PEX16 contributes to peroxisome maintenance by constantly trafficking PEX3 via the ER

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Running Title
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ABSTRACT

The endoplasmic reticulum (ER) is required for the de novo biogenesis of peroxisomes in mammalian cells. However, its role in peroxisome maintenance is unclear. In order to explore ER involvement in the maintenance of peroxisomes, we redirect a peroxisomal membrane protein (PMP), PEX3, to directly target to the ER using the N-terminal ER signal sequence from preprolactin. Using biochemical techniques and fluorescent imaging, we find that the ER-targeted ssPEX3 is continuously imported into pre-existing peroxisomes. This suggests that the ER constitutively provides membrane proteins and associated lipids to pre-existing peroxisomes. Using quantitative time-lapse live-cell fluorescence microscopy applied to cells either depleted of or exogenously expressing PEX16, we find that PEX16 mediates the peroxisome trafficking of two distinct peroxisomal membrane proteins, PEX3 and PMP34, via the ER. These results not only provide insight into peroxisome maintenance and PMP trafficking in mammalian cells but also highlight important similarities and differences in the mechanisms of PMP import between the mammalian and yeast systems.
INTRODUCTION

Peroxisomes are ubiquitous metabolic organelles found in most eukaryotic cells. In the mammalian system, they are required for the metabolism of very long chain fatty acids, purines, polyamines and hydrogen peroxides in addition to the biosynthesis of key components such as plasmalogens and bile acids (van den Bosch et al., 1992). Defects in any of the peroxisome biogenesis genes, called Peroxins (PEX), result in a group of hereditable genetic diseases called Peroxisome Biogenesis Disorders (PBD) (Steinberg et al., 2006).

Studies on PBDs and the use of yeast genetics have revealed the protein import mechanisms of peroxisomes to be distinct among other single-membrane bound organelles. Like mitochondria and chloroplasts, peroxisomes are able to import all of their matrix (lumen) proteins directly from the cytosol (Ma et al., 2011; Rucktaschel et al., 2011). However, peroxisomal membrane proteins (PMPs) can be imported via two distinct pathways. The Group I import pathway targets newly synthesized PMPs to the ER before routing them to peroxisomes, whereas Group II PMPs are imported directly into peroxisomes from the cytosol (South et al., 2001; Titorenko and Rachubinski, 2001).

While the Group I pathway is necessary for de novo biogenesis of peroxisomes from the ER in cells without pre-existing peroxisomes, conflicting evidence exists on the extent that these two pathways are used in normal cells (Theodoulou et al., 2013). Using both fluorescent imaging and biochemical techniques, PMPs in the yeast, S. cerevisiae, have been shown to target to the ER in conditions where there are no pre-existing peroxisomes, while in normal cells, they appear to be imported directly to peroxisomes (Motley and Hettema, 2007). Similarly, after cell division in S. cerevisiae, a key peroxisome biogenesis factor, Pex3p, appears to be directly imported to pre-existing peroxisomes (Menendez-Benito et al., 2013). These results apparently conflict with a recent demonstration that most PMPs in S. cerevisiae can be targeted directly to the ER via the post-translational import system, which suggests that most PMPs use the Group I import pathway even in S. cerevisiae with pre-existing peroxisomes (van der Zand et al., 2010; Thoms et al., 2011).

Similar conflicting results are also reported in mammalian systems. There, PEX16, an essential PMP involved in peroxisome biogenesis, is targeted to the ER before it is transported to peroxisomes (Kim et al., 2006). Nevertheless, based on colocalization and in vitro targeting assays, others have argued that mammalian PMPs only target to peroxisomes via the Group I
pathway in cells without pre-existing peroxisomes, and that the ER does not contribute to the maintenance of mammalian peroxisomes (Matsuzaki and Fujiki, 2008; Huybrechts et al., 2009).

It has been rather suggested that all PMPs in normal cells are targeted directly to peroxisomes without accessing the ER (Lazarow and Fujiki, 1985; Sacksteder et al., 2000; Matsuzaki and Fujiki, 2008; Huybrechts et al., 2009; Schmidt et al., 2012).

We believe that the role of the ER in targeting PMPs to pre-existing peroxisomes has been erroneously discounted due to the difficulty in detecting PMPs in the ER at steady state. Rather than being completely absent from the ER, PMPs may be rapidly exported from the ER to peroxisomes resulting in their short residence in the ER (Nuttall et al., 2011; Schmidt et al., 2012). To test this hypothesis, we have developed a biophysical imaging technique to quantify the kinetics of PMP import into peroxisomes. With the assumption that import rates of PMPs that are directly imported to peroxisomes from the cytosol will differ from those routed through the ER, quantification of import rates of various PMPs provides a method to determine whether multiple pathways of PMP import into peroxisomes exist. We report here that the PMPs explored are imported into peroxisomes at two distinct rates: a fast import rate similar to matrix proteins (Group II pathway); and a slower rate similar to that of a PMP forced into the Group I pathway. We find that PEX16 is imported into peroxisomes via the Group I pathway, and may also play a direct role in regulating this pathway. Furthermore, we present evidence that the Group I pathway may be the default route to peroxisomes for all PMPs. Based on these results, we propose a model for the mammalian PMP import system in which the ER constitutively provides both lipids and proteins for the maintenance of pre-existing mature peroxisomes.
RESULTS

ER-targeting PEX3 is routed to peroxisomes via the ER

It is not clear whether the ER is involved in the maintenance of peroxisomes in normal mammalian cells with pre-existing peroxisomes. In order to determine whether such cells can transport PMPs to peroxisomes via the ER (i.e. the Group I PMP pathway), we designed a PMP that is ‘forced’ to target to the ER co-translationally. Previously, PEX3 containing an ER-targeting signal sequence was shown to complement a PEX3-deficient cell line that lacked peroxisomes, suggesting that ER-localized PEX3 can form peroxisomes (Toro et al., 2009). Using a similar construct, we asked whether an ER-localized PEX3 could be transported to pre-existing functional peroxisomes. The ER-targeting PMP, named ssPEX3-GFP, consists of PEX3 with a cleavable ER-targeting Signal Sequence (ss) from bovine preprolactin at its N-terminus and a monomerized EGFP at its C-terminal end (Fig. 1A). The preprolactin signal sequence (ss) was selected, as it is a well-characterized signal sequence that is shown to have a high ER-targeting fidelity (Jungnickel and Rapoport, 1995).

To assess whether ssPEX3-GFP is targeted to the ER, we looked for the cleavage of the ss portion of the polypeptide, which is cleaved by the ER signal sequence, peptidase, inside the ER lumen during translation-coupled translocation into the ER (Jungnickel and Rapoport, 1995). The cleavage of ss in ssPEX3-GFP expressed in HeLa cells was determined by comparing its molecular weight on SDS-PAGE to PEX3-GFP (without ss) and that of NssPEX3-GFP (with a nonsense ss) (Fig. 1A). The NssPEX3-GFP construct is similar to ssPEX3-GFP except for four mutations in the ss making it less hydrophobic and therefore unrecognizable by the Signal Recognition Particle that would target it to the ER (von Heijne, 1985). A SDS-PAGE comparison between these three constructs showed a single band for ssPEX3-GFP with mobility in between that of PEX3-GFP and NssPEX3-GFP (Fig. 1B). This was consistent with cleavage at the predicted cleavage site (Fig. 1A). The presence of a single band confirmed that the majority of the synthesized proteins were targeted to the ER.

We next used confocal microscopy to examine the sub-cellular localization of the three PEX3 constructs transiently transfected in HeLa cells (Fig. 1C-E). UB-RFP-SKL was used to visualize pre-existing peroxisomes: ssRFP-KDEL for the ER, and MitoTracker Red for the
mitochondria. UB-RFP-SKL is a chimera of mRFP fused to ubiquitin at the N-terminus and a peroxisome matrix targeting signal tripeptide, Ser-Lys-Leu (PTS1), at the C-terminus (Kim et al., 2008). The ubiquitin motif in UB-RFP-SKL was used to minimize the accumulation of non-targeted protein in the cytosol through degradation via the ubiquitin-proteasome degradation pathway. PEX3-GFP without an ER signal sequence localized mainly to peroxisomes as previously shown, but it was also found to localize to mitochondria in the few cells expressing at higher levels (Fig. 1C) (Soukupova et al., 1999; Sacksteder et al., 2000).

The sub-cellular localization of the non-ER-targeting NssPEX3-GFP was similar to PEX3-GFP. At low expression levels, it co-localized with the peroxisome marker, UB-RFP-SKL; at higher expression levels, it was found on both peroxisomes and mitochondria (Fig. 1D). The localization of ssPEX3-GFP, however, differed from the wild type PEX3 and NssPEX3. As shown in the representative cells in Figure 1E, ssPEX3-GFP almost exclusively localized to peroxisomes at lower expression levels, but in the few cells with very high expression levels, it was also found on the ER but not mitochondria (Figs. 1E & S1). Since the majority of the signal sequence on ssPEX3-GFP was cleaved under similar transfection conditions (Fig. 1B), these results suggested that ssPEX3-GFP initially targeted to the ER before routing to peroxisomes. The presence of ssPEX3-GFP in the ER in cells with high expression levels implied that the export of ssPEX3-GFP from the ER to peroxisomes was saturable. Similar ER accumulation was previously reported for PEX16 (Kim et al., 2006).

**ssPEX3 targets to pre-existing peroxisomes**

Close examination of the images in Figure 1 showed ssPEX3-GFP co-localized to all punctate structures positive for the matrix protein, UB-RFP-SKL (Fig. 1E), which suggested that ssPEX3-GFP could target to pre-existing peroxisomes. To confirm that ssPEX3-GFP is targeted to pre-existing peroxisomes, we transiently co-expressed a photoactivatable RFP with PTS1, PARFP-SKL, with a plasmid construct that placed ssPEX3-GFP under a tetracycline-response element inducible system. PARFP-SKL was photoactivated before ssPEX3-GFP expression was induced with doxycycline (Fig. 2A). As PARFP-SKL was not re-photoactivated, all PARFP-SKL positive puncta were considered peroxisomes that existed before the induction of ssPEX3-GFP. After 18 hours of induction, ssPEX3-GFP were found co-localized with the photoactivated
PARFP-SKL, demonstrating that ssPEX3-GFP was able to target to pre-existing peroxisomes (Fig. 2B).

**ssPEX3-GFP complements PEX3 mutation**

Next, we asked whether the ER-targeting ssPEX3-GFP was functional by examining whether it could complement the PEX3-deficient human cell line, PBD400-T1 (South et al., 2000). In the early stages of expression (24h after transfection), PEX3-GFP was found localized to mitochondria, whereas ssPEX3-GFP localized to the ER, suggesting a difference in their membrane targeting mechanisms (Fig. 3A, B). As previously shown, complementing these cells with full length PEX3 for 72 hours resulted in the formation of new peroxisomes as confirmed by the co-localization of PEX3-GFP with endogenous catalase in punctate structures (Fig. 3C) (South et al., 2000). Similarly, ssPEX3-GFP was able to complement the PEX3 mutation since its expression resulted in the formation of catalase positive structures 72 hours after transfection (Fig. 3D).

**PMP peroxisome import rates quantification**

The observation that ssPEX3-GFP readily located to pre-existing peroxisomes suggested that the Group I PMP import pathway occurs in normal mammalian cells. We quantified the kinetics of PMP import into pre-existing mature peroxisomes to determine whether other PMPs also targeted to peroxisomes via the Group I pathway. The ER trafficking pathway should be significantly slower than the direct import pathway due to the inherent complexity of protein trafficking between two organelles (Lodish, 1988; Zaal et al., 1999). Thus, we assert that PMPs routed through the ER to peroxisomes (Group I pathway) will manifest a slower import rate into peroxisomes compared to directly targeted Group II PMPs or peroxisomal matrix proteins.

We measured the increase in the fluorescent signal of various GFP chimera PMPs with respect to an artificial peroxisomal matrix protein, UB-RFP-SKL, using time-lapse imaging of living cells (see Methods and Supplementary). PTS1 containing polypeptides are directly imported into mature import-competent peroxisomes from the cytosol (Santos et al., 1988). Figure 4 shows the quantification of the apparent import rates of ssPEX3-GFP ($k_{\text{GFP}}$) and UB-RFP-SKL ($k_{\text{RFP}}$) into functional mature pre-existing peroxisomes using UB-RFP-SKL as a peroxisome marker. Time-lapse images were acquired starting at an early stage of the protein
expression to analyze the apparent import rates of these proteins into peroxisomes (Fig. 4A). In the representative cell (Fig. 4A), GFP and RFP signals within peroxisomes were quantified and corrected for background (Fig. 4B).

For ssPEX3-GFP expressing cells, the entire population of UB-RFP-SKL positive punctate structures were observed to show an increase in both RFP and GFP signals over time (Fig. 4A, B), which suggested that ssPEX3-GFP was readily imported into pre-existing peroxisomes. The GFP (Fig. 4B: black circles) and RFP (Fig. 4B: white circles) fluorescent signals within peroxisomes were plotted against time. Both ssPEX3-GFP and UB-RFP-SKL import into peroxisomes show a linear increase with time ($R^2>0.98$) (Fig. 4B).

**The import rate of PEX16 is similar to ssPEX3-GFP but distinct from other PMPs**

Using this quantitative import rate technique, we quantified the relative import rates for three distinct PMPs (PMP34, PEX16 and PEX3) then compared them to the relative rates of the ER-targeting ssPEX3 and a matrix protein, UB-GFP-SKL. PMP34 was chosen since it was not required for peroxisome biogenesis and its membrane topology differed from the other two PMPs. PMP34 has 6 transmembrane domains (TMs) with both of its ends at the cytosolic side (Honsho and Fujiki, 2001). In contrast, PEX3 is a Type I membrane protein with a single TM near its N-terminus that places its N-terminal end inside the matrix of peroxisomes; PEX16 has been predicted to have two TMs with both of its ends at the cytosolic side (Soukupova et al., 1999; Honsho et al., 2002). ssPEX3-GFP was used as a control protein as it traffics to peroxisomes via the ER, and UB-GFP-SKL was used as a direct trafficking control (Fig. 4C).

In order to compare the import rates of these GFP-fused proteins, we used the relative rate of import of the GFP-fused proteins to the rate of UB-RFP-SKL import ($k_{\text{GFP}}/k_{\text{RFP}}$; Fig. 4B). The relative import rates of PEX3-GFP and PMP34-GFP were not significantly different from each other or with the matrix protein, UB-GFP-SKL (Fig. 4C). In contrast, the relative import rates of the ER-targeting PEX3, ssPEX3-GFP, and PEX16-GFP were both less than half of the rates of UB-GFP-SKL and the other two PMPs (Fig. 4C). To ensure that the difference in rates of these various PMPs was not due to variation of the imaging parameters, we also performed the time-lapse image acquisition of cells expressing either PEX3-GFP or PEX16-GFP in parallel (Fig. S2A) as well as cells expressing PMP34-GFP or ssPEX3-GFP in parallel (Fig. S2B).
enabled a direct comparison of the apparent import rates ($k_{\text{GFP}}$ and $k_{\text{RFP}}$ (AU/h)) of PEX3-GFP to PEX16-GFP, and PMP34-GFP to ssPEX3-GFP. Similar to the relative import rates ($k_{\text{GFP}}/k_{\text{RFP}}$), we found that PEX16-GFP and ssPEX3-GFP rates were approximately twice lower than PEX3-GFP and PMP34-GFP, respectively (Fig. S2A, B).

Next, we examined whether the difference in the rates was due to differences in the expression levels of the various PMP constructs. No correlation was found when the apparent rate for both ssPEX3-GFP and PEX3-GFP were plotted against the non-peroxisomal (Cyto-ER) fluorescent signal within a corresponding cell (Fig. S2C, D). These results suggested that the apparent import rate of GFP-fused protein into peroxisomes was independent of the protein expression level. Furthermore, the co-expression of PMP-GFP constructs did not affect the apparent import rate of UB-RFP-SKL into peroxisomes (Fig. S2A, B), suggesting that the import of matrix proteins was independent of expression of the co-transfected PMPs.

Overall, these results showed that the import rate of PEX16-GFP into pre-existing peroxisomes was significantly slower than that of PEX3-GFP and PMP34-GFP. Instead, the PEX16-GFP import rate was similar to the ER-targeting ssPEX3-GFP. These results would suggest that the majority of exogenously expressed PEX16 is targeted to the ER before being transported to peroxisomes. In contrast, in our system, the majority of exogenously expressed PEX3 and the multi-TM protein, PMP34, are imported directly to pre-existing peroxisomes.

**ER-targeted ssPEX3-GFP does not complement PEX16 mutation**

In mammalian cells, PEX16 is able to recruit PEX3 into both the ER and peroxisomal membranes (Kim et al., 2006; Matsuzaki and Fujiki, 2008). However, most yeast strains, such as *S. cerevisiae* and *H. polymorpha*, do not express a PEX16 homologue. Instead, the *S. cerevisiae* homologue, PEX3p, is targeted directly to the ER via the post-translational translocon complex Sec 61/62/63 (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; van der Zand et al., 2010; Thoms et al., 2011). Therefore, it is possible that the sole function of PEX16 in the mammalian system is to import PEX3 into peroxisomal membranes. Since the mammalian PEX3 is thought to be involved in the import of PMPs into peroxisomes, recruiting PEX3 to the ER could allow for the subsequent import of other PMPs. If this is the case, then targeting PEX3 to
the ER by means other than PEX16 may be sufficient for *de novo* biogenesis of peroxisomes from the ER in cells deficient in PEX16 function.

To test this hypothesis, we exogenously expressed ssPEX3-GFP in the peroxisome-free PEX16-deficient cell line, GM06231 (Brocard et al., 2005). Given that ssPEX3-GFP is functional due to its ability to complement a PEX3 mutant cell line (Fig. 3D), ssPEX3-GFP should be able to complement GM06231 cells if the sole function of PEX16 was to import PEX3 to ER membranes. For these experiments, we used the 3×myc-tagged versions of ssPEX3 and PEX3 in the unlikely possibility that the GFP motif might cause steric hindrance. The ability of PEX16-GFP to complement PEX16 function was used as a control (Fig. 5A, B). Similar to the PBD400-T1 cell line, 24 hours after transfection, PEX3-3×myc was found localized to mitochondria whereas ssPEX3-3×myc was predominately found on the ER (Fig. 5C-E). In fact, the localization of ssPEX3-3×myc at 24 hours after transfection was similar to that of PEX16-GFP (Fig. 5A, C). However, unlike PEX16-GFP, which was able to complement the PEX16 mutation (Fig. 5B), ssPEX3-3×myc was not able to complement the PEX16 mutant cells even at 96 hours after transfection and remained localized to the ER (Fig. 5D). This result suggests that PEX16 has other function(s) in addition to its role as a receptor for PEX3 import.

**Knockdown of PEX16 retards ssPEX3 transport from ER to peroxisomes**

An additional possible function of PEX16 could be its involvement in the process of trafficking PMPs from the ER to peroxisomes. To test this hypothesis, we quantified the import rates of various PMPs into pre-existing peroxisomes in cells depleted of PEX16 expression using RNAi compared to control cells. If PEX16 is involved in the transport of PMPs from the ER to peroxisomes, then the amount and the rate of import of these PMPs should decrease in cells depleted of PEX16. Conversely, PMPs that are targeted directly to peroxisomes should not be affected.

To deplete PEX16 expression levels, cells were treated with siRNA against PEX16 (siPEX16) or with non-targeting siRNA (siCNTR) as the negative control. The depletion of PEX16 mRNA was verified by quantifying PEX16 mRNA levels using qRT-PCR (Fig. S3A). For these experiments, we employed HeLa cells stably expressing RFP-SKL as it allowed for easier identification of pre-existing peroxisomes. To compare the distribution of PMPs the
peroxisomal and non-peroxisomal compartments at various cell conditions, the relative amount
of PMPs in peroxisomes was determined by dividing the average fluorescent intensity of PMP-
GFP within peroxisomes \( (I_{\text{per}}) \) by the average fluorescent intensity within the whole cell \( (I_{\text{cell}}) \) 20
hours after transfection. In this assay, a higher \( I_{\text{per}}/I_{\text{cell}} \) ratio indicates a higher fraction of PMPs
in peroxisomes. The distributions of the relative amount of PMPs in peroxisomes of 150 cells
from 3 independent experiments are summarized as a histogram for cells treated with siPEX16 or
siCNTR (Figs. 5A-C & S3C-E). These results illustrated a significant decrease in the amount of
ssPEX3-GFP within peroxisomes in cells depleted of endogenous PEX16 compared to control
cells \( (p<<0.001; \) Fig. 6A), suggesting that depletion of PEX16 disrupted the localization of
ssPEX3-GFP into peroxisomes. This decrease was probably not due to off-target effects that the
PEX16 siRNA may have on the cell, since co-expressing a siRNA-resistant PEX16 (PEX16*-mCerulean) was able to rescue the phenotype (Fig. S3B). Unexpectedly, siPEX16-treated cells
had a significantly higher relative PMP34-GFP and PEX3-GFP signal within peroxisomes
compared to control \( (p<<0.001; \) Fig. 6B, C), suggesting an increase of these protein fractions in
peroxisomes.

To test whether the difference in the amount of PMPs in peroxisomes in siPEX16 versus
siCNTR-treated cells was due to changes in their peroxisomal import rates, we compared the
apparent import rate of PMP-GFP into peroxisomes in siPEX16 \( (k_{\text{siPEX16}}) \) treated cells against
that of siCNTR-treated cells \( (k_{AV(siCNTR)}) \). For these experiments, siCNTR and siPEX16-treated
cells were imaged in parallel to allow for direct comparison between the two conditions (Fig. 6).
In agreement with the distribution data for ssPEX3-GFP within peroxisomes (Fig. 6A), PEX16
depletion resulted in a significant decrease in the rate of ssPEX3-GFP import into pre-existing
peroxisomes compared to control cells. This lower rate for ssPEX3-GFP in the PEX16
knockdown cells was not due to a difference in peroxisome numbers since the depletion of
PEX16 expression did not change the peroxisome number \( (Fig. S3F) \). Since ssPEX3-GFP is
targeted to peroxisomes via the ER, this suggests that PEX16 could be required for the
trafficking of ssPEX3-GFP from the ER to peroxisomes. Interestingly, the import rates of PEX3-
GFP and PMP34 did not decrease upon depletion of PEX16 expression, but rather showed
significant increase compared to control.
Exogenously expressed PEX16 retards PEX3 and PMP34 import rates

One explanation for the increase in PEX3-GFP and PMP34-GFP import rates into peroxisomes in cells depleted of PEX16 expression is that these PMPs may be imported into peroxisomes by both the Group I and Group II pathways. Previously, we reported that ER-localized PEX16 was able to recruit both PEX3-GFP and PMP34-GFP to the ER (Kim et al., 2006). Here, we found that most of the newly synthesized PEX16 initially targeted to the ER before being routed to peroxisomes (Fig. 4). Hence, it is possible that newly synthesized PEX16 is diverting newly synthesized PEX3-GFP and PMP34-GFP to the ER before being transported to peroxisomes. Thus, depleting the cells of PEX16 using siRNA reduces the Group I pathway, resulting in Group II pathway to compensate. Since the overall import rate of Group II pathway is faster than the Group I pathway, the loss of the ER pathways may be causing the observed increase in the rate of import of the two PMPs (Fig. 6D).

To test this possibility, we measured the import rate of both PMP-GFP proteins in cells co-transfected with PEX16-mCerulean, or mCerulean alone (control). As shown in Fig. 6E, the ssPEX3-GFP apparent import rate was found unaffected by PEX16-mCerulean overexpression compared to control cells. However, the import rates of PMP34-GFP and PEX3-GFP co-transfected with PEX16-mCerulean were about half the rates of those co-transfected with mCerulean alone (p<0.005). This decrease in rates was not due to an import competition between PEX16-mCerulean and PMP-GFPs since the import rate of PEX16-GFP did not change when co-expressed with PMP34-mCerulean (Fig. 6E). To further test the hypothesis that the increasing PEX16 expression (via PEX16-mCerulean expression) promotes the Group I pathway for PMPs, we examined whether the rate of PEX3-GFP import inversely correlated with PEX16 expression. Indeed, we found that as the PEX16/PEX3 expression ratio increased, the peroxisomal import rate of PEX3-GFP decreased (Fig. 6F). This difference in rates in cells overexpressing PEX16-mCerulean was not due to a change in peroxisome number since the exogenous expression of PEX16 or PEX3-GFP had no effect on peroxisome numbers (Fig. S3G,H). Together with the PEX16 depletion data, our results suggest that PMP34 and PEX3 traffick to peroxisomes by both Group I and Group II pathways.
DISCUSSION

We have demonstrated that the ER is directly involved in maintaining peroxisomes in normal mammalian cells. Using an ER targeting signal sequence on a peroxisomal membrane protein, ssPEX3, we show that it targets to the ER prior to being relocalized to pre-existing peroxisomes. By comparing its peroxisome import kinetics to those of other peroxisomal membrane proteins (PMPs), we demonstrate that exogenously expressed PMPs are targeted to peroxisomes by both the Group I and Group II import pathways. The exception is PEX16, which targets to peroxisomes exclusively via the Group I import pathway (via the ER).

The direct evidence that ssPEX3-GFP first targets to the ER before being routed to peroxisomes is the efficient cleavage (>90%) of its preprolactin signal sequence. It has been difficult to observe ssPEX3-GFP on the ER during its early expression, and only with very high expression has it been visualized on the ER (Fig. 1E). One interpretation is that it is efficiently transported to peroxisomes after targeting to the ER. Alternatively, it may be released to the cytosol upon the cleavage of its signal sequence at the ER. For example, calreticulin is an ER resident protein that also has a small cytosolic fraction due to its abortive translocation after the cleavage of its signal sequence (Shaffer et al., 2005). However, we believe ssPEX3-GFP is efficiently targeted to peroxisomes via the ER after the cleavage of its signal sequence for three reasons. First, the signal sequence of preprolactin has been shown to be highly efficient in the complete translocation of various chimera proteins, including calreticulin, across the ER membrane. Replacing the signal sequence of calreticulin with that of preprolactin prevents any cytosolic fraction due to abortive ER translocation (Shaffer et al., 2005). Second, PEX16, which is transported to peroxisomes from the ER, also shows a peroxisomal colocalization profile at low expression, and on ER as well as peroxisomes (but not cytosolic) at high expression (Kim et al., 2006). Third, if ssPEX3-GFP is efficiently released from the ER into the cytosol, then in the absence of peroxisomes, ssPEX3-GFP should be localized to the cytosol or mitochondria much like the wild type PEX3-GFP. However, we observe ssPEX3-GFP on the ER in cells without pre-existing peroxisomes (Fig. 3 & Fig. 5). Similarly, we find that the import kinetics of ssPEX3-GFP is significantly different from that of PEX3-GFP suggesting a difference in their targeting mechanism. Together these results support the model that ssPEX3-GFP is transported to pre-existing peroxisomes from the ER.
In this study, we have found the import rate of PEX16 to be similar to ssPEX3-GFP, while that of PEX3 was comparable to PMP34 (Fig. 4C). The difference in the import rate of PEX16/ssPEX3 to PEX3/PMP34 is unlikely due to differences in their membrane topologies since the faster importing PMP34 contains six transmembrane domains while PEX16 possesses only two, and both PEX3 and ssPEX3 have only one (Honsho and Fujiki, 2001; Honsho et al., 2002). Similarly, the rate difference between wild type PEX3 and ER-targeting ssPEX3 is unlikely to be the result of the processing of the signal sequence on ssPEX3, as the removal of the signal sequence is rapid and occurs during translation (Hortin and Boime, 1980; Ibrahimi, 1987; Jungnickel and Rapoport, 1995). Nor is the disparity due to extra residues on the N-terminal end of ssPEX3 since we have found that the import rate of the non-ER targeting construct, NssPEX3-GFP, has a similar import rate as PEX3-GFP (data not shown). Instead, we suggest that two different import rates represent two distinct mechanisms of importing peroxisomal proteins to peroxisomes. Given that exogenously expressed PEX3 can target directly to peroxisomes in vitro (Matsuzaki and Fujiki, 2008), we propose that the majority of the exogenously expressed PEX3 and PMP34 are directly targeted to peroxisomes (via the Group II pathway) while PEX16 and ssPEX3 are targeted via the Group I pathway.

However, a significant fraction of exogenously expressed PEX3 and PMP34 was targeted to peroxisomes in a PEX16-dependent manner. Depleting endogenous PEX16 expression significantly increased the import rates of the overexpressed PEX3-GFP and PMP34-GFP into peroxisomes (Fig. 6B-D). Conversely, co-expression with PEX16-mCerulean decreased the rates of their import (Fig. 6E) in a PEX16 concentration-dependent manner (Fig. 6F). Since PEX16 is targeted to peroxisomes via the Group I pathway, this implies that a portion of newly synthesized PEX3 and PMP34 are being targeted to peroxisomes via the Group I pathway and the measured peroxisomal import rate of the exogenously expressed PEX3 and PMP34 (Fig. 4) is the combination of both the Group I and II pathways.

Although our observations suggest that both PEX3 and PMP34 are able to target to peroxisomes via two pathways, it is not clear whether both pathways are used at endogenous levels or if one pathway dominates over the other. It is possible that dual targeting pathways for PEX3 and PMP34 can be a result of their overexpression. High expression levels of these PMPs may overwhelm one pathway and "spill over" into the other pathway. One possibility is that
Group II is the preferred pathway. In this scenario, the predominate pathway is the direct targeting of PMPs to peroxisomes and the ER pathway is compensating for excess PMP expression. Alternatively, the PEX16-dependent ER pathway (Group I pathway) could be the predominate pathway while the Group II pathway serves as an overflow. Of the two, our data supports the latter scenario. This is based on our observation that the endogenous level of PEX16 is sufficient to retard the import rates of overexpressed PEX3-GFP and PMP34-GFP (Fig. 6B-D). If the ER pathway was the secondary pathway, then the knockdown of endogenous PEX16 should not result in an increase of both PEX3 and PMP34 import rates. In addition, we find that PEX16 overexpression affects the import rates of these proteins in a concentration-dependent manner (Fig. 6 E-F).

Another possible explanation for the changes in PMPs import rates upon PEX16 overexpression or depletion is that there may be a common factor shared by both pathways. If this common factor or chaperone is involved in both the Group I and II pathways, then overexpressing both PMPs and PEX16 may saturate it, thus resulting in a decrease in import rates for both PMPs and PEX16. However, this is unlikely since the co-expression of PMP34 with PEX16 did not slow down PEX16 import but only retarded PMP34 import (Fig. 6E). Therefore, our data suggests that the PMPs favour the PEX16-dependent Group I pathway over the Group II pathway; and we propose that the Group I pathway is the default import pathway for PMP import to peroxisomes.

Generalizing these findings, we put forward the following model for the maintenance of peroxisomes in the mammalian cell (Fig. 7). We propose that most PMPs are capable of being targeted to peroxisomes via two distinct pathways, the Group I and Group II, and the extent to which each pathway is used is dependent on the ratio of newly synthesized PEX16/PMP concentration. We suggest that the Group I pathway is the dominant pathway, meaning that most PMPs are targeted initially to the ER by PEX16 before being routed to peroxisomes. Significantly increasing the expression of PMPs can saturate the ER recruitment pathway, due to the limited amount of PEX16, in which case, they would be imported directly to peroxisomes via the Group II pathway. Although we depict a small vesicle between the ER and peroxisomes (Fig. 7), the true nature of the communication between the two organelles is not known in the mammalian system.
This model shares some similarities to current models of PMP import into *S. cerevisiae*. PMPs in *S. cerevisiae* have been reported to be able to import by both the Group I and II pathways (van der Zand et al., 2010; Thoms et al., 2011; Huybrechts et al., 2009). However, unlike in the mammalian system where the ER provides new PMPs to pre-existing peroxisomes, the ER in *S. cerevisiae* does not provide new PMPs to pre-existing peroxisomes, but rather is involved in forming pre-peroxisomal vesicles that fuse to form new peroxisomes form the ER (Titorenko et al., 2000; van der Zand et al., 2012). In *S. cerevisiae*, PMPs are targeted to the ER exclusively for the de novo biogenesis of peroxisomes, whereas the Group II targeting mechanism is only involved in the maintenance of pre-existing peroxisomes. This mechanism differs from our model of peroxisome maintenance in the mammalian system where the ER constitutively provides PMPs to peroxisomes. Furthermore, we suggest in our model that during high peroxisome proliferation events such as the activation of nuclear receptor peroxisome proliferator-activated receptors (PPAR), where peroxisome numbers are rapidly increased 4-10 fold increase in a very short time frame (Reddy, 2004), the excess PMPs synthesized are likely being targeted via the Group II pathway. However, even in high peroxisome proliferation conditions, the ER is probably heavily involved in maintaining peroxisomes by providing lipids and key PMPs, such as PEX16, to the dividing peroxisomes. In fact, PEX16 expression has been shown to be upregulated upon the activation of PPARγ (Karnik et al., 2009). Since we find that the majority of the newly synthesized PEX16 are first targeted to the ER, we suggest that the ER plays an essential role in facilitating peroxisome proliferation by providing the PMPs required for peroxisome proliferation. However, in conditions where the total amount of PMPs exceed the capacity of PEX16, PMPs can also be targeted directly to peroxisomes via the Group II pathway.

In summary, we show two distinct pathways of PMPs import into pre-existing peroxisomes in mammalian cells: a direct targeting to peroxisomes and an indirect route via the ER. In addition, we show evidence that the ER constitutively provides at least two very distinct PMPs, PEX3 and PMP34, to pre-existing peroxisomes, and that this mechanism is dependent on PEX16.
MATERIALS AND METHODS

Cell lines and reagents

HeLa human epithelial carcinoma cells were obtained from American Type Culture Collection (ATCC). PBD400-T1 cells (South et al., 2000) were a gift from S.J. Gould (Johns Hopkins University, Baltimore). GM06231 cells were purchased from NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ, USA. Rabbit anti-GFP was a gift from RS Hegde (NIH, Bethesda).

Plasmid Constructs

The construction of pPEX16-GFP, pPMP34-GFP, pUB-GFP-SKL, pPEX16-mCerulean, pmRFP-SKL, pssRFP-KDEL pPEX3-GFP and pcDNA3-PEX3–3×myc, and Venus-Omp25TM were described previously (Kim et al., 2006; South et al., 2000; Wang et al., 2012). Both plasmid construct strategies used and the Primer sequences are available from the authors upon request.

Culture conditions and transfections

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/High without L-glutamine (Thermo Scientific HyClone) supplemented with 10% Fetal Bovine Serum (GIBCO/Invitrogen) and 2mM L-glutamine (Thermo Scientific HyClone). Cells were grown at 37°C in 5% CO2 in humidified air. Transient transfections were performed using Lipofectamine™ 2000 reagent according to the manufacturer’s standard protocol (Invitrogen). PBD400-T1 or GM06231 cells were cultured in freshly prepared DMEM media supplemented with 15% of Fetal Bovine Serum and 2mM L-glutamine and transfected by electroporation (Amaxa Nucleofection, program V-13) using the Amaxa Basic Nucleofector kit for primary fibroblasts (Lonza).

For live cell microscopy, cells were seeded onto 4-well Lab-Tek™ (Nunc) chambered coverglass and cultured under standard conditions for 24 hours prior to transfection. Cells were then imaged at the indicated time in CO2 independent medium (GIBCO/Invitrogen) at 37°C. For the induction of Tet-ON system, pTET-ON-ssPEX3-GFP transfected into HeLa Tet-on advanced cells was induced using 0.1 µg/ml of doxycycline (Sigma) as suggested by manufacture (Clontech).
Fluorescence microscopy in living cells; time-lapse experiments

All fluorescence images were acquired using a Zeiss LSM 710 laser scanning confocal microscope. For analysis of protein import into peroxisomes the images were acquired using a 40×1.3NA Plan-Neofluar oil immersion objective with LSM710 ZEN2009 software. GFP signal was acquired using a 488nm Argon laser with a 493-565nm or 515-565 nm (in presence of mCerulean) bandpass filter. RFP was acquired using a 561nm diode laser with a 600-700nm bandpass. mCerulean images were acquired using a 405nm diode laser with a 450-495 nm bandpass filter. PARFP-SKL was photoactivated using the 405nm diode laser and imaged using the RFP setting. Brightness and contrast in representative images shown in the article were adjusted on ImageJ program to improve visibility, with the same brightness and contrast adjustments for all frames of time-lapse images. All analysis was performed on the original (unadjusted) 8-bit images.

Quantification of Import rate of peroxisomal protein into peroxisomes

The quantitative analysis was performed using ImageJ program (Rasband, W.S., ImageJ, NIH, http://rsb.info.nih.gov/ij/, 1997-2009). The maximal area of each cell enriched with GFP signal, excluding nucleus, was selected for the ROI. Peroxisomes within the cell were identified by masking UB-RFP-SKL signal using a threshold. The threshold was chosen to maximize the number of peroxisomal punctuate structures with area between 0.1-1µm². The average pixel intensity of the background of GFP (C_G) and RFP (C_R) fluorescent signals were quantified by averaging the signal from 10 non-peroxisomal regions distributed evenly throughout the selected area (ROI) in close proximity to peroxisomes. The fluorophore pairs that we choose for our studies were monomerized GFP and monomerized RFP. They were selected due to their non-overlapping spectra, their monomeric nature, and also the similarity in their maturation rate (approx 1 hour) (Shaner et al., 2004; Sniegowski et al., 2005).

Quantification of relative import rates for PMP-GFP and UB-GFP-SKL.

HeLa cells were transfected with PMP-GFP and UB-RFP-SKL. The imaging was started 20h after transfection. The following criteria were used when selecting cells for analysis: (i) cells showing very low expression of both GFP and RFP signal in early time frames; (ii) cells with GFP and RFP signal within the dynamic range of the photomultiplier tube (PT) detection; and
(iii) cells where their peroxisomal signal for both RFP and GFP proteins increased over time without saturating the PT. The calculation of RFP (PR) and GFP (PG) signal in peroxisomes was as follows:

\[ P_R = T_R - C_R \]  
\[ P_G = T_G - C_G \]

where \( T_R \) and \( T_G \) are average pixel intensity of RFP and GFP in peroxisomes respectively, and \( C_R \) and \( C_G \) are average pixel intensity of RFP and GFP background respectively.

To ensure the same fluorescent intensity signal units for GFP and RFP, we converted the intensity of GFP expressed in AU of GFP into AU of RFP. For these conversions we used the GFP-RFP-SKL construct, which has GFP and RFP in tandem with a PTS1 at its C-terminus. This construct should give a constant RFP/GFP signal ratio (r):

\[ r = \frac{TP'_R - C'_R}{TP'_G - C'_G} \]

where \( TP'_R \) and \( TP'_G \) are average pixel intensity of RFP and GFP in peroxisomes, respectively, of GFP-RFP-SKL construct. \( C'_R \) and \( C'_G \) are average pixel intensity of RFP and GFP background, respectively, of GFP-RFP-SKL construct.

We used this ratio as a coefficient between GFP and RFP signal units. The images of HeLa cells expressing GFP-RFP-SKL were acquired in the same way as described previously and the ratio was determined each day of the experiment.

The GFP signal units conversion in peroxisomes was calculated as follows:

\[ c_{PG} = P_G \times r \]

The fluorescence signal for both GFP \( (c_{PG}) \) and RFP \( (P_R) \) protein was plotted against time and fitted with linear regression:

\[ \text{peroxisomal signal}=kt+b \]
where k is the apparent import rate of labeled protein into peroxisomes in AU/h, t is time in hours and b is an initial signal value of measured fluorescent protein at time zero.

In order to compare the kinetic data between different days and different PMP-GFPs, the import rate of GFP (k_{GFP}) was normalized to the import rate of RFP (k_{RFP}) within the same cell. The import ratio of GFP and RFP was designated as the relative rate of import (k_{GFP}/k_{RFP}) and calculated for every cell. The relative rates for all analyzed cells from all independent trials are pooled together and presented as a mean±standard error in Fig. 3C.

**Import rate of PMP-GFPs in cells treated with siRNA and exogenously PEX16 expressing cells.**

HeLa cells stably expressing RFP-SKL protein were treated with siCNTR or siPEX16 prior to transfection with the plasmid encoding the PMP-GFP protein of interest. The imaging was started 7h after transfection. Peroxisomes within the cell were identified by RFP-SKL signal. GFP signal in peroxisomes was calculated using Eq. 1b.

The fluorescence signal for PMP-GFP (P_G) was plotted against time and fitted with linear regression (Eq. 3). To make the data comparable between the experiments, average import rate of siCNTR treated cells (k_{AV(siCNTR)}) in a given experiment was calculated as follows:

\[ k_{AV(siCNTR)} = \frac{\sum k_{(siCNTR)i}}{n} \]  

where \( k_{(siCNTR)i} \) is the import rate of individually analyzed siCNTR treated cell in a given experiment and n is the number of analyzed siCNTR treated cells in this experiment. The import rate for each siPEX16 treated cell (k_{(siPEX16)}) was normalized to the average import rate for siCNTR treated cells (k_{AV(siCNTR)}) from the same experiment and presented as the relative import rate (k_{(siPEX16)}/k_{AV(siCNTR)}). The relative import rates for all analyzed siPEX16 treated cells from 3 independent trials (20 cells per trial per treatment) were pooled together and presented as a mean±standard error in Fig. 5D.

The same measurement and calculation strategy was used in experiments where various PMP-GFP constructs were co-expressed with PEX16-mCerulean or PMP34-mCerulean (Fig.
Cells co-expressing with mCerulean instead of PEX16-mCerulean or PMP34-mCerulean were used as a control. For a given PMP-GFP construct, its import rate ($k_{(\text{PMP-GFP})}$) for each individual cell coexpressing PEX16-mCerulean or PMP34-mCerulean was normalized to the average import rate for cells coexpressing mCerulean control ($k_{AV(CNTR)}$) from the same experiment. The resulting relative import rates ($k_{(\text{PMP-GFP})/\text{AV(CNTR)}}$) for all analyzed cells from 3 independent trials (20 cells per trial per treatment) were pooled together and presented as mean ± standard error in Fig. 5E.

**Distribution of PMP-GFP signal in the cell at fixed time point.**

HeLa cells stably expressing RFP-SKL protein were treated with siCNTR or siPEX16 prior to transfecting the cells with the plasmid encoding the PMP-GFP protein of interest as described below. The GFP signal inside peroxisome was determined using RFP-SKL for peroxisome detection. pssPEX3-GFP, pPEX3-GFP or pPMP34-GFP were transfected on the 3rd day after the first siRNA treatment and the images were acquired 20h later (on the 4th day). The average GFP fluorescent intensity within peroxisomes ($I_{\text{per.}}$) and in the whole cells ($I_{\text{cell}}$) was determined 20 hours after transfection. PMP-GFP distribution was presented as the intensity ratio ($I_{\text{per.}}/I_{\text{cell}}$) (Fig. 5A-C). In each of the 3 performed experiments, the peroxisomal import rate of PMP-GFP protein was determined for at least 50 cells for every treatment (150 cells in total).

**Western Blots**

For western blot analysis, cells grown in 6-well plates were washed twice with PBS and lysed in 100µl of Lysis buffer (0.1M Tris-HCl, pH 9; 1 % SDS). Lysate was collected and incubated at 100°C for 15min with intervals of vigorous vortexing to shred the genomic DNA. Protein concentration was determined with a BCA protein assay kit (Novagen). Lysates containing 10µg of total protein was analyzed separated in 8% SDS-PAGE (Laemmli, 1970), transferred to PVDF membrane, and membrane was probed for GFP using a rabbit anti-GFP antibody at dilution 1:5,000 and subsequently goat anti-rabbit antibody conjugated to horseradish peroxidase at dilution 1:15,000 (Cedarlane Laboratories Ltd). The blot was visualized using an enhanced chemiluminescence kit from Pierce.
Immunofluorescence assay

Immunofluorescence was performed as previously described (Kim et al., 2006). Briefly, the cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% TX-100 in PBS. Catalase was probed using a rabbit polyclonal anti-catalase antibody at dilution 1:2000, and GFP using a mouse anti-GFP antibody at dilution 1:2000. Alexa antibody (Invitrogen) was used for visualization, as indicated, at dilution 1:1000.

Knockdown assay

PEX16 expression in HeLa cells was depleted using the siRNA sequence sense 5’-UGACGGGAUCCUACGGAAGdTdT-3’ (Shanghai GenePharma Co., Ltd.) as previously described (Fang et al., 2004; Kim et al., 2008). Briefly, HeLa cells constantly expressing RFP-SKL were transfected with either siRNA PEX16 or non-targeting control twice in a 24-h interval using Lipofectamine™ 2000 reagent.

For rate determination experiments, pssPEX3-GFP, pPEX3-GFP or pPMP34-GFP were transfected on the 3rd day after the first siRNA treatment and the time-lapse imaging experiment was started 7h later (3rd day). siRNA PEX16 and control treated cells were tested in parallel on 20 different fields each. In each of the 3 performed experiments, the peroxisomal import rate of GFP-labeled protein was determined in at least 20 cells for every treatment (in total about 60 cells). The RFP-SKL was used to determine the GFP signal in peroxisomes as described above.

The effect of siRNA on the relative transcription level of pex16 was determined with real-time PCR using Mastercycler ep realplex software (Eppendorf) for analysis using SYBR Green PCR Master Mix (ABI). Total RNA was isolated using SV Total RNA Isolation System (Promega), and Go Script Reverse Transcriptase (Promega) with specific primers for pex16 5’-aaaagtgacccccaaactgtagaatgatatttc-3’ and for β-actin 5’-aatgtcaagcgagttgcc-3’ (reference gene) was used. Each sample was measured in triplicate.

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Abbreviations list

GFP - monomeric enhanced green fluorescent protein; ER – endoplasmic reticulum; PBD - peroxisome biogenesis disorder; PMP – peroxisomal membrane protein; ROI – region of interest; S. cerevisiae - Saccharomyces cerevisiae; siRNA – small interfering RNA; ss – preprolactin signal sequence; TM - transmembrane domain; UB-RFP-SKL - ubiquitin-red fluorescent protein- Ser-Lys-Leu; Y. lipolytica - Yarrowia lipolytica


**FIGURE LEGENDS**

**Figure 1.** ssPEX3-GFP protein is targeted to peroxisomes via the ER. (A) Schematic representation of N-terminal ends of PEX3-GFP, ssPEX3-GFP and NssPEX3-GFP. GFP tagged at the C-terminus of each PEX3 construct is not shown. The amino acid sequences correspond to the signal sequence (ss) of preprolactin. The cleaved portion of ss is bolded and the arrow indicates the cleavage site. The calculated molecular weights of each construct in kDa including the cleavage form of ssPEX3-GFP are shown. The mutations made on NssPEX3-GFP to abolish the ER-targeting nature of the SS are highlighted in red. (B) ss cleavage assay. Cells expressing PEX3-GFP, ssPEX3-GFP or NssPEX3-GFP were lysed 20 hours after transfection and analyzed by western blot using an anti-GFP antibody. (C-E) The sub-cellular localization of each PEX3 construct exogenously expressed in HeLa cells was analyzed using fluorescent live-cell microscopy 20 hours after transfection. Representative confocal fluorescence microscopy images for PEX3-GFP (C), NssPEX3-GFP (D), and ssPEX3-GFP (E) of both low and high expression levels are shown. For the low PEX3 expressing cells, co-expression with the peroxisomal marker, UB-RFP-SKL, is shown. For the higher expressing images, cells stained with either MitoTracker Red (C, D) or co-expression with the ER targeting ss-RFP-KDEL (E), are shown. See supplementary Figure 1S for magnification of high ssPEX3-GFP. All images were taken with the same settings and brightness for each panel was enhanced equally for presentation. Scale bars are 10µm.

**Figure 2.** ssPEX3-GFP targets to pre-existing peroxisomes. HeLa Tet-On cells transfected with plasmids encoding ssPEX3-GFP under a TRE-Tight promoter and photoactivatable RFP-SKL (PARFP-SKL) under a CMV promoter. (A) Representative HeLa Tet-On cells before photoactivation of PARFP-SKL. Channels for PARFP-SKL, ssPEX3-GFP and DIC images are indicated. (B) Image of the same cell immediately after photoactivation of PARFP-SKL (red) followed by the addition of doxycycline to induce ssPEX3-GFP (green) expression (approx 5 min). (C) The same cells as above 18 hours after photoactivation and induction. Scale bars are 10µm.

**Figure 3.** ssPEX3-GFP and PEX3-GFP complement PEX3 deficiency in PBD400-T1 cells. Representative immunofluorescent confocal images of PEX3-deficient PBD400-T1 cells transiently co-transfected with pPEX3-GFP and pmCerulean-Omp25TM (a mitochondrial
marker) (A) or pssPEX3-GFP and pmCerulean-cb5TM (an ER marker) (B) and immunostained for endogenous catalase 24 hours (A, B) and 72 hours after transfection (C, D), as indicated. The analyses of 300 cells expressing the PEX3 constructs 72 hours after transfection from 3 independent experiments resulted in almost 100% peroxisome recovery. Scale bars are 10µm.

**Figure 4: PMP import kinetic quantification assay.** (A) Time-lapse imaging experiment of HeLa cells co-expressing ssPEX3-GFP with UB-RFP-SKL. GFP and RFP signals were acquired at 37°C for 10h in CO₂ independent media in several different fields of view. Shown are representative time-lapse images at different time points. For presentation, the brightness of the images was enhanced equally for all frames. Scale bar is 10µm. The boundaries of the analyzed cell are lined with a white line on ssPEX3-GFP images. (B) The change in the fluorescent intensity within peroxisomes of ssPEX3-GFP (●) and UB-RFP-SKL (○) in the representative cell shown in (A), is plotted against time to illustrate the rates of ssPEX3-GFP and UB-RFP-SKL import. The apparent peroxisome import kinetics is shown for both proteins. AU = arbitrary unit. Data were fit with linear regression ($R^2>0.98$). The error bars are for the standard deviation of signal between peroxisomes at a given time. Both the mean and the slopes were found to be significantly different (p>0.0001) by Student t-test. (C) Summary for relative import rates of the PMPs, PEX3-GFP, PMP34-GFP, ssPEX3-GFP and PEX16-GFP respective to the import rate of UB-RFP-SKL ($k_{GFP}/k_{RFP}$) are shown in the bar graph. UB-GFP-SKL was used as a control. The analyses were performed on cells at early stages of protein expression. At least 17 cells from 3 independent experiments were analyzed for each GFP protein construct. The error bars are standard error of the mean. The p-value was determined using Student t-test. Both the relative import rates of PEX16 and ssPEX3 were significantly different from the GFP-SKL control (p<0.005), whereas PEX3 and PMP34 showed no significant difference.

**Figure 5. Targeting of PEX3 to ER does not complement PEX16 deficient cells.** Representative immunofluorescent confocal images of the PEX16-deficient cell line, GM06231, transiently transfected with pPEX16-GFP (A, B), pssPEX3×3myc (C, D) or pPEX3×3myc (E). PEX16-GFP was found initially in the ER (A) at 24 hours; however, by 96 hours it was found co-localized with catalase positive punctate structures suggesting complementation of PEX16 function (B). In contrast, ssPEX3×3myc is unable to complement PEX16 deficiency in GM06231 cells (C, D). ssPEX3×3myc is localized to ER on 24 (C) and 96 (D) hours after
transfection. No catalase positive punctuate structures were detected. (E) PEX3×3myc is localized to mitochondria in GM06231 cells. Scale bars are 10µm.

Figure 6. Depletion of PEX16 expression delays ssPEX3-GFP import into peroxisome, while overexpression of PEX16 retards the peroxisome import of PEX3-GFP and PMP34-GFP. (A-C) The distribution of PMP-GFP signal within peroxisomes (I_{per.}) with respect to the average fluorescent signal, within the whole cell (I_{cell}) treated with either siPEX16 or siCNTR, are plotted on a histogram as a ratio (I_{per.}/I_{cell}) for (A) ssPEX3-GFP, (B) PMP34-GFP, and (C) PEX3-GFP. The distributions for each PMP in siPEX16-treated cells were significantly different from that in control cells (siCNTR) (p<<0.001, n=50). A scatter plot of the same data (A-C) is shown in Figure S3 (C-E). (D) The peroxisomal import rates (k_{siPEX16}) of ssPEX3-GFP, PMP34-GFP, and PEX3-GFP in PEX16-depleted cells compared to control non-targeting siRNA-treated (k_{AVsiCNTR}) cells are shown in a bar graph. (E) The peroxisomal import rates (k_{PMP-GFP}) of ssPEX3-GFP, PMP34-GFP, PEX3-GFP or PEX16-GFP in cells co-expressing PEX16-mCerulean or PMP34-mCerulean, as indicated, compared to the average rates of the corresponding PMP-GFP construct in cells co-expressing mCerulean (k_{AV(CNTR)}) are shown in a bar graph. (F) The rate of PEX3-GFP import into peroxisomes decreased with increasing PEX16-mCerulean expression. Shown here is a different representation of (E) for PEX3-GFP relative import rate compared to control plotted against the ratio of PEX16-mCerulean over the total PEX3-GFP fluorescent signal. The rates are subdivided into three groups (with a comparable number of cells in each group) from low ratio to high ratio. The error bars are standard error of the mean. *: p<0.05, **: p<0.005, and ns: not significant.

Figure 7. A schematic model of PMP import into peroxisomes in mammalian cells. PMPs can be targeted to peroxisomes via two distinct pathways: the Group I pathway where PMPs initially target to the ER before being routed to peroxisomes; or the Group II pathway where PMPs are directly imported to peroxisomes. The pathway utilized by the PMP depends on the level of PEX16 in the ER, which is co-translationally targeted to the ER. On the ER, PEX16 can recruit other PMPs to the ER where they are rapidly transported to pre-existing peroxisomes. The mechanism of the transport between ER to peroxisomes has yet to be determined. However, based on studies on both yeast and plants, PEX16 and PMPs may accumulate in a specialized domain on the ER that is enriched for PMPs and peroxisomal lipids (grey membrane) in order to
generate pre-peroxisomal vesicles. In conditions where PMPs are in excess compared to PEX16, such as an ectopic expression of PMPs, they can also target directly to peroxisomes via the Group II pathway. Matrix proteins (empty triangles) are directly targeted to mature peroxisomes from the cytoplasm.

**ONLINE SUPPLEMENTAL MATERIAL:**

**Figure S1.** At high expression level, ssPEX3-GFP co-localized with ER and peroxisome signals but not mitochondrial signal. Representative confocal fluorescence microscopy images of high ssPEX3-GFP expressing living cell. HeLa cells stably expressing RFP-SKL were co-transfected with pssPEX3-GFP and pmCerulean-cb5(TM) (an ER marker). On the day after transfection, the cells were exposed to 50 µM of MitoTracker Deep Red (MTDR: a mitochondria marker) 20 mins before imaging. Shown is each marker in pseudo colours: ER (red), Mitochondria (cyan), peroxisomes (white) and ssPEX3-GFP (green). Arrows indicate peroxisomes. Scale bar is 10µm.

**Figure S2.** (A,B) The apparent import rate of UB-RFP-SKL is independent of GFP-fused proteins. (A) HeLa cells co-transfected with UB-RFP-SKL/PEX3-GFP or UB-RFP-SKL/PEX16-GFP were imaged and analyzed in parallel as described in Fig. 4. The averages of apparent peroxisomal import rates are plotted versus the corresponding GFP and RFP signals. (B) Same as in (A) but cells were co-transfected with the plasmids UB-RFP-SKL/ssPEX3-GFP or UB-RFP-SKL/PMP34-GFP. For each pair of PMPs (A or B) images were obtained in parallel in a single time-lapse experiment to ensure same microscope settings so that their apparent import rates can be readily compared. The GFP and RFP signals in peroxisomes were calculated and the apparent import rates were determined. In all cases, the GFP-fused proteins do not change the apparent import rate of UB-RFP-SKL. The error bars are the apparent import rate standard error of the mean. (C, D) Apparent import rate of GFP-fused protein into peroxisomes is independent of the protein expression level. Time-lapse experiment of HeLa cells co-expressing ssPEX3-GFP (C) or PEX3-GFP (D) together with UB-RFP-SKL. GFP and RFP fluorescent signals were determined in peroxisomal and non-peroxisomal (cytoplasm-ER) space for each cell (see Materials and methods). The apparent import rate (not normalized against RFP/GFP ratio from GFP-RFP-SKL) for each cell is plotted versus corresponding cyto-
ER signals. All represented cells were from the same experiment performed on the same day in order to minimize signal variations. Each point on the graphs represents one cell.

**Figure S3. PEX16 knockdown.** (A) Evaluation of PEX16 knockdown efficiency. Level of knockdown was evaluated by transcription efficiency of PEX16 on 3rd and 4th days after transfection using quantitative real-time PCR at the indicated days (see Materials and Methods). Each bar represents the mean ± standard error of the mean of three independent experiments. (B) PEX16 knockdown recovery assay by the co-expression of the siRNA-resistant PEX16 construct (PEX16*-mCerulean). The peroxisomal import rates of ssPEX3-GFP in PEX16-depleted cells exogenously co-expressing ssPEX3-GFP and mCerulean are compared to those cells co-expressing ssPEX3-GFP and PEX16*-mCerulean. In the presence of PEX16*-mCerulean the import rate is approximately doubled, which is in agreement with Fig. 6 (D, E).

(C-E) Scatter plot presentation of results shown in Fig. 6 A-C respectively.

(F-H) PEX16 depletion/overexpression or PEX3 overexpression does not significantly affect peroxisome number in cells. (F) HeLa cells treated with siCNTR or siPEX16 were fixed on 4th day after 1st siRNA treatment. (G) HeLa cells were transfected with either pPEX16-mCerulean or pCerulean-N1 plasmids and incubated for 2 days. (H) HeLa cells were transfected with either pPEX3-GFP or EGFP-N1 plasmids and incubated for 2 days. (F-H) At the indicated time, cells were fixed and probed with rabbit anti-PMP70 antibody followed by staining with goat anti-rabbit ALEXA-633 secondary antibody. The z-stacks were collected and the number of peroxisomes was counted in 100 cells for each treatment using the ImageJ program. The error bars are the peroxisome number standard deviation.
Fig. 5_Aranovich et al