B mutant D803N (D2), failed to reestablish this topology, thus exhibiting strong punctate α -Pex14N staining apparently comparable to mock-transfected cells (panels a, c–e). Scale bar, 20 μ m. *B*, protease protection assay assessing the membrane orientation of the Pex14 N-terminal domain in the same transfected cells as shown in (*A*). Post-nuclear supernatants (PNS) were treated on ice with 1 mg/mL proteinase K for 30 min in the absence or presence of 1% Triton X-100. Samples were separated by SDS-PAGE and analyzed by immunoblotting using α -Pex14N antibody. A solid arrowhead denotes full-length Pex14, and an open arrowhead indicates the $\sim$ 18 kDa protease-protected fragment corresponding to the N-terminal domain, either embedded in the membrane or lumen-facing orientation. All experiments were independently repeated at least three times with similar results.

Figure 3.

ATP is required to maintain the membrane-protected topology of the Pex14 N-terminal domain.

A, immunofluorescence analysis of the Pex14 N-terminal domain in human fibroblasts under ATP-depleted conditions. Cells were incubated for 6 h at 37°C in ATP-depletion medium (DMEM without glucose, supplemented with 10 mM sodium azide, 10 mM sodium fluoride, and 50 mM 2-deoxy-D-glucose), then selectively permeabilized with 60 μ g/mL digitonin (panels a, b) or fully permeabilized with 1% Triton X-100 (panels c, d). Samples were stained with α -Pex14N antibody. Scale bar, 20 μ m. *B*, protease protection assay to assess the membrane topology of the Pex14 N-terminal domain under ATP-depleted conditions. PNS fractions were treated on ice with 1 mg/mL proteinase K for 30 min in the absence or presence of 1% Triton X-100. Samples were analyzed by SDS-PAGE and immunoblotting using α -Pex14N antibody. A solid arrowhead denotes full-length Pex14, and an open arrowhead indicates the $\sim$ 18-kDa protease-resistant fragment corresponding to the membrane-embedded N-terminal domain. All experiments were independently repeated at least three times with comparable results.

Figure 4.

Pharmacological inhibition of AAA+ ATPases impairs peroxisomal matrix protein import and alters the membrane topology of the Pex14 N-terminal domain.

A, immunofluorescence analysis of peroxisomal matrix protein import in ZP164 cells (*PEX6*-deficient CHO cells) transfected with wild-type *PEX6*. Cells were treated with DMSO (vehicle control; panels b, f), 100 nM CB-5083 (panels c, g), or 100 nM NMS-

873 (panels d, h) for 72 h, fixed, permeabilized with 1% Triton X-100, and stained with antibodies against PTS1-containing proteins (panels a–d) and catalase (panels e–h). Mock-transfected cells (*pex6* ZP164, panels a, e) served as a negative control. Scale bar, 10 μ m. *B*, quantitative analysis of import activity shown in (*A*). Import efficiency was calculated as the percentage of Pex14-positive cells exhibiting punctate signals for PTS1-containing proteins or catalase. Data represent mean $\pm$ SD from three independent biological experiments, each quantified from six technical measurements ($n = 6$). Statistical significance was determined using unpaired two-tailed *t*-tests (** $p < 0.01$, *** $p < 0.001$). *C* and *D*, immunofluorescence analysis of the membrane orientation of the Pex14 N-terminal domain in CHO-K1 cells (*C*) and human fibroblasts (*D*) following ATPase inhibition. Cells were treated with DMSO (vehicle control; panels a, b), 50 nM CB-5083 (panels c, d), or 10 μ M NMS-873 (panels e, f) for 72 h, then subjected to selective permeabilization with 60 μ g/mL digitonin (left panels) or full permeabilization with 1% Triton X-100 (right panels). Samples were stained with α -Pex14N antibody. Scale bars: 10 μ m (*C*), 20 μ m (*D*).

Figure 5.

Ubiquitination of Pex5 promotes cytosolic exposure of the Pex14 N-terminal domain and regulates its membrane topology.

A, immunofluorescence analysis of peroxisomal matrix protein import in ZP164 cells (*PEX6*-deficient CHO cells) transfected with either mock (panel a) or wild-type *PEX6* (panels b–d). Cells were treated for 6 h with DMSO (vehicle control; panel b), 10 μ M MLN-4924 (panel c), or 10 μ M MLN-7243 (panel d), then fixed, permeabilized with 1% Triton X-100, and stained with an antibody against the PTS1 epitope. Scale bar, 10 μ m. *B*, quantification of import activity shown in (*A*), calculated as the percentage of Pex14-positive cells exhibiting punctate PTS1 signals. Data represent mean $\pm$ SD from three independent biological experiments, each quantified using six technical replicates ($n = 6$). Statistical significance was determined by unpaired two-tailed *t*-tests (*** $p < 0.001$). *C*, immunofluorescence analysis of the membrane orientation of the Pex14 N-terminal domain in control, Δ *PEX6*, Δ *PEX26*, and ATP-depleted human fibroblasts. Cells were treated for 6 h with DMSO (vehicle control; panels a–d) or 10 μ M MLN-7243 (panels e–h), selectively permeabilized with 60 μ g/mL digitonin, and stained with α -Pex14N antibody. Scale bar, 20 μ m. *D*, quantitative analysis of cytosolic exposure of the Pex14 N-terminal domain shown in (*C*). Data represent the percentage of cells with detectable α -Pex14N signal following digitonin permeabilization. Results are shown as mean $\pm$ SD from three biological replicates, each assessed in six technical replicates ($n =$

6). *** $p < 0.001$ by unpaired two-tailed t -tests. *E*, protease protection assay assessing the membrane topology of the Pex14 N-terminal domain in control and $\Delta PEX6$ fibroblasts with or without MLN-7243 treatment. PNS fractions were incubated on ice with 1 mg/mL proteinase K for 30 min in the absence or presence of 1% Triton X-100. Samples were analyzed by SDS-PAGE and immunoblotting using α -Pex14N. Solid and open arrowheads indicate full-length Pex14 and the ~ 18 kDa protease-resistant fragment, respectively. *F*, protease protection assay of Pex5 under the same conditions as (*E*). Full-length Pex5 (solid arrowhead) and partial digestion products (open arrowhead) were detected in control cells and increased following MLN-7243 treatment. *G*, immunoblot analysis of Pex5 ubiquitination in $\Delta PEX6$ fibroblasts. PNS (P) fractions were prepared from *PEX6*-deficient human fibroblast cells treated with or without 1 μ M MLN-7243 for 6 h. Samples were incubated with 10 mM N-ethylmaleimide (NEM) to preserve ubiquitin conjugates, fractionated by centrifugation into the cytosolic (C) and organelle (O) fractions, and subjected to SDS-PAGE. Where indicated, samples were further treated with 50 mM dithiothreitol (DTT) to reduce cysteine-linked ubiquitin conjugates. An aliquot of the C fraction equivalent to the P fraction and seven aliquots of the O fraction were loaded. Immunoblotting was performed using anti-Pex5N antibody. Open and closed arrowheads indicate monoubiquitinated and unmodified Pex5, respectively (50).

Figure 6.

Proposed model for ATP- and ubiquitination-dependent topological conversion of the Pex14 N-terminal domain.

Under basal conditions, Pex14 is integrated into the peroxisomal membrane with its N-terminal domain embedded and oriented toward the lumen, where it facilitates matrix protein translocation. Upon cargo delivery, the cytosolic receptor Pex5, carrying a PTS1-containing protein, docks onto the Pex14-containing docking/translocation complex (Step 1). Following cargo release, Pex5 undergoes monoubiquitination at its N-terminal cysteine residue, which is proposed to trigger a topological rearrangement that reorients the N-terminal domain of Pex14 toward the cytosolic side (Steps 2 and 3). This cytosol-oriented conformation may facilitate the recruitment and recycling of Pex5. The AAA+ ATPase Pex6, anchored to the membrane via Pex26, recognizes ubiquitinated Pex5 and extracts it from the peroxisomal membrane in an ATP hydrolysis-dependent manner (Step 4). This extraction process is concurrently coupled to the restoration of the lumen-facing topology of the Pex14 N-terminal domain, effectively resetting the docking/translocation complex for subsequent rounds of protein import. Pharmacological inhibition of ubiquitination using MLN-7243 blocks the outward topological switch, while inhibition

of ATPase activity by NMS-873 prevents the inward reorientation. Together, these findings support a model in which dynamic and reversible regulation of Pex14 topology is integral to the peroxisomal protein import cycle. CC, coiled-coil domain; TM, transmembrane domain.

Figure S1.

Pex2, Pex10 and Pex12 are not required to maintain the proper membrane topology of the Pex14 N-terminal domain.

Immunofluorescence analysis of the membrane orientation of the N-terminal (N) and C-terminal (C) domains of Pex14 in control, Δ PEX2 (*pex2*), Δ PEX10 (*pex10*), and Δ PEX12 (*pex12*) human fibroblasts. Cells were selectively permeabilized with 60 μ g/mL digitonin (left panels), which preserves peroxisomal membrane integrity while allowing access to cytosolically exposed epitopes, or fully permeabilized with 1% Triton X-100 (right panels). Samples were stained with antibodies directed against the N-terminal domain (α -Pex14N; panels a, c, e, g, i, k, m, and o) or the C-terminal domain (α -Pex14C; panels b, d, f, h, j, l, n, and p) of Pex14. Scale bar, 20 μ m.

Figure S2.

Pex6 and Pex26 are required to maintain the proper membrane topology of the Pex14 N-terminal domain in Chinese Hamster Ovary cells.

Immunofluorescence analysis of the membrane orientation of the N-terminal (N) and C-terminal (C) domains of Pex14 in control, CHO mutants, ZP107 (*pex1*), ZP164 (*pex6*), ZP167 (*pex26*), Z65 (*pex2*) and ZP109 (*pex12*). Cells were selectively permeabilized with 60 μ g/mL digitonin (left panels), which preserves peroxisomal membrane integrity while allowing access to cytosolically exposed epitopes, or fully permeabilized with 1% Triton X-100 (right panels). Samples were stained with antibodies directed against the N-terminal domain (α -Pex14N; panels a, c, e, g, i, k, m, o, q, s, u and w) or the C-terminal domain (α -Pex14C; panels b, d, f, h, j, l, n, p, r, t, v and x) of Pex14. Scale bar, 20 μ m.

Figure S3.

Protease protection assay of the Pex14 N-terminal domain in cells expressing a monoubiquitination-defective Pex5 mutant (C11A).

ZP101 cells, which lack endogenous Pex5 and exhibit marked instability of endogenous Pex14, were first transiently transfected with PEX14, followed by expression of either wild-type PEX5 or the monoubiquitination-defective mutant PEX5(C11A). Post-nuclear supernatant fractions were treated on ice with 1 mg/mL proteinase K in the absence or

presence of 1% Triton X-100, and samples were analyzed by SDS-PAGE and immunoblotting using α -Pex14N antibody. In cells expressing Pex5(C11A), the Pex14 N-terminal domain remained predominantly protease-protected in the absence of detergent, similar to wild-type Pex5, indicating that loss of Pex5 monoubiquitination does not cause cytosolic exposure of the Pex14 N-terminus.

Figure S4.

Pex14 is inserted on peroxisomal membrane in *PEX6*-deficient cells.

Δ *PEX6* (*pex6*) human fibroblasts were fractionated into postnuclear supernatant, cytosolic, and organelle fractions. The organelle fractions (T, total) were treated with 0.1 M Na₂CO₃ and separated into soluble (S, supernatant) and membrane (P, pellet) fractions. Equal aliquots of respective fractions were analyzed by SDS-PAGE and immunoblotting with α -Pex14N antibody.

Figure S5.

Pex14 topological remodeling is Not driven by dual-topology de novo insertion.

Immunofluorescence analysis of the Pex14 N-terminal domain in human fibroblasts with cycloheximide under ATP-depleted conditions. Cells were incubated for 6 h at 37°C in ATP-depletion medium (DMEM without glucose, supplemented with 10 mM sodium azide, 10 mM sodium fluoride, and 50 mM 2-deoxy-D-glucose) with 25 μ g /ml cycloheximide, then selectively permeabilized with 60 μ g/mL digitonin (panels a, b) or fully permeabilized with 1% Triton X-100 (panels c, d). Samples were stained with α -Pex14N antibody. Scale bar, 20 μ m.

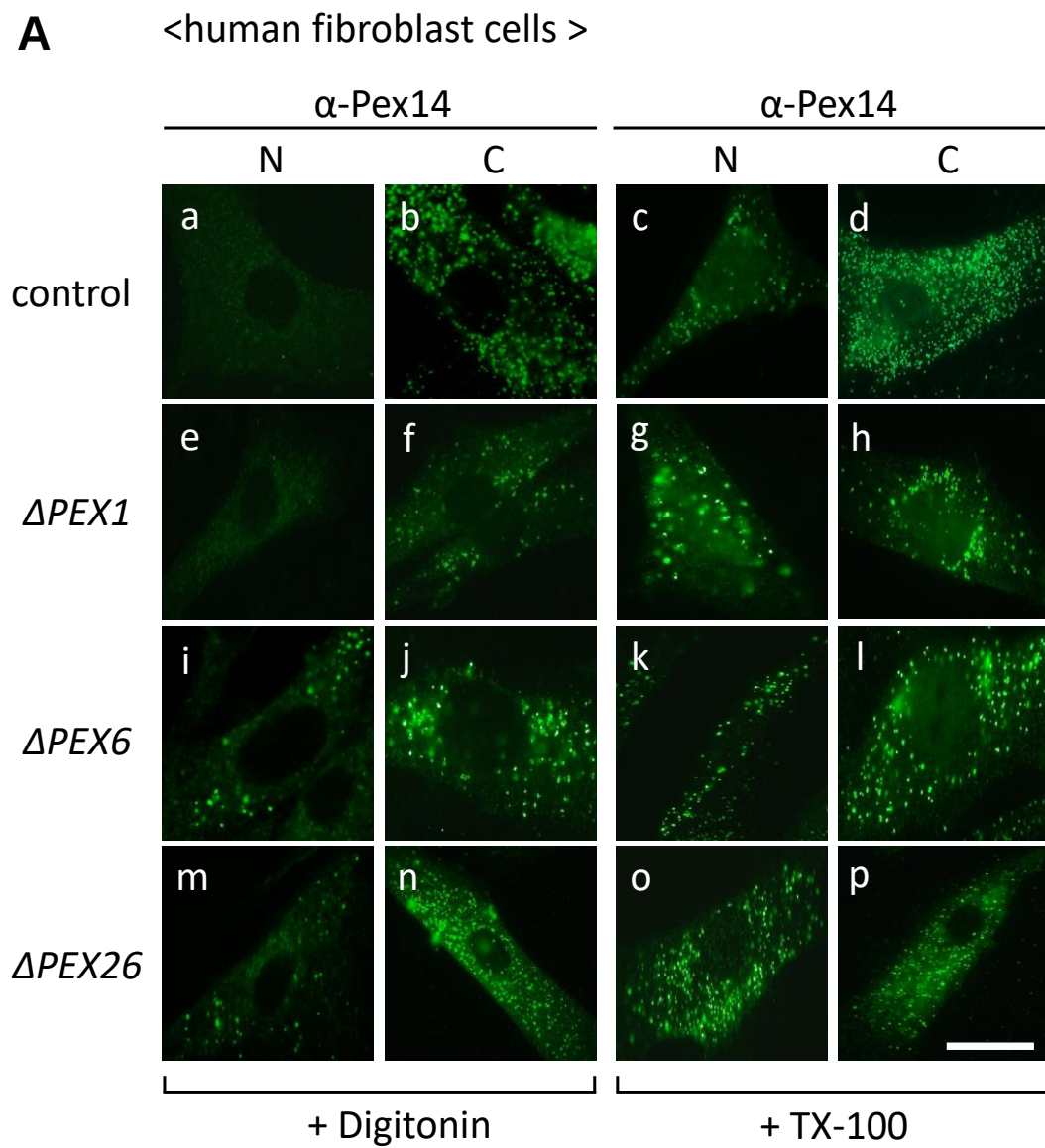
Figure S6.

Proposed model for dynamic topological remodeling of Pex14 during peroxisomal matrix protein import.

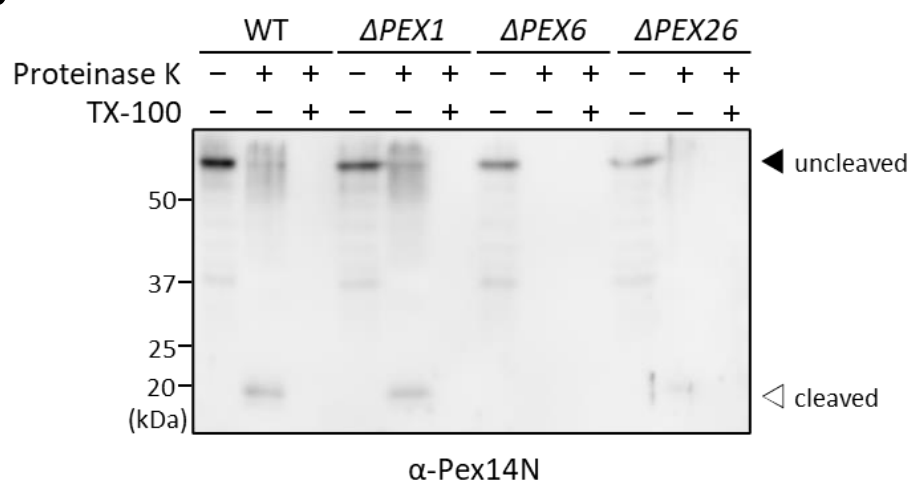
This schematic summarizes a working model in which the membrane topology of the N-terminal region of Pex14 is dynamically regulated in coordination with the receptor cycle of Pex5. Pex14 is a peroxisomal membrane peroxin that serves as a major docking component for the cytosolic PTS1 receptor Pex5 and is thought to participate in assembly of the transient translocation machinery. Pex26 is depicted as a membrane anchor for the cytosolic AAA+ ATPase Pex6 and is proposed to organize a functional membrane subcomplex.

- (❶) Basal state. Pex14 is integrated into the peroxisomal membrane, and its N-terminal region is oriented toward the cytosolic side under steady-state conditions. Pex26 is proposed to stabilize Pex14 and/or maintain the architecture of the Pex14-containing membrane subcomplex.
- (❷) Engagement with the docking/translocation machinery. Pex14 can dynamically associate with the Pex13-containing docking/translocation apparatus, thereby contributing to formation of a transient import conduit. In this state, Pex14 is positioned to receive incoming receptor–cargo complexes.
- (❸,❹) Docking and translocation. Cargo-loaded Pex5 (bound to a PTS1-containing matrix protein) docks to Pex14 on the cytosolic face of the peroxisomal membrane. This docking step promotes subsequent translocation of the cargo into the lumen/matrix, followed by cargo release.
- (❺) Receptor ubiquitination. After cargo release, membrane-associated Pex5 is recognized by the cytosol-facing RING peroxins, which catalyze ubiquitination of Pex5 as a key step of the receptor recycling pathway.
- (❻) Topological remodeling of Pex14. Ubiquitination of Pex5 is proposed to trigger or accompany a conformational/membrane rearrangement that alters the accessibility/orientation of the Pex14 N-terminal region, representing the topological remodeling event highlighted in this model.
- (❼) AAA-ATPase–driven extraction and reset. The Pex26-anchored AAA+ ATPase Pex6 recognizes ubiquitinated Pex5 and extracts it from the peroxisomal membrane in an ATP hydrolysis–dependent manner. This extraction step is proposed to be coupled to resetting of the Pex14-containing docking/translocation machinery for subsequent rounds of matrix protein import.

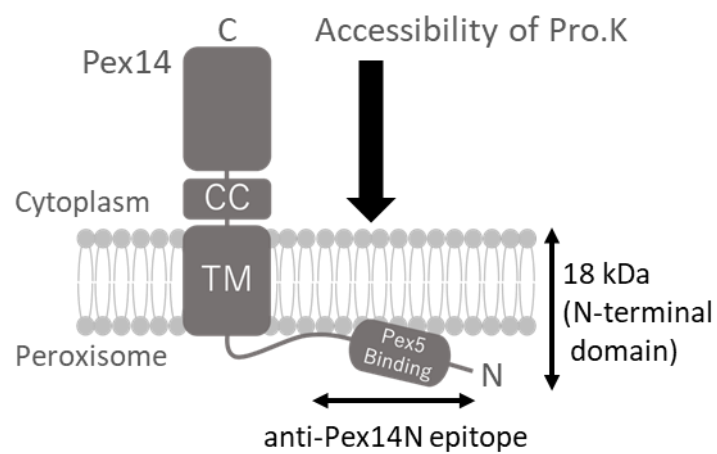
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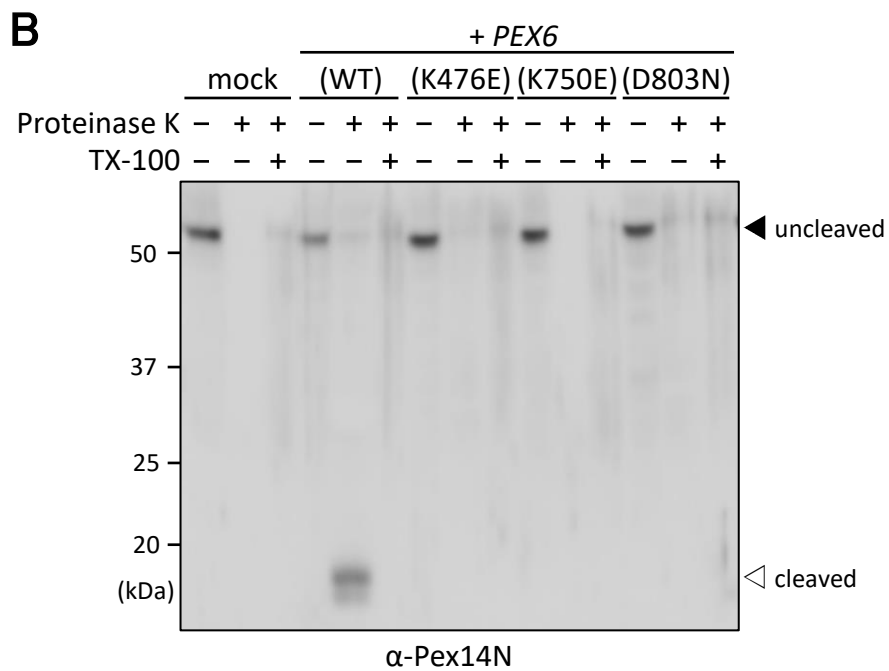
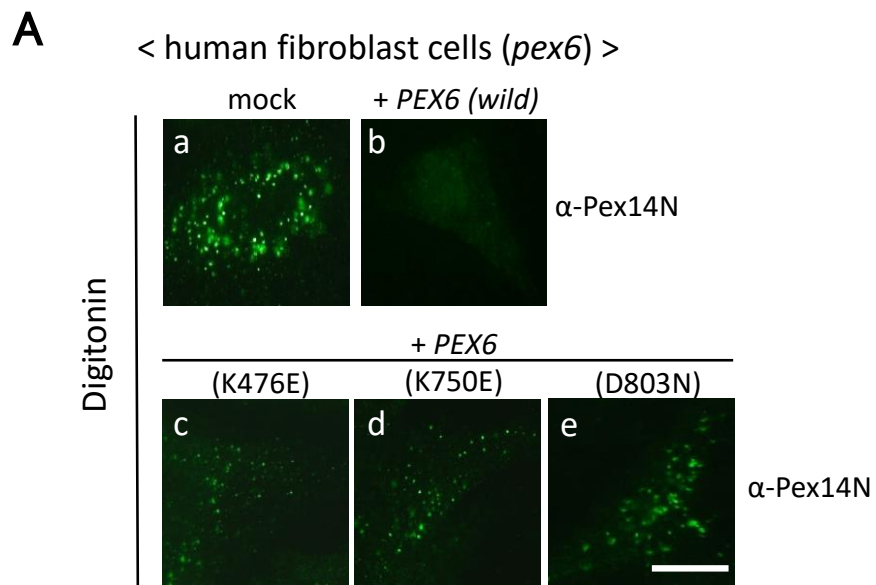


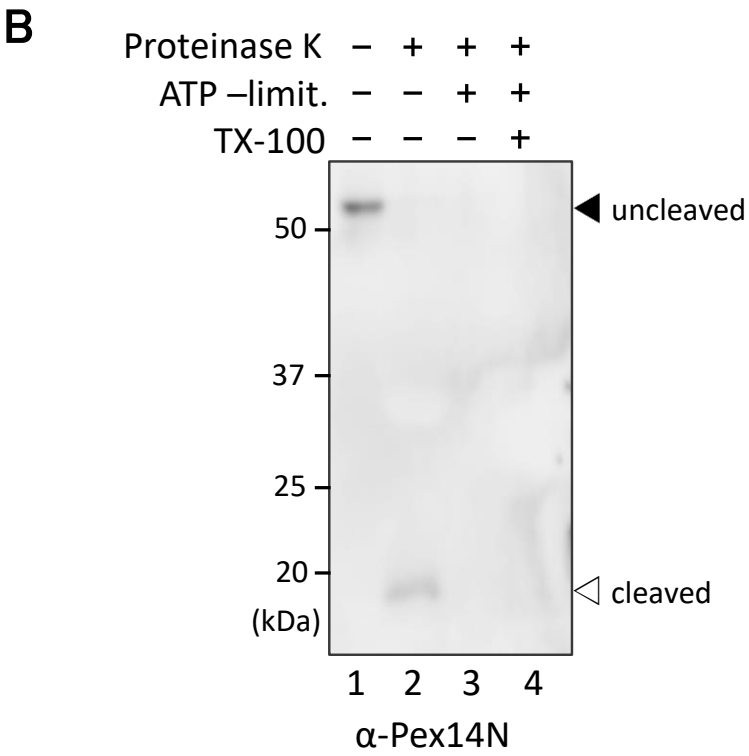
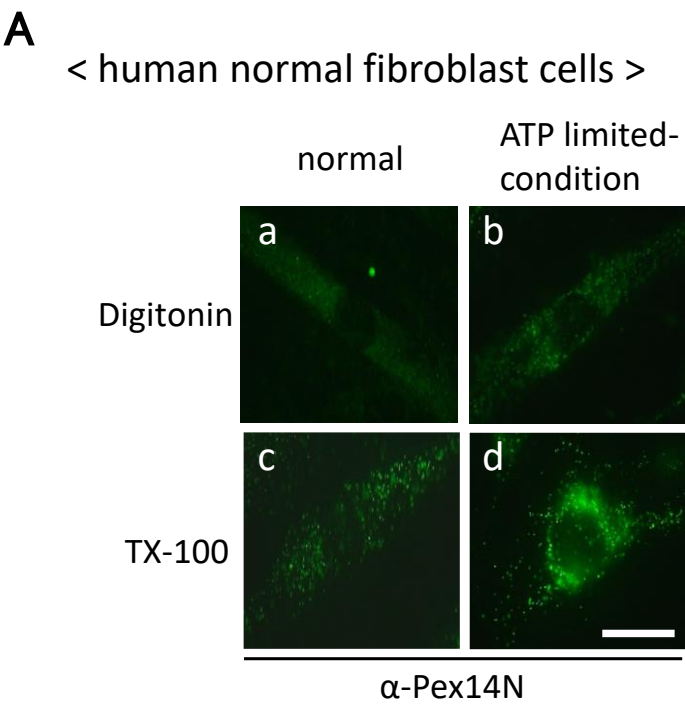
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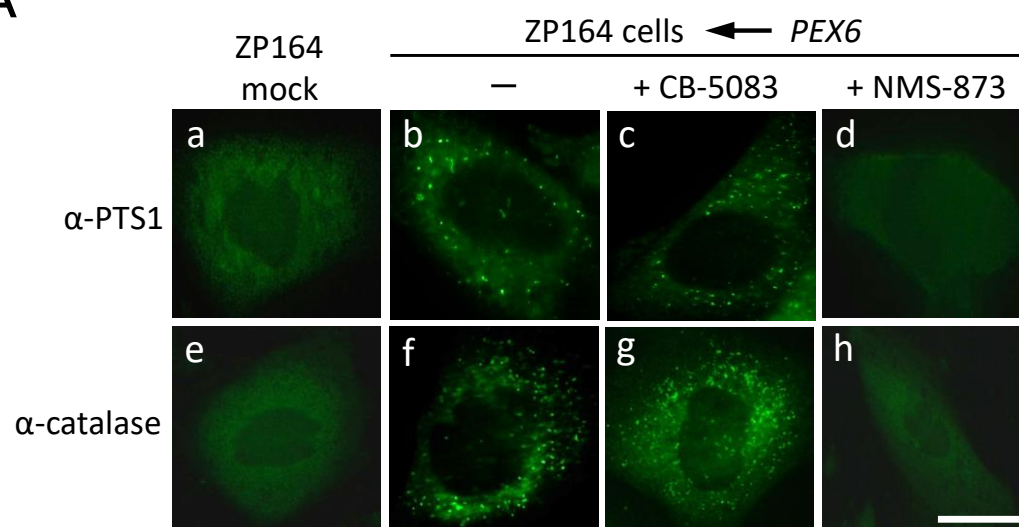
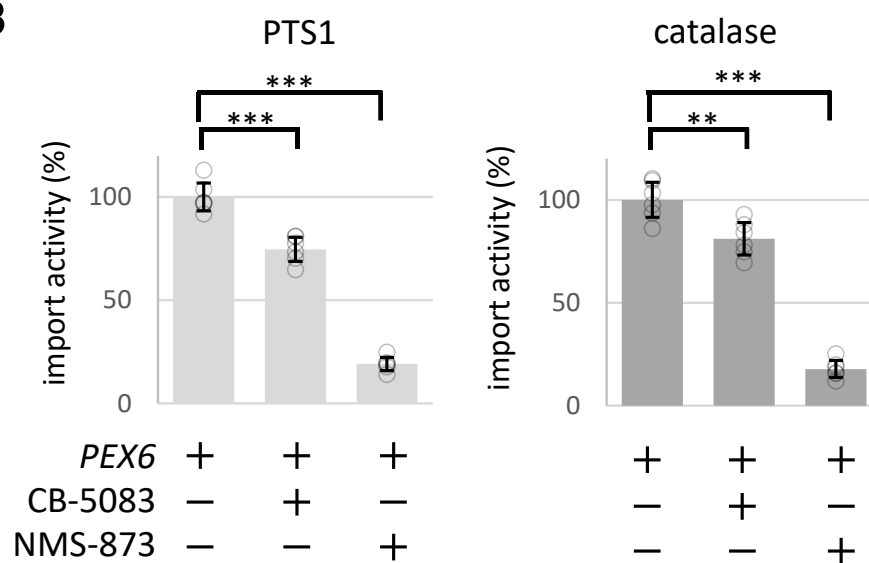


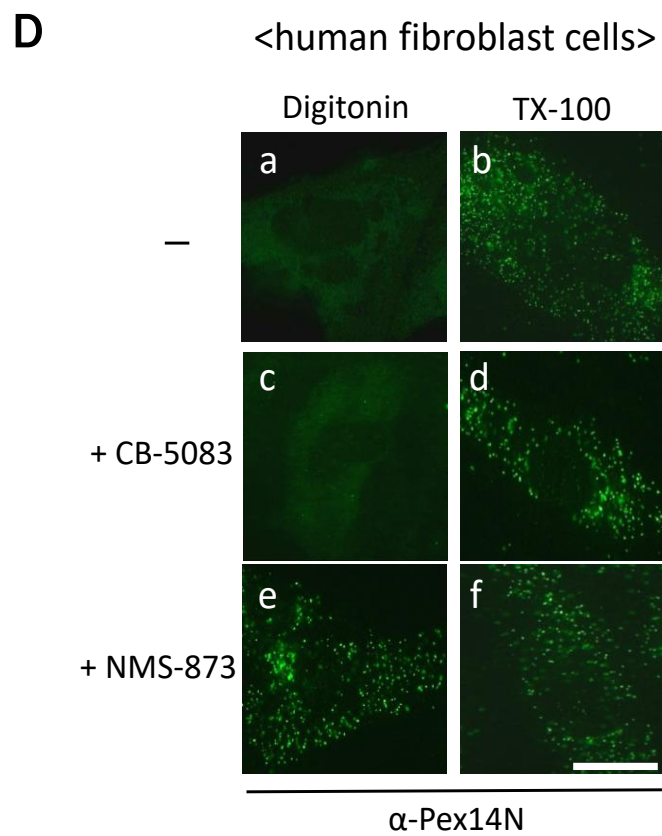
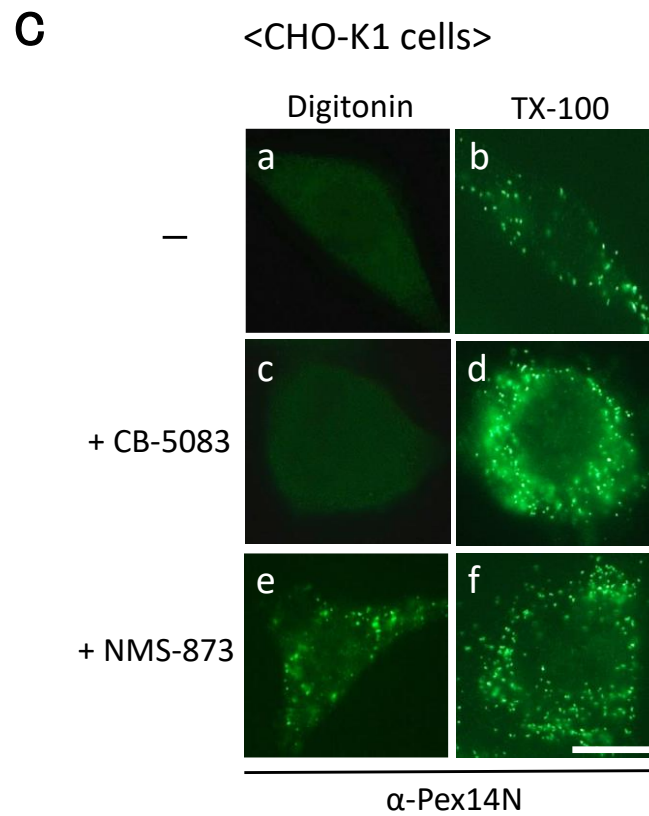
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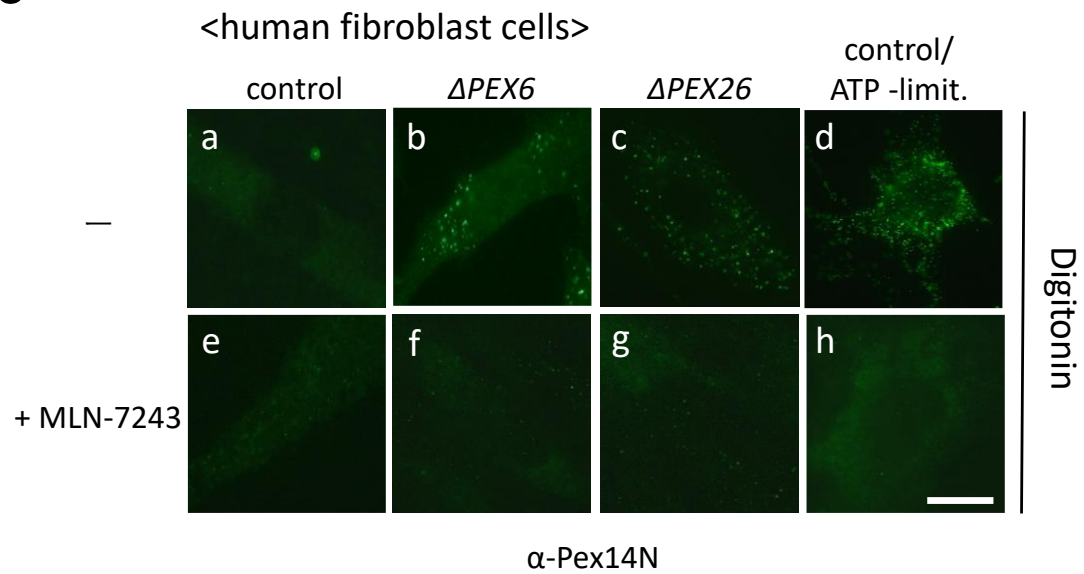
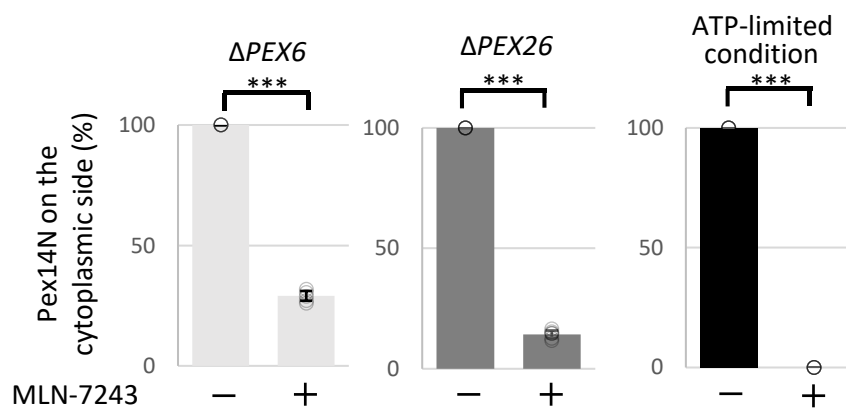


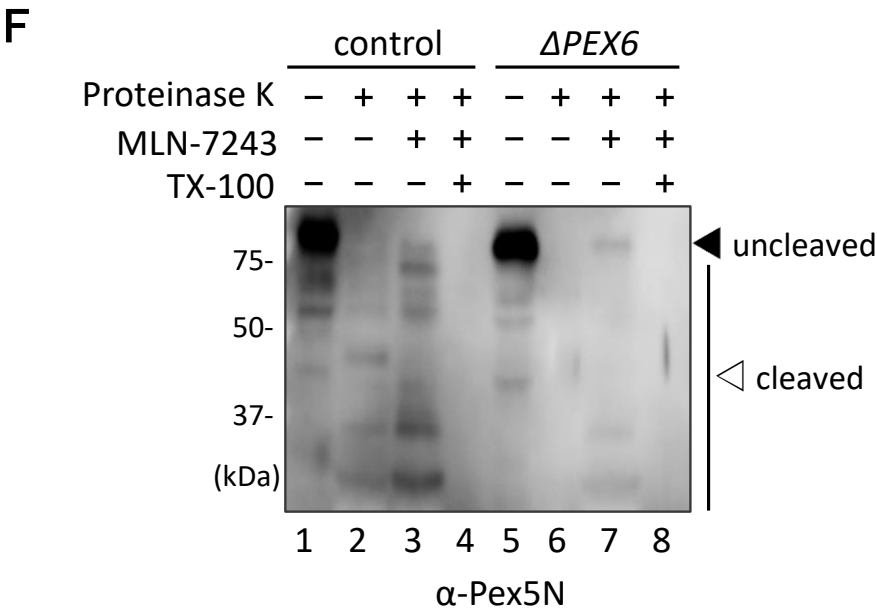
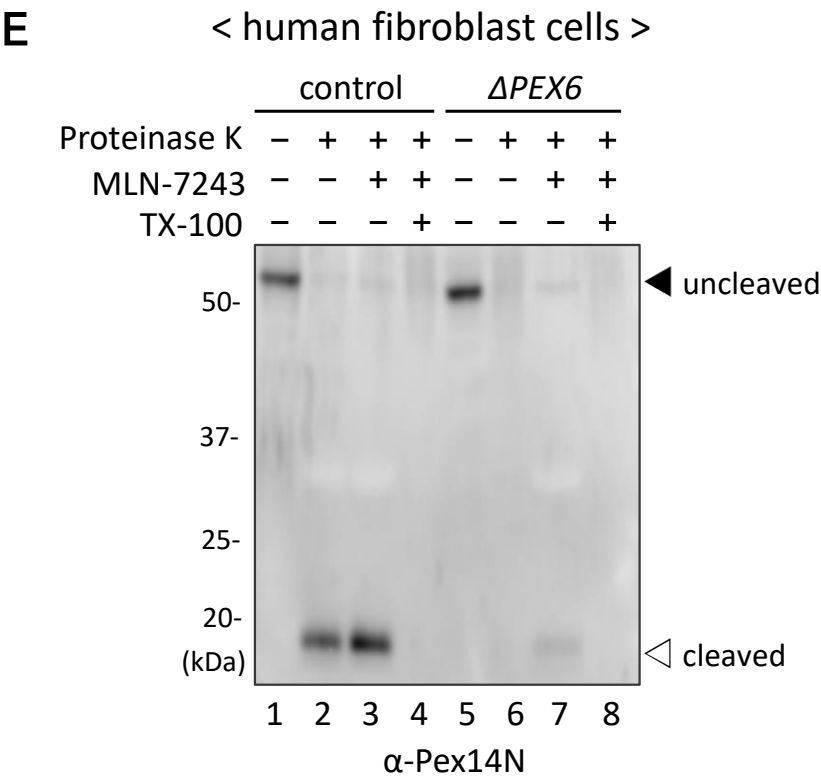




A**B**



C**D**



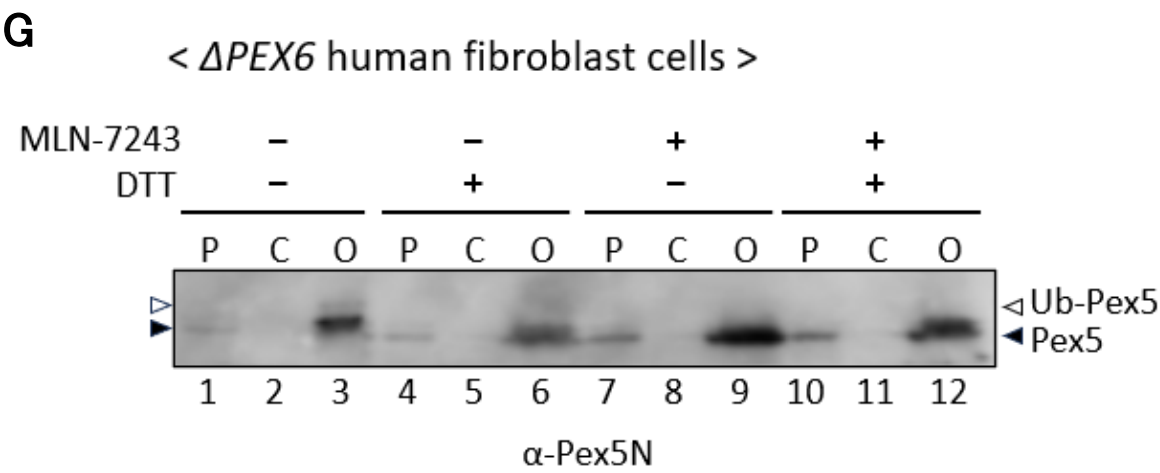
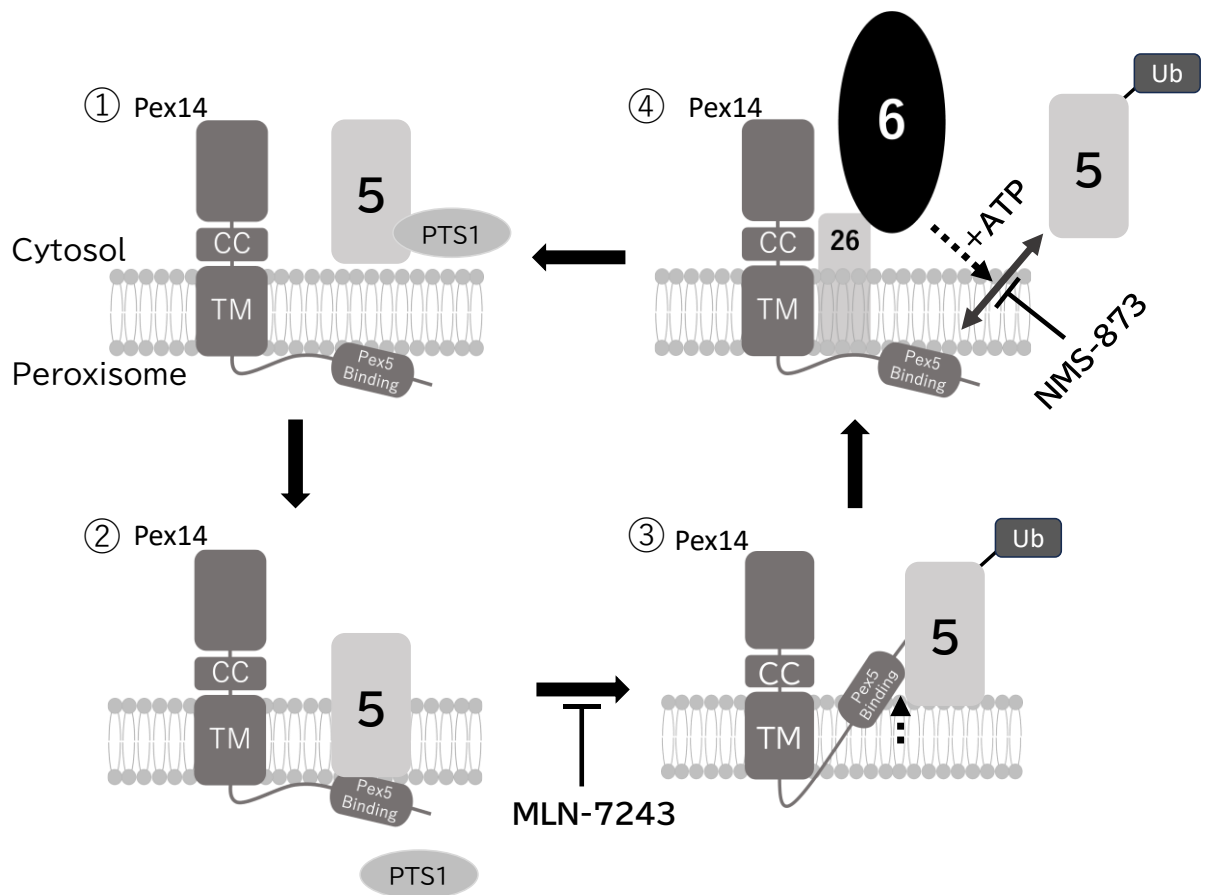


Figure 6 Yasumitsu



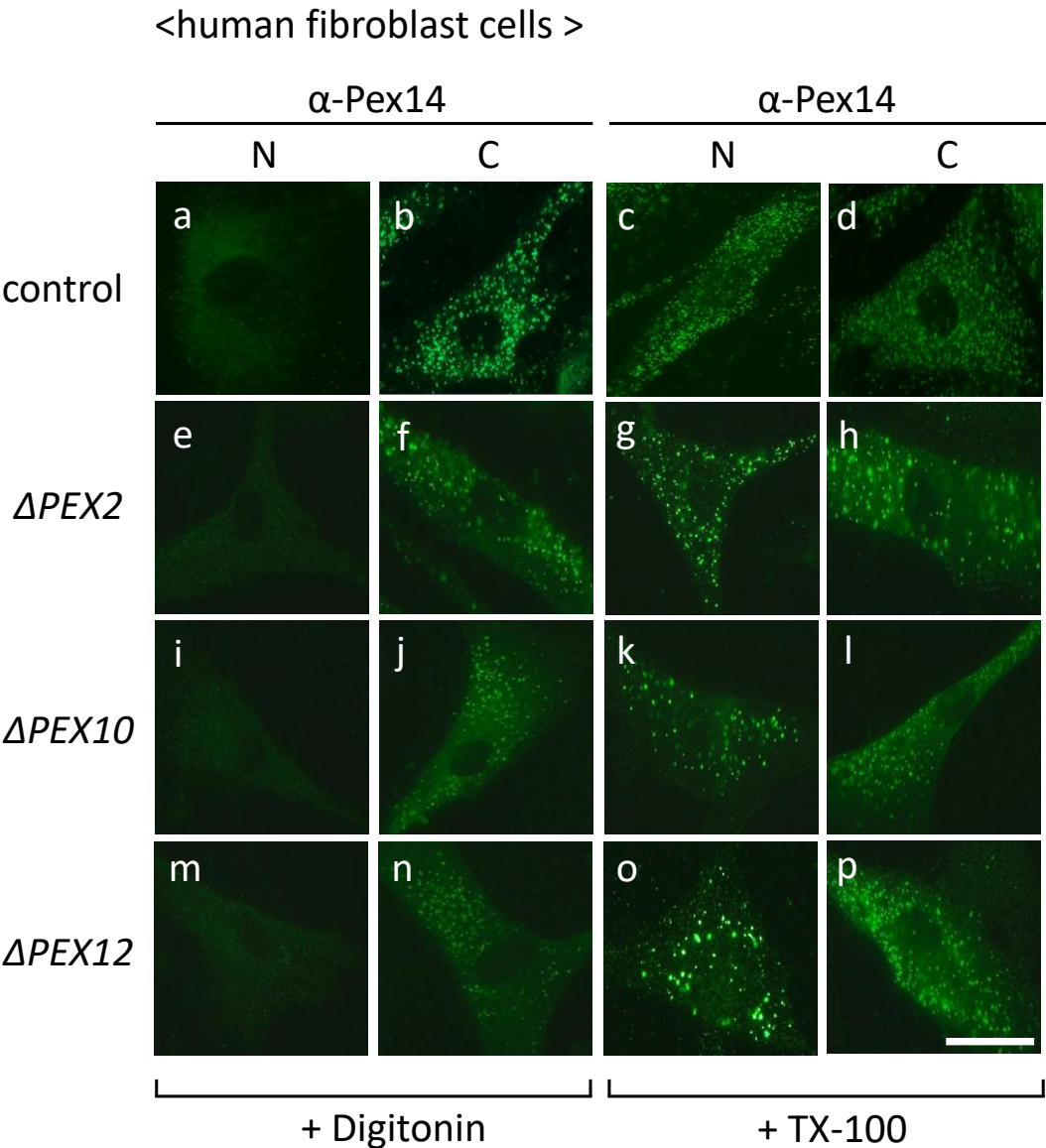
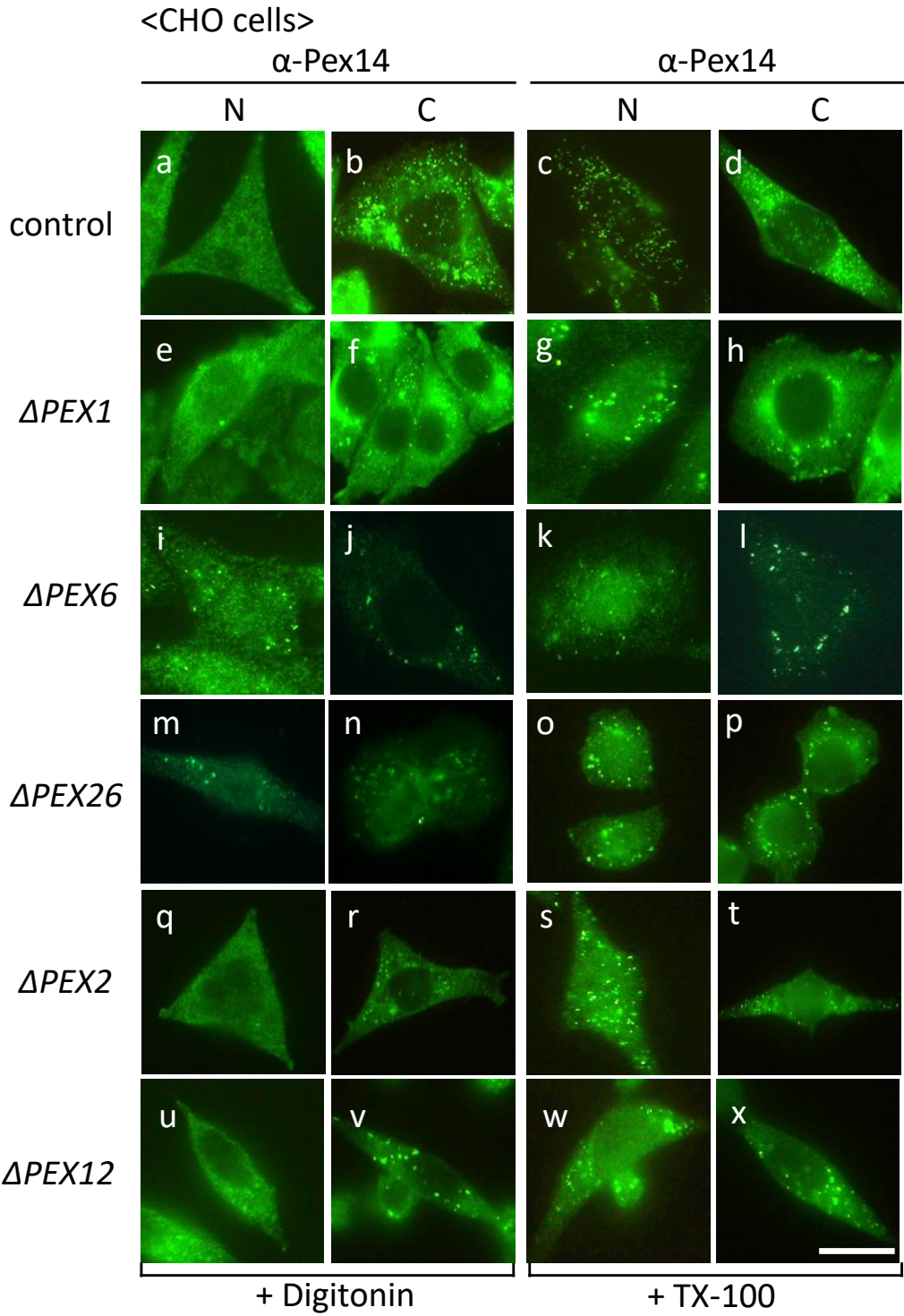


Figure S2 Yasumitsu



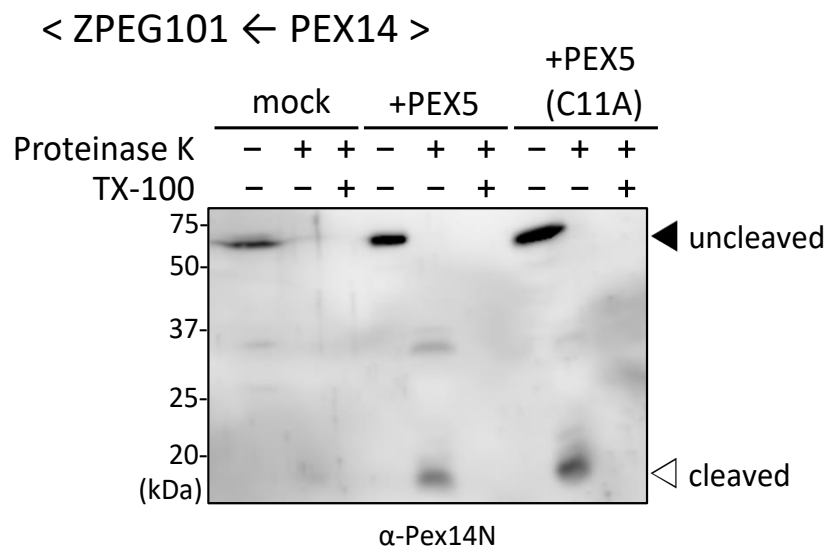


Figure S3

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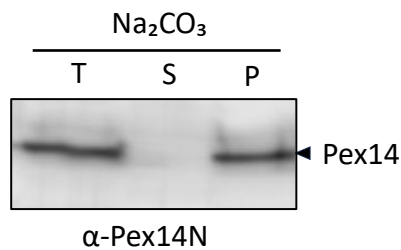


Figure S4

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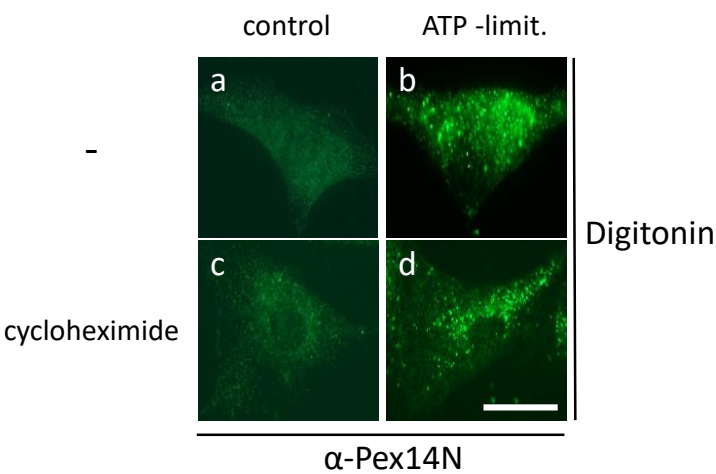
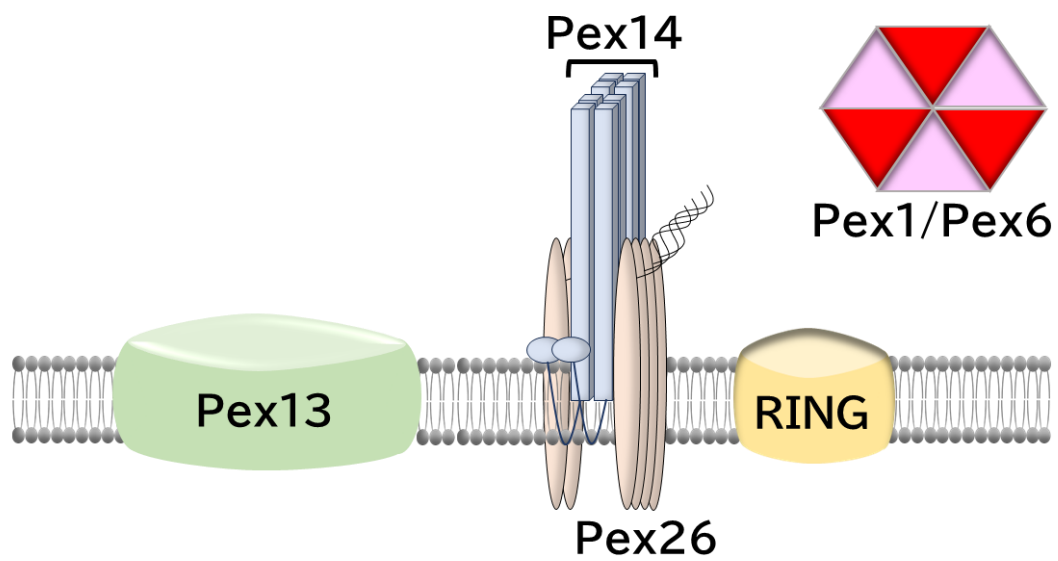


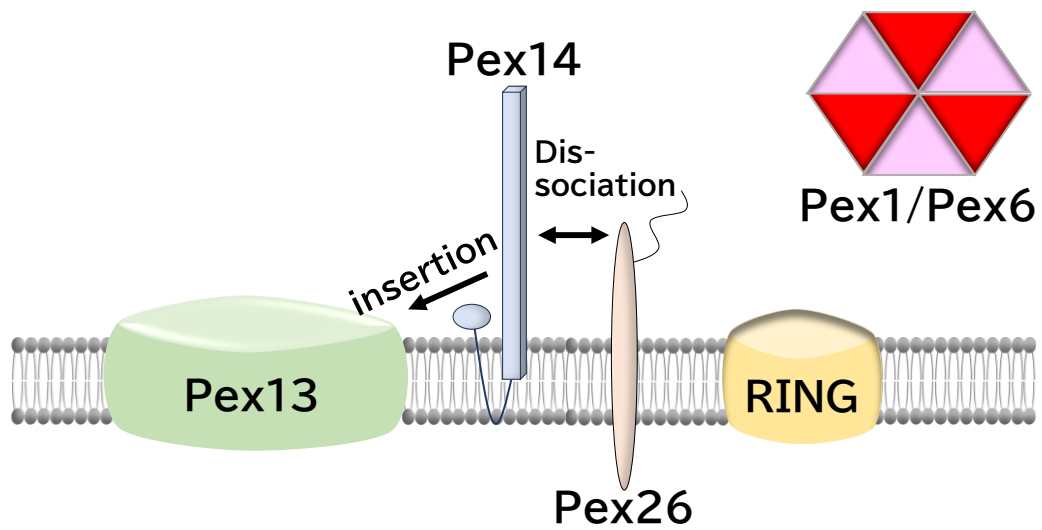
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Figure S6 Yasumitsu

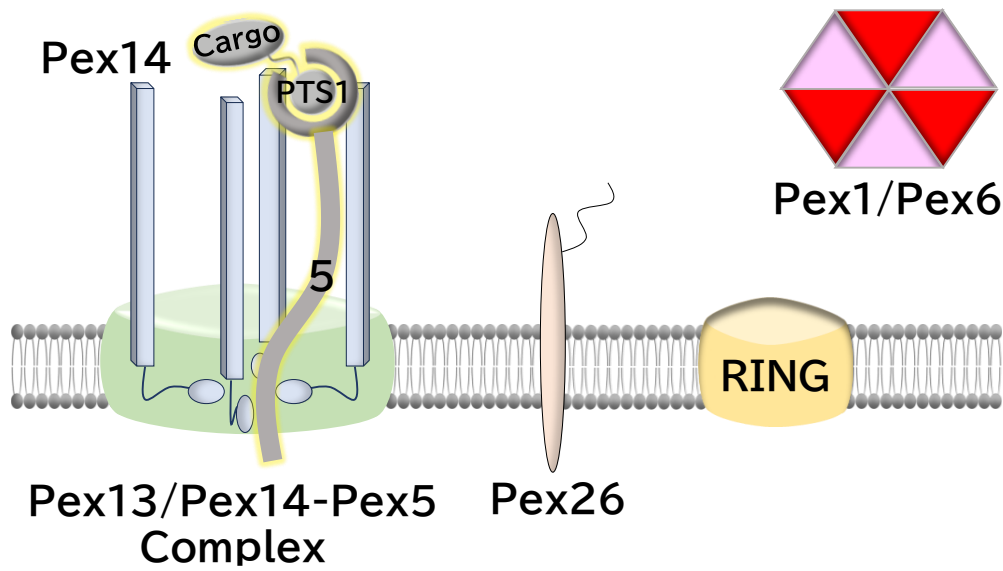
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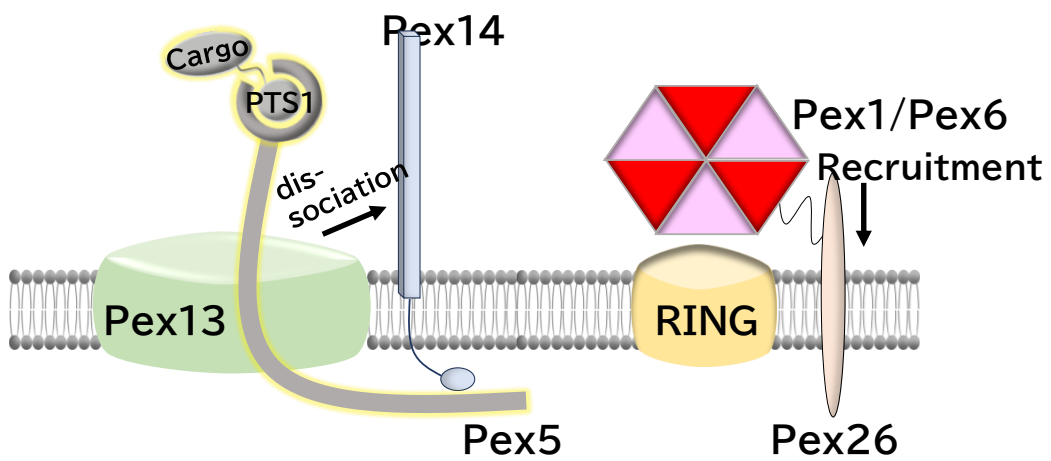
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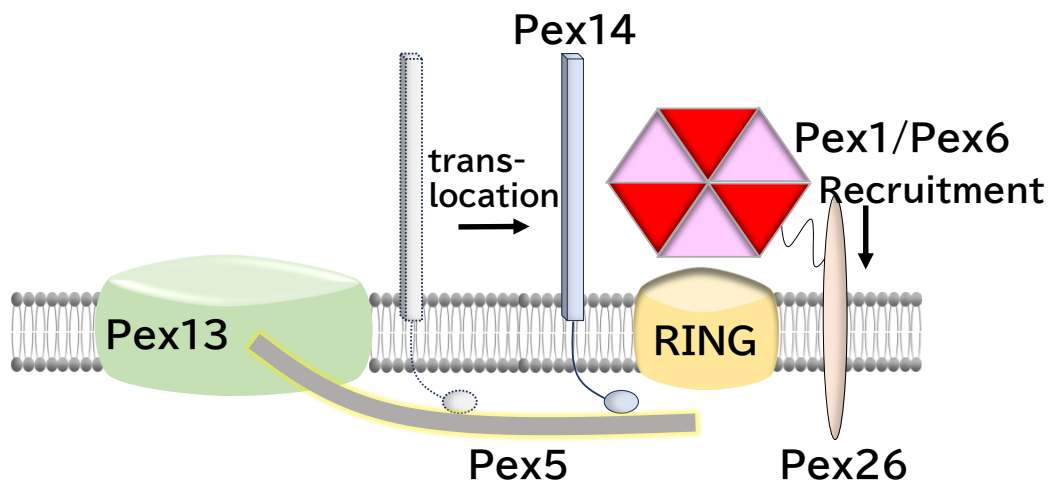
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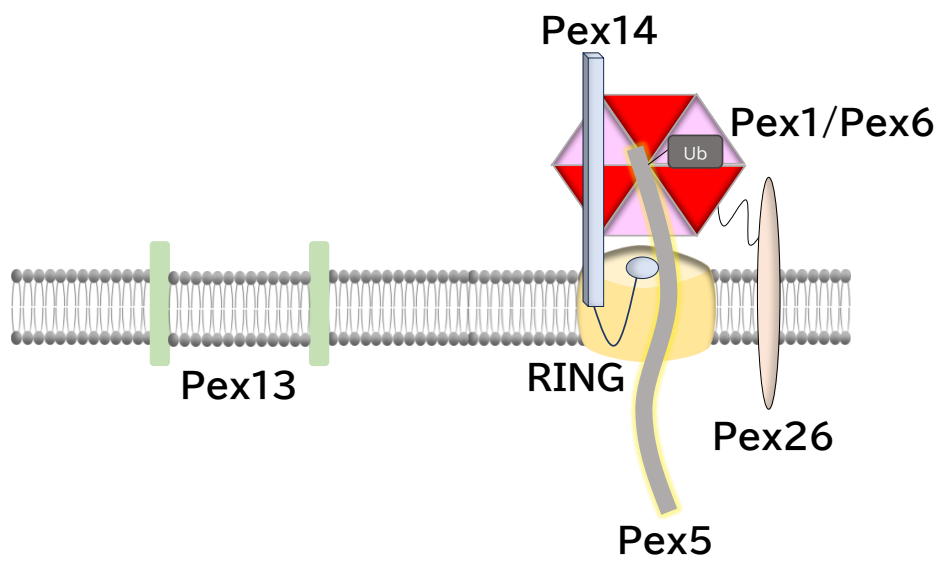


Figure S6 Yasumitsu

