Mff functions with Pex11pβ and DLP1 in peroxisomal fission

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Summary
Peroxisomal division comprises three steps: elongation, constriction, and fission. Translocation of dynamin-like protein 1 (DLP1), a member of the large GTPase family, from the cytosol to peroxisomes is a prerequisite for membrane fission; however, the molecular machinery for peroxisomal targeting of DLP1 remains unclear. This study investigated whether mitochondrial fission factor (Mff), which targets DLP1 to mitochondria, may also recruit DLP1 to peroxisomes. Results show that endogenous Mff is localized to peroxisomes, especially at the membrane-constricted regions of elongated peroxisomes, in addition to mitochondria. Knockdown of MFF abrogates the fission stage of peroxisomal division and is associated with failure to recruit DLP1 to peroxisomes, while ectopic expression of MFF increases the peroxisomal targeting of DLP1. Co-expression of MFF and PEX11β, the latter being a key player in peroxisomal elongation, increases peroxisome abundance. Overexpression of MFF also increases the interaction between DLP1 and Pex11pβ, which knockdown of MFF, but not Fis1, abolishes. Moreover, results show that Pex11pβ interacts with Mff in a DLP1-dependent manner. In conclusion, Mff contributes to the peroxisomal targeting of DLP1 and plays a key role in the fission of the peroxisomal membrane by acting in concert with Pex11pβ and DLP1.

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Introduction
Peroxisome is a ubiquitous, spherical organelle present in virtually all eukaryotes, from yeast to mammals. The growth and division model of peroxisome biogenesis predicts that peroxisomes grow and multiply by taking up newly synthesized proteins from the cytosol (Lazarow and Fujiki, 1985).

Peroxisomal division comprises three stages: elongation, constriction, and fission (Itoyama et al., 2012; Koch et al., 2003; Li and Gould, 2003; Schrader et al., 1998). Pex11p is a peroxisome-specific division factor conserved from yeast to humans (Thoms and Erdmann, 2005). High-level expression of PEX11 promotes the proliferation of peroxisomes (Marshall et al., 1995; Schrader et al., 1998), while deletion of PEX11 reduces the number of peroxisomes (Erdmann and Blobel, 1995; Li et al., 2002b), thereby suggesting that Pex11p plays a key role in peroxisomal division. Pex11p also functions in peroxisomal elongation, which is the first step in peroxisomal division (Marshall et al., 1995; Opaliński et al., 2011; Schrader et al., 1998). In mammalian cells, three isoforms have been identified: PEX11α (Abe et al., 1998; Li et al., 2002a), PEX11β (Abe and Fujiki, 1998; Li et al., 2002b; Schrader et al., 1998), and PEX11γ (Li et al., 2002a; Tanaka et al., 2003). PEX11β is expressed in almost all types of human cells (Schrader et al., 1998), in contrast to PEX11α and PEX11γ, which are expressed in a tissue-specific manner (Li et al., 2002a; Schrader et al., 1998), thus strongly suggesting that Pex11pβ plays a fundamental role in peroxisome division.

Dynamin-like protein 1 (DLP1), a member of the large GTPase family, promotes the maintenance of peroxisomal and mitochondrial morphology, especially during membrane fission (Ishihara et al., 2009; Tanaka et al., 2006; Waterham et al., 2007). DLP1 is predicted to mediate the fission of peroxisomes and mitochondria via the formation of large multimeric spirals, in a molecular machinery similar to that of dynamin at the site of endocytosis (Danino and Hinshaw, 2001; Ford et al., 2011; Zhang and Hinshaw, 2001). DLP1 and dynamin have several common multidomains including the GTPase, middle, and GTPase effector domains. In particular, the middle domain functions in the higher-order assembly of both proteins, which is required for the formation of functional multimeric spirals (Ingerman et al., 2005; Ramachandran et al., 2007). Therefore, mutations in the DLP1 middle domain result in the abnormal elongation of peroxisomes and hyperturbation of mitochondria (Tanaka et al., 2006; Waterham et al., 2007). Translocation of
DLp1 from the cytosol to peroxisomes and mitochondria is a prerequisite for membrane fission.

Fission1 (Fis1) and mitochondrial fission factor (Mff) are thought to be involved in the peroxisomal targeting of DLp1 in mammalian cells (Gandre-Babbe and van der Bliek, 2008; Kobayashi et al., 2007; Koch et al., 2005; Otera et al., 2010). Fis1 is a tail-anchored protein that functions in the fission of peroxisomes and mitochondria (Kobayashi et al., 2007; Koch et al., 2005). Fis1 interacts with DLp1 and ectopic expression of Fis1 increases the interplay between Pex11pβ and DLp1 (Kobayashi et al., 2007), suggesting that Fis1 recruits DLp1 to peroxisomes. Furthermore, Pex11pβ, Fis1, and DLp1 coordinately regulate the fission step of peroxisomal division (Kobayashi et al., 2007). Meanwhile, Mff, another tail-anchored protein, is involved in the maintenance of peroxisomal and mitochondrial morphology (Gandre-Babbe and van der Bliek, 2008). A recent study reported that the mitochondrial targeting of DLp1 was mediated via direct binding of Mff (Otera et al., 2010), and Mff was recently found to be involved in Pex11p-mediated peroxisomal fission (Koch and Brocard, 2012); however, the precise function of Mff in peroxisomal division remains unclear.

The present study shows that Mff recruits DLp1 to peroxisomes and suggests that a functional complex comprising Pex11pβ, Mff, and DLp1 promotes Mff-mediated fission during peroxisomal division.

Results

Dual localization of Mff to peroxisomes and mitochondria

To investigate the function of Mff, rabbit polyclonal antiserum was raised against the N-terminal region of human Mff splicing variant 8 (residues 27–173) (Fig. 1A). Western blot analysis revealed that the Mff antibody specifically recognized the endogenous Mff protein in organelle fractions from HeLa, HEK293, and Chinese hamster ovary (CHO) cells (Fig. 1B); several bands were detected, including six bands in HEK293 cells. All bands were eliminated by the transfection of siRNA targeting MFF (Fig. 1C), possibly reflecting some of the nine Mff splicing variants previously reported (Gandre-Babbe and van der Bliek, 2008).

The subcellular localization of endogenous Mff was investigated by immunostaining with Mff-specific antibody. In control fibroblasts, Mff was mostly localized to Tom20-positive mitochondria and Pex14p-positive peroxisomes (Fig. 1D). In addition, the localization of endogenous Mff was also assessed in post-heavy mitochondrial fractions obtained from control fibroblasts by isopycnic ultracentrifugation (Fig. 1E). Mff was detected in Pex14p-positive mitochondrial fractions (lanes 12 and 13, open arrowheads), which were devoid of Tom20-positive mitochondria or P450r-positive smooth microsomes. Collectively, these results strongly suggest that Mff is localized to peroxisomes as well as mitochondria.

Mff is essential for peroxisome membrane fission

Mff was suggested to be involved in the division of peroxisomes (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010). To clarify the functional role of Mff in peroxisomal division, the effect of MFF knockdown on the division of peroxisomes was assessed in fibroblasts deficient in acyl-CoA oxidase 1 (AOx) encoding the enzyme catalyzing the first step in peroxisomal β-oxidation. We recently reported that docosahexaenoic acid (DHA, C22:6n-3) induces the division of peroxisomes in cells defective in peroxisomal β-oxidation in a Pex11pβ-dependent manner (Itoyama et al., 2012). This is a useful physiological system for inducing peroxisome proliferation. Seventy-two hours after adding MFF dsRNA, the Mff protein level was significantly reduced in AOX-deficient fibroblasts (Fig. 2B). Peroxisome abundance was greater in AOX-deficient fibroblasts treated with control dsRNA and supplemented with DHA (157±39) than in

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Fig. 1. Mff is localized to peroxisomes and mitochondria. (A) The domain structure of human Mff splicing variant 8 is presented. The red, blue, and green boxes indicate the two repeat regions, coiled-coil domain and TMD. (B) Cytosol and organelle fractions prepared from HeLa, HEK293, and CHO-K1 cells were analyzed by SDS-PAGE and immunoblotting using antibodies to Mff and Tom20. (C) HEK293 cells were treated for 72 h with two different dsRNAs (MFF #1 and MFF #2). Mff levels were assessed by immunoblotting with anti-Mff antibody. Actin was used as a loading control. (D) Control fibroblasts were stained with antibodies to Mff (a), Pex14p (b), and Tom20 (c); the merged view of the three proteins is shown (d). Scale bar: 10 μm. Insets, higher magnification images of the boxed regions, scale bar: 2 μm. (E) PHM fraction from control fibroblasts was fractionated by Opti-prep density gradient ultracentrifugation. The distribution of peroxisomes, mitochondria, and smooth ER was assessed by immunoblotting using antibodies to the marker proteins Pex14p, Tom20, and P450o redactase (P450r), respectively. Downward solid arrowheads indicate the peak fractions of peroxisomes; the upward open arrowhead indicates Mff (Ps; lane 12 and 13).
mock-treated cells (76±19). By contrast, DHA-inducible peroxisomal division was strongly inhibited by MFF knockdown in two independent experiments using dsRNA MFF#1 (74±18) and MFF#2 (84±22), respectively, rather giving rise to numerous elongated peroxisomes (Fig. 2Ac,d,C). These results strongly demonstrate that Mff is essential to peroxisome membrane fission.

Mff is involved in the recruitment of DLP1 to peroxisomes

Mff functions in the mitochondrial recruitment of DLP1 (Otera et al., 2010). To investigate the potential involvement of Mff in the peroxisomal recruitment of DLP1, the intracellular localization of DLP1 was assessed upon MFF knockdown in fibroblasts from a healthy control. Knocking down MFF in control fibroblasts significantly reduced the Mff level (Fig. 3B). In cells treated with
control RNAi, DLP1 was observed as dot-like structures and partially localized to punctate peroxisome structures (Fig. 3Aa–d); however, knockdown of MFF reduced the translocation of DLP1 to the numerous elongated peroxisomes (Fig. 3Ae–h). Furthermore, to investigate whether Mff promotes the translocation of DLP1 to the numerous elongated peroxisomes (Fig. 3Ae–h), we transfected HA2-DLP1 into HeLa cells and assessed its intracellular localization 24 h post-transfection. HA2-DLP1 was mostly diffused throughout the cytoplasm (Fig. 3Ca–d). By contrast, in cells co-expressing HA2-DLP1 and FLAG-Mff, HA2-DLP1 colocalized with FLAG-Mff, which is consistent with earlier results (Otera et al., 2010), to Pex14p-positive peroxisomes (Fig. 3Ce–h). Translocation of DLP1 to peroxisomes was not observed in cells co-expressing HA2-DLP1 and FLAG-Mff mutants such as MffD_TMD, which lacks a transmembrane domain (TMD), and MffD_N, which lacks amino acids 1–87 including two repeat regions (Fig. 3Ci–p). Next, we assessed the interaction of endogenous Mff and DLP1 by co-immunoprecipitation with Mff-specific antibody. DLP1 was co-immunoprecipitated with Mff from the lysates of HEK293 cells treated with the cross-linker dithiobis[succinimidyl propionate] (DSP) (Fig. 3D), strongly suggesting that endogenous Mff and DLP1 interact. Collectively, these results suggest that Mff recruits DLP1 to peroxisomes.

Peroxisome elongation is required for Mff-mediated membrane fission

The expression of Mff induces the fragmentation of mitochondria (Otera et al., 2010). To investigate whether the expression of MFF induces the proliferation of peroxisomes, we transfected FLAG-MFF into HEK293 cells and measured peroxisome abundance. Twenty-four hours post-transfection, imaging results showed that part of the FLAG-MFF-positive particles could be overlaid onto Pex14p-positive peroxisomes, while peroxisome abundance was not significantly altered (Fig. 4Aa–f,B). In addition, we sought to determine whether the elongation of peroxisomes was required for the fission mediated by Mff. FLAG-MFF was co-expressed with PEX11β-Myc in HEK293 cells. Pex11pβ-Myc induced peroxisomal elongation and a modest increase in peroxisome abundance (Fig. 4Ag–i,B). Furthermore, the number of peroxisomes was more abundant in cells dually expressing Pex11pβ-Myc and FLAG-Mff, resulting in numerous punctate peroxisomes (Fig. 4Aj–l,B). These results suggest that peroxisomal elongation is required for Mff-mediated peroxisome membrane fission.

Mff localizes to membrane-constricted regions in elongated peroxisomes

Next, we investigated the peroxisomal localization of endogenous Mff in dlp1 mutant ZP121 CHO cells (Tanaka et al., 2006). ZP121 cells show abnormal tubular peroxisomes due to the expression of a dominant-negative DLP1 mutant; this phenotype permits the assessment of the localization of membrane proteins on elongated peroxisomes. In ZP121 cells, Mff was indeed localized to extended peroxisomes and to mitochondria and partially accumulated in the limited area, which is devoid of Pex14p (Fig. 5). Thus, Mff is localized at the membrane-constriction sites of elongated peroxisomes and functions in peroxisomal fission.

Pex11pβ, Mff, and DLP1 coordinate peroxisomal fission

To address how Pex11pβ, Mff, and DLP1 function during peroxisomal division, we assessed the interaction of Pex11pβ with Mff and DLP1 by immunoprecipitation. Forty-eight hours after adding control or MFF dsRNA to HeLa cells, we expressed FLAG-Pex11pβ, HA2-DLP1, HA2-Mff, and siRNA-resistant HA2-Mff (HA2-MffR); immunoprecipitation was then performed with anti-FLAG IgG-conjugated agarose upon DSP treatment. As shown in Fig. 6A, FLAG-Pex11pβ was found to interact with endogenous Mff, HA2-Mff, and HA2-DLP1, and expression of HA2-Mff increased the interplay between FLAG-Pex11pβ and HA2-DLP1 (Fig. 6A, lanes 6–8). By contrast, the interplay...
between FLAG-Pex11pβ and HA2-DLP1 was decreased in cells treated with MFF dsRNA and restored by the expression of HA2-MffRβ (Fig. 6A, lanes 9 and 10), indicating that Pex11pβ interacts with DLP1 via Mff. Fis1 was suggested to function in the fission step of peroxisomal division and to form ternary complexes with Pex11pβ and DLP1 (Kobayashi et al., 2007). Thus, we assessed the effect of siRNA targeting FIS1 on the formation of the complex containing Pex11pβ, Mff, and DLP1. The interplay between FLAG-Pex11pβ and HA2-DLP1 was not affected by FIS1 knockdown, suggesting that Fis1 is not essential for the formation of the Pex11pβ/Mff/DLP1 complex (Fig. 6B). Taken together, these results suggest that Pex11pβ, Mff, and DLP1 cooperate to achieve peroxisome membrane fission.

The middle domain of DLP1 is involved in the mitochondrial recruitment and high-order assembly of DLP1 (Chang et al., 2010). Therefore, we assessed whether Pex11pβ forms a complex with the DLP1 middle domain mutants G363D and A395D, both defective in higher-order assembly and GTPase activity (Tanaka et al., 2006; Waterham et al., 2007). As shown in Fig. 7A, the middle domain mutations decreased the translocation of DLP1 to peroxisomes stimulated by the expression of MFF in HeLa cells (Fig. 7Ae–l). We transfected FLAG-PEX11β, HA2-MFF, HA2-DLP1, HA2-DLP1 G363D, and HA2-DLP1 A395D in HeLa cells, and performed immunoprecipitation with anti-FLAG IgG-conjugated agarose after DSP treatment. Wild-type HA2-DLP1, but not the HA2-DLP1 mutants, was detected in FLAG-Pex11pβ immunoprecipitates (Fig. 7B, lanes 4–6), suggesting that the middle domain of DLP1 is required for the formation of the Pex11pβ/Mff/DLP1 complex. To elucidate the interplay between Pex11pβ, Mff, and DLP1 further, the effect of DLP1 knockdown on that interaction was assessed. The interplay between FLAG-Pex11pβ and Mff was decreased strikingly in cells treated with DLP1 dsRNA (#1 and #2) compared to cells treated with control RNAi (Fig. 7C), indicating that DLP1 promotes the interaction between Pex11pβ and Mff. Taken together, it is likely that the complex formed by Mff and DLP1 interacts with Pex11pβ, leading to the formation of large multimeric DLP1 spirals and peroxisome membrane fission.

Pex11pβ is not required for the localization of Mff to the membrane-constriction site

Furthermore, we verified whether Pex11pβ recruits Mff at the membrane-constriction sites, by making use of mouse embryonic fibroblasts (MEF) cells from a PEX11β-knocked out mice (Li et al., 2002b) (Fig. 8). In control MEF cells, immunofluorescence staining with Mff antibody showed typical mitochondrial tubular

**Fig. 5. Intracellular localization of endogenous Mff in dlp1 ZP121 cells.** dlp1 ZP121 cells were immunostained with antibodies to Pex14p (a) and Mff (b); the merged view of the two proteins is shown (c). Scale bar: 10 μm. Insets, higher magnification images of the boxed regions; scale bar: 2 μm. Arrowheads indicate regions enriched for Mff compared to Pex14p-positive regions of peroxisomes; arrows show mitochondria.

**Fig. 6. Pex11pβ interacts with DLP1 via Mff.** (A) After 48h treatment with control dsRNA or MFF #2 dsRNA, HeLa cells were transfected for 24h with FLAG-PEX11β, HA2-DLP1, HA2-MFF, or HA2-MFFR. Cells were then treated with 1mM DSP, lysed and subjected to immunoprecipitation using anti-FLAG IgG-conjugated agarose. Immunoprecipitates were analyzed by SDS-PAGE and immunoblottting with antibodies to HA, Mff, and DLP1. Arrowhead designates HA2-Mff or HA2-MffR. Input (10%) was loaded in lanes 1–5. (B) HeLa cells treated for 48h with control dsRNA or two different dsRNAs (FIS1 #1 and FIS1 #2) were transfected for 24h with FLAG-PEX11β and HA2-DLP1. Cells were treated with 1mM DSP and analyzed as in panel A, except that antibodies to HA, Mff, FLAG, and Fis1 were used. Lanes 1–5, input (10%). (C) HeLa cells treated for 48h with MFF #2 dsRNAs were transfected for 24h with HA2-MFFR, and stained with antibodies to Pex14p (a–c) and Mff (d–f). Scale bar: 10 μm. Insets, higher magnification images of the boxed regions; scale bar: 2 μm. Note that peroxisome morphology was restored by the expression of HA2-MFFR.
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Fig. 7. DLP1 is required for the interaction between Pex11pβ and Mff and the middle domain of DLP1 promotes the formation of the Pex11pβ/Mff/DLP1 complex. (A) FLAG-MFF was co-expressed with HA-DLP1 (upper panels), HA-DLP1G363D (middle panels), or HA-DLP1A395D (lower panels) in HeLa cells. After 24 h, cells were stained with antibodies to Pex14p (a,e,i), FLAG (b,f,j), and HA (c,g,k); the merged view of the three proteins is shown (d,h,l). Scale bar: 10 μm. Insets, higher magnification images of the boxed regions, scale bar: 2 μm. Arrowheads indicate the sites of colocalization of Pex14p, FLAG-Mff, and HA-DLP1. (B) HeLa cells were transfected with HA-DLP1, HA-DLP1-

Discussion

Mff was identified in an siRNA screen in Drosophila cells and shown to be involved in the morphogenesis of mitochondria and peroxisomes (Gandre-Babbe and van der Bliek, 2008). Mff recruits DLP1 to mitochondria (Otera et al., 2010); however, the function of Mff, especially that of endogenous Mff, in peroxisomes remains elusive. In this study, we showed that endogenous Mff localizes to peroxisomes in addition to mitochondria and is responsible for peroxisomal division (Figs 1, 2). Peroxisomal targeting of DLP1 is decreased upon knockdown of MFF and is conversely increased by ectopic expression of MFF (Fig. 3). Thus, we conclude that Mff recruits DLP1 to peroxisomes.

Pex11pβ plays a key role in peroxisomal division and mainly supports membrane elongation in peroxisomes (Opalinski et al., 2011; Schrader et al., 1998). In the PEX11β<sup>−/−</sup> family, only PEX11β is expressed in almost all the types of human cells (Schrader et al., 1998), in contrast to PEX11α and PEX11γ, which are expressed in a tissue-specific manner (Li et al., 2002a; Schrader et al., 1998). Therefore, understanding Pex11pβ function is key to understanding the mechanisms underlying peroxisome division. In this report, we show that Pex11pβ interacts with DLP1 via Mff (Fig. 6A), suggesting that Pex11pβ forms a ternary complex with Mff and DLP1 during the fission process of peroxisomal division. We reported very recently that Pex11pβ localizes to the constricted regions of elongated peroxisomes, which are devoid of Pex14p (Itoyama et al., 2012). In the present study, we also found that Mff is similarly localized to the constricted regions of elongated peroxisomes (Fig. 5). Therefore, it is likely that the ternary complex comprising Pex11pβ, Mff, and DLP1 fission at the constricted region of elongated peroxisomes. Intriguingly, a recent report showed that Mff stimulates the GTPase activity of DLP1 <span class="red" in vitro</span> (Otera and Mihara, 2011), suggesting that the self-assembly of DLP1 is facilitated by Mff. We found here that the middle domain DLP1 mutants, which are defective in self-assembly, decrease DLP1/Mff/Pex11pβ

Fig. 8. Localization of Mff to the elongated peroxisomes in PEX11β<sup>−/−</sup> MEF cells. Control MEF and PEX11β<sup>−/−</sup> MEF cells were stained with antibodies to Pex14p (a,d) and Mff (b,e); the merged view of the two proteins is shown (c,f). Scale bar: 10 μm. Insets, higher magnification images of the boxed regions; scale bar: 2 μm. The arrowhead indicates regions enriched for Mff compared to Pex14p-positive regions.
complex formation (Fig. 7B). Taken together, we conclude that DLP1 forms a ternary complex with Mff and Pex11pβ at the constricted regions of elongated peroxisomes; this event is followed by higher-order assembly, resulting in the fission of the peroxisomal membrane.

The interaction of Pex11pβ with Mff was very recently shown by co-immunoprecipitation from cells expressing Flag-tagged Pex11pβ and EGFP-fused Mff (Koch and Brocard, 2012). However, physiological significance of the interaction between Pex11pβ and Mff still remains elusive. A previous study reported that the ectopic expression of PEX11β targets DLP1 to peroxisomes (Li and Gould, 2003), implying that Pex11pβ could recruit DLP1 to peroxisomes by interacting with Mff; however, Otera et al. reported that a Mff mutant in which the TMD was replaced with the plasma membrane-targeted CAAX motif elicits the translocation of DLP1 to the plasma membrane (Otera et al., 2010), suggesting that Mff is sufficient to recruit DLP1 complex.

Peroxisomes (Li and Gould, 2003), implying that Pex11pβ/b may be involved in the localization of Mff to the membrane-constriction site of peroxisomes. Very recently, GDAPI (ganglioside-induced differentiation associated protein 1) was suggested to be required for peroxisome fission at the downstream of Pex11pβ and the upstream of fission steps mediated by Mff and DLP1 (Huber et al., 2013), inferring that GDAPI likely mediates the interaction between Pex11pβ and Mff/DLP1 complex.

The regulatory mechanism underlying peroxisomal division remains elusive. In mitochondria, Fis1, Mff, MiD49, and MiD51 can each recruit DLP1 in one of the rate-limiting steps of mitochondrial fission (Cereghetti et al., 2008; Losón et al., 2013; Otera and Mihara, 2011; Palmer et al., 2011; Zhao et al., 2011). For instance, overexpression of MFF facilitates the mitochondrial targeting of DLP1, resulting in the fragmentation of mitochondria (Otera et al., 2010). By contrast, peroxisomal proliferation, resulting from peroxisomal fission, was not increased by the overexpression of MFF despite massive recruitment of DLP1 to the peroxisomal membrane (Fig. 3C, Fig. 4). Moreover, the proliferation of peroxisomes is significantly suppressed in AOX-deficient fibroblasts, although DLP1 localizes to peroxisomes (Itoyama et al., 2012). These findings strongly suggest that the recruitment of DLP1 is not a rate-limiting step for peroxisomal division. Interestingly, the fission of peroxisomes in the methylotrophic yeast Pichia pastoris is regulated by the interaction of Pex11p with Fis1 via phosphorylation of Pex11p in oleate medium, not methanol (Joshi et al., 2012). In mammalian cells, DHA is one of the mediators of peroxisomal division and induces elongation of peroxisomes in a Pex11pβ-dependent manner (Itoyama et al., 2012). Here we demonstrated that DHA promotes the proliferation of peroxisomes in AOX-deficient fibroblasts in a manner dependent on Mff and DLP1 (Fig. 2). Furthermore, co-expression of MFF and PEX11β, but not expression of MFF alone, promotes peroxisomal proliferation (Fig. 4). These data suggest that the elongation of peroxisomes, giving rise to the formation of Pex11pβ-enriched and membrane-constricted regions (Itoyama et al., 2012), is a prerequisite for peroxisomal fission via activation of DLP1. Taken together, the membrane elongation of peroxisomes is likely to be a rate-limiting step in peroxisomal division and might facilitate the formation of the DLP1 spiral structures at the constricted regions, leading to division.

Materials and Methods

Cell culture and DHA supplementation

Human skin fibroblasts from a healthy subject (Tig120) were purchased from the Human Science Research Resources Bank (Osaka, Japan). Fibroblasts from a patient with AOX deficiency (PDLS0092) were described previously (Ferdinandusse et al., 2007; Poll-The et al., 1988). Control MFF and PEX11β−/− MEF cells were a generous gift from Dr. J. Gould (Li et al., 2002b). Fibroblasts, MEF, HeLa cells, and HEK293 cells were cultured at 37°C in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Rockville, MD) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO) in 5% CO2 (Okamoto et al., 1998). CHO cell lines, including CHO-K1 and dlp1/ZP121 (Tanaka et al., 2011) cells, were cultured as described previously (Tsukamoto et al., 1990). DHA (Nacalai Tesque, Kyoto, Japan) dissolved in DMEM supplemented with 0.4% fatty acid-free bovine serum albumin (Nacalai Tesque) was used in cell cultures at a final concentration of 150 µM as described previously (Itoyama et al., 2012).

Antibodies

The antibodies used were rabbit antisera to rat Pex14p (Shimizu et al., 1999), HA peptide (Otera et al., 2000), and guinea pig antisera to rat Pex14p (Mukai et al., 2002). Polyclonal antibodies to FLAG and human Fis1 were purchased from Sigma and Enzo Life Sciences (Farmingdale, USA), respectively. Monoclonal antibodies to human DLP1 (BD Biosciences, Franklin lake, NJ), human Tom20 (Santa Cruz Biotech, Santa Cruz, CA), PS40 reductase (Santa Cruz Biotech), actin

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(Chemonic, Temecula, CA), HA (Covance, Princeton, NJ), and e-Myc (Santa Cruz) were purchased.

Rabbit antiserum against human Mff was raised as follows: an expression vector encoding the primary sequence (residues 27 to 173) of human (Hs) Mff fused to GST, termed GST-HsMff (27–173), was constructed as previously described (Shimizu et al., 1999) using FLAG-MFF as a template and the primers GST-HsMff (27–173)-Fw 5′-CCGCGATCCCATCGCAAGAATTCTGCAATT-3′ and GST-HsMff (27–173)-Rv 5′-CAAGGGCGGCGCCATACAGAGGAATCTTC-3′. A BamHI-EcoRI fragment of the PCR product was ligated into the corresponding sites of pGEX6p-1 (GE Healthcare, Chalfont, UK). Escherichia coli BL21 cells were transformed with pGEX6p-1-GST-HsMff (27–173) and grown according to the manufacturer’s instructions. BL21 cells were then harvested in lysis buffer (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride in PBS) and sonicated. The lysate was centrifuged at 20,000 × g for 10 min and the supernatant was subjected to purification using glutathione-Sepharose beads (GE Healthcare). After a thorough wash, purified GST-HsMff (27–173) was cleaved with PreScission protease (GE Healthcare) to remove the GST moiety, and the eluted Mff (27–173) was further purified with Q-Sepharose Fast Flow ion-exchanger columns (GE Healthcare). The peak fractions were recovered as recombinant HsMff (27–173).

The rabbit anti-Mff antibody was raised by conventional subcutaneous injection of HsMff (27–173) in PBS/0.1% Triton X-100 (Okamoto et al., 1998).

Reference

For RNAi, six sets of complementary antisense oligonucleotides were designed (Invitrogen, Carlsbad, CA). The sequences were as follows: human MFF #1 oligonucleotides 5′-UUCAUCACUAUUGGAAACUC-3′ and 5′-GGAG-UUUAUUGAAGGCACAUUUGGAAUA-3′; human MFF oligonucleotides 5′-UAUUGAAGCCAAUCCUGACCGG-3′; and 5′-CGUGCAGUUAAUCAAUAUAUA-3′; human FISI #1 oligonucleotides 5′-UUAACGUAGCAUGUACG-3′ and 5′-GAGCAACAGGGAACGGCGAAUAUA-3′; human FISI #2 oligonucleotides 5′-AAUCCCGUUCCUCUUCGCGC-3′ and 5′-GGAGCAAGGAAGAAGCAGGCGAAUA-3′; human PEX11 oligonucleotides 5′-AACCCCAGGGAGAGACGGGAA-3′; human PEX11 oligonucleotides 5′-CACUGUAAACUCCUGCUAAGGACG-3′; and 5′-CCUG-CUUUAUGUUGCGCGGGUUAUA-3′; human PEX11 oligonucleotides 5′-AAUUGAAGCCAAUCCUGACCGG-3′ and 5′-GAGCAACAGGGAACGGCGAAUAUA-3′; human PEX11 oligonucleotides 5′-AACCCCAGGGAGAGACGGGAA-3′; and 5′-CCUG-CUUUAUGUUGCGCGGGUUAUA-3′.

DNA construction

For FLAG-MFF, FLAG-MFF-ATMD, HA-MFF, and HA-DLPI, human MFF (splice variant 8) (Gandile-Babbe and van der Biek, 2008) and DLPI cDNA were amplified by RT-PCR using total RNA isolated from HeLa cells and the primer pairs described below; MFF-Fw 5′-AGTGGCATGCGGACAGAATTGCTGAATG-3′, MFF-Rv 5′-CAAGGGCGGCGGCGGGAAACACCG-3′, MFF-ATM-Rv 5′-GGTGGCACCGGCCTTCTATTTAGACG-3′, MFF-ATN-Rv 5′-AAGAAGTCGGAATTAAGAGATTGTTCTTTCTC-3′, DLPI-Fw 5′-GTTGGCCCGGTCAAATAGTCCTGTG-3′, and DLPI-Rv 5′-AAAGGCGGCGGCTCAACAGATGGTCTC-3′. PC products were cloned into pcDNA3.1 Zeo+/-Flag-Ubiquitin (Okamoto et al., 2011b) or pcDNA3.1 Zeo+/-HA-Ubiquitin (Okamoto et al., 2011a) by replacing the BamHI-NotI fragments of vectors. We also used pCtD32H yg/FLAG-PExEI 11 (Abe and Fujiki, 1998) and pExEI 11-Myc (Y. Y. and Y. F., unpublished).

Site-directed mutagenesis was performed to introduce substitutions in DLPI and MFF using the following primers: DLPI G363-Fw 5′-AATCTTGGAGGATGGTGGTGGT-3′ and DLPI G363-Rv 5′-AAATAATTAAACAAATCTTCAACACGAGATGCTGAATG-3′; DLPI A395D-Fw 5′-CACCCTGAGATTTTCGTCGCGTGTTT-3′ and DLPI A395D-Rv 5′-AAAGGCGGCGGCTCAACAGATGGTCTC-3′. The resulting constructs were transformed into E.coli DH5α and sequenced to confirm the presence of the desired substitutions.

Morphological analysis

Cells were fixed with 4% paraformaldehyde (ph 7.4) for 15 min at room temperature. Peroxisomes were visualized by indirect immunofluorescence staining with the indicated antibodies as described previously (Mukai et al., 2002). Antigen–antibody complexes were detected with goat anti-mouse and anti-rabbit IgG conjugated to Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes, Eugene, OR). Cells were observed under a fluorescence light microscope (Axioskop2) and by confocal laser microscopy (LSM710, Carl Zeiss, Oberkochen, Germany).

The number of peroxisomes was counted in at least 50 randomly selected cells (Kim et al., 2006). Optical images obtained by confocal fluorescence microscopy were converted into grayscale images and then the number of peroxisomes was calculated using the Particle Analysis package of ImageJ. Values are means ± S.D. of three independent experiments.