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Peroxisome proliferation in *Hansenula polymorpha* requires Dnm1p which mediates fission but not *de novo* formation

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**Abstract**

We show that the dynamin-like proteins Dnm1p and Vps1p are not required for re-introduction of peroxisomes in *Hansenula polymorpha pex3* cells upon complementation with PEX3-GFP. Instead, Dnm1p, but not Vps1p, plays a crucial role in organelle proliferation via fission. In *H. polymorpha DNM1* deletion cells (*dnm1*) a single peroxisome is present that forms long extensions, which protrude into developing buds and divide during cytokinesis. Budding *pex11.dnm1* double deletion cells lack these peroxisomal extensions, suggesting that the peroxisomal membrane protein Pex11p is required for their formation. Life cell imaging revealed that fluorescent Dnm1p-GFP spots fluctuate between peroxisomes and mitochondria. On the other hand Pex11p is present over the entire organelle surface, but concentrates during fission at the basis of the organelle extension in *dnm1* cells. Our data indicate that peroxisome fission is the major pathway for peroxisome multiplication in *H. polymorpha*.

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Keywords: Peroxisome; Yeast; Dynamin-like protein; Dnm1p; Pex11p

1. Introduction

Peroxisomes are single membrane bound organelles that are ubiquitously present in eukaryotic cells. Depending on species and environmental conditions, peroxisomes display an unprecedented diversity in protein composition and metabolic functions like fatty acid oxidation and ether–lipid biosynthesis in man, methanol oxidation in methylotrophic yeast and penicillin biosynthesis in fungi [1]. In man inherited disorders are known that result in malfunctioning of peroxisomes and lead to severe abnormalities which often are lethal [2].

Peroxisomes have long been considered to be solely formed by growth and division of pre-existing ones (reviewed in [3–5]). However, recently peroxisome research has made exciting advancements, as the general view that peroxisomes are autonomous organelles – like mitochondria and chloroplasts – has been challenged by data supporting that they may be formed from the endoplasmic reticulum [6]. Also proteins have been identified that are suggested to play a role in peroxisome fission, among others dynamin-like proteins (DLPs), Pex11p and Pex11p-related proteins [5,7].

DLPs are GTPases that play a role in various membrane fusion and fission events. Data from plant, human and yeast cells provide evidence for the involvement of DLPs in peroxisome proliferation as well [8–12]. The baker's yeast genome encodes 3 DLPs, namely Vps1p, Dnm1p and Mgm1p. Vps1p has initially been identified as a protein essential for vacuolar protein sorting [13], but later studies also revealed functions in vacuole fusion and fission events [14] and in peroxisome proliferation [9].

It is well documented that Dnm1p is involved in mitochondrial division and is recruited to the mitochondrial outer membrane by Fis1p [15–18]. Recent observations revealed that both proteins have a dual location and are also localized to peroxisomes, where they play a role in peroxisome proliferation in cells grown at peroxisome-inducing conditions (oleate [8]).

Mgm1p is localized to the mitochondrial inner membrane, where it is involved in mitochondrial fusion [19], but does not function in peroxisome proliferation [9].
Higher eukaryotes contain a single DLP, designated Dlp1p in mammals and DRP3A in plant [10]. Together with Fis1p, these DLPs are involved in fission of both mitochondria and peroxisomes [11,12].

Pex11p is a peroxisomal membrane protein, whose function so far is mainly speculative. Comparison of genome sequence information of various organisms indicated that most species contain multiple Pex11-like proteins. For instance, *S. cerevisiae* contains Pex11p, Pex25p and Pex27p, whereas PEX11α, β and γ have been identified in mammals [20].

Based on elegant studies performed with mammalian cells, Schrader and colleagues proposed a model predicting that peroxisome fission involves 3 consecutive steps, namely elongation, constriction and fission, and that Pex11p is involved in the first step [11,21,22]. Interestingly, recent data indicated that Pex11β interacts with Fis1p, suggesting that Fis1p, Dlp1p and Pex11β together regulate peroxisome proliferation in mammals [23].

Data that peroxisomes may arise from the ER in yeast and mammals [6,24], raises the question whether peroxisomes are generally formed from the ER with a putative function of DLPs and Pex11p’s in e.g. pre-peroxisomal vesicle formation, or whether proliferation of organelles by fission of pre-existing peroxisomes also indeed contributes to a significant extent to the total peroxisome population in induced yeast cells. This study addresses these questions using the methylotrophic yeast *Hansenula polymorpha* as model organism.

### 2. Materials and methods

#### 2.1. Micro-organisms and growth conditions

The *H. polymorpha* strains used in this study are listed in Table 1. The *dnm1*, *vps1* and *dnm1.pex3* double mutants were obtained by crossing [25] the *dnm1* and *vps1* or *pex3* (leu1.1) mutants. Strain *vps1.pex3* was obtained by crossing *pex3* (ura3) and *vps1*. Diploids were subjected to random spore analysis, and prototrophic segregants were subjected to complementation analysis to determine their genotypes.

Yeast cultures were grown at 37 °C on 1) YPD media containing 1% yeast extract, 1% peptone and 1% glucose, 2) selective media containing 0.67% yeast nitrogen base without amino acids, supplemented with 1% glucose (YNB) or 0.5% methanol (YNM) or 3) mineral media (MM) [26] supplemented with 0.5% glucose or 0.5% methanol as carbon sources, and 0.25% ammonium sulphate or 0.25% methylamine as nitrogen sources. When required, amino acids or uracil were added to a final concentration of 30 μg/ml. For growth on agar plates the medium was supplemented with 2% agar. For the selection of resistant transformants YPD plates containing 100 μg/ml zeocin or 100 μg/ml nourseothricin (Invitrogen, Breda, the Netherlands) were used.

For cloning purposes *Escherichia coli* DH5α was used. Cells were grown at 37 °C in LB [27] supplemented with 100 μg/ml ampicillin when required.

#### 2.2. Molecular techniques

Standard recombinant DNA techniques were carried out essentially according to Sambrook et al. [27]. Transformation of *H. polymorpha* cells [28] and site specific integration in the *H. polymorpha* genome [29] were performed as described. DNA modifying enzymes were used as recommended by the suppliers (Roche, Almere, the Netherlands; Fermentas, St. Leon-Rot, Germany). *Pwo* polymerase was used for preparative polymerase chain reactions (PCR). Oligonucleotides were synthesized by Biolegio (Nijmegen,
The Netherlands). DNA sequencing reactions were performed at BaseClear (Leiden, The Netherlands) using a LiCor automated DNA-sequencer and dye primer chemistry (LiCor, Lincoln, NB). For DNA sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, USA) was used.

2.3. Identification of the H. polymorpha VPS1, DNM1, and PEX11 genes

The H. polymorpha VPS1 gene (GenBankAY780359) and the H. polymorpha PEX11 gene (GenBank DQ645582) were previously identified (Kuravi, K., unpublished data; [20]). To identify the H. polymorpha DNM1 gene the BLAST algorithm [30] was used to screen the H. polymorpha genome [31] for protein sequences showing similarity to the S. cerevisiae Dnm1p. This resulted in a single candidate gene, which was designated H. polymorpha DNM1. The DNM1 ORF consists of 2262 bp that codes for a protein of 753 amino acids with a calculated mass of approximately 84 kDa that shows 61% identity to the S. cerevisiae Dnm1p. The nucleotide sequence of DNM1 is deposited at the GenBank with accession number EF682093.

2.4. Construction of a H. polymorpha dnm1 deletion mutant

A dnm1 deletion strain was constructed by replacing the genomic region of DNM1 comprising nucleotides +341 to +1855 by the auxotrophic marker LEU2. To this end pDEST-DNM1-LEU was made using Gateway® Technology (Invitrogen, Breda, the Netherlands). Two DNA fragments comprising the LEU2 genomic region were obtained by PCR using respectively primers attDNM15F/attDNM15R and attDNM13F/attDNM13R and H. polymorpha genomic DNA as a template. The PCR fragments were cloned into the vectors pDONR P4-1R and pDONR P2-P3, respectively, resulting in the entry vectors pENTR DNM1 5' and pENTR DNM1 3'. Additionally, a DNA fragment comprising the Candida albicans LEU2 gene was obtained by PCR using primers Entr221_LEUca_F and Entr221_LEUca_R and plasmid pB-LEU2Ca as a template. The resulting PCR fragment was recombined into vector pDONR221 resulting in the entry vector pENTR LEU2Ca2. Recombination of the entry vectors pENTR DNM1 5', pENTR LEU2Ca2 and pENTR DNM1 3', and the destination vector pDEST R4-R3, resulted in pDESTDMN1-LEU. Subsequently, H. polymorpha WT leu1::ura3 cells were transformed with the 3 kb DNM1 deletion fragment, which was obtained by PCR using primers Dnm15' and Dnm13', and pDESTDMN1lea as template. Correct integration was confirmed by PCR and Southern blot analysis. The resulting strain was designated dnm1.

2.5. Plasmid construction

Plasmids and primers used in this study are listed in Table 2 and 3. For the construction of plasmid pHl-DsRed-SKL (pSNA03) the 1.2 kb Nsi-I-Smal fragment from pHipZ4-DsRed-SKL was inserted between the NsiI and Smal of pH-lac-FL. For stable integration of the plasmid into the H. polymorpha genome, the plasmid was linearized with SacII in the P<sub>GAL1</sub> region and transformed to various strains.

Plasmid pSNA01 was constructed by amplification of the 1425 bp 3' end of the DNM1 gene, lacking the stop codon, using primers DNM1GFPfw and DNM1GFPrev and H. polymorpha genomic DNA as template. The resulting PCR product was digested with HindIII and BglII, and ligated between the HindIII and BglII sites of pANL31 [32]. The resulting plasmid pSNA01 was linearized with BsrI to enable integration into the DNM1 locus of the H. polymorpha genome. For the construction of pSNA02 the full length DNM1 gene, lacking the stop codon, was obtained by PCR using primers DNM1GFP-Fw and DNM1GFP-Rv and transformed to various strains.

Table 2

<table>
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<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>pHl-Z5</td>
<td>Plasmid containing H. polymorpha AMO promoter and AMO terminator regions; zeo&lt;sup&gt;R&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[28]</td>
</tr>
<tr>
<td>pANL31</td>
<td>Plasmid containing GFP without start codon; zeo&lt;sup&gt;R&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[32]</td>
</tr>
<tr>
<td>pANL29</td>
<td>pHlZ4 containing P&lt;sub&gt;GAL1&lt;/sub&gt;GFP-SKL, amp&lt;sup&gt;R&lt;/sup&gt;, zeo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[32]</td>
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<tr>
<td>pHipZ4-DsRed-TI-SKL</td>
<td>Plasmid containing P&lt;sub&gt;GAL1&lt;/sub&gt;DsRed-SKL, amp&lt;sup&gt;R&lt;/sup&gt;, zeo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[50]</td>
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<tr>
<td>pHlX3-GFP-SKL</td>
<td>Plasmid containing P&lt;sub&gt;GAL1&lt;/sub&gt;GFP-SKL, kan&lt;sup&gt;R&lt;/sup&gt;, LEU2</td>
<td>[51]</td>
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<td>pFEM156</td>
<td>Plasmid containing P&lt;sub&gt;GAL1&lt;/sub&gt;GFP-SKL, amp&lt;sup&gt;R&lt;/sup&gt;, URA3</td>
<td>[48]</td>
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<tr>
<td>pHipZ5-Pex3-GFP</td>
<td>pHlZ5 containing PEX3-GFP, zeo&lt;sup&gt;R&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSNA01</td>
<td>pANL31 containing fusion gene between GFP and C-terminal part of DNM1; zeo&lt;sup&gt;R&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td>pSNA02</td>
<td>pHlZ4 containing PEX3-GFP; zeo&lt;sup&gt;R&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pSNA03</td>
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<td>This study</td>
</tr>
<tr>
<td>pRBG3</td>
<td>Plasmid containing PEX3 deletion cassette; URA3, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[33]</td>
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</table>
| pSNA04       | pRBG3 with URA3 replaced with Ura

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BglII and cloned into the HindIII–BglII digested pANL31 [32] resulting in the plasmid FL-DNM1-GFP. Subsequently, the BamHI–SphI fragment from the plasmid pH1525, containing the P_{AMO} region, was ligated into the BamHI–SphI digested FL-DNM1-GFP plasmid. The resulting plasmid pSNA02 was linearized with NarI in the P_{AMO} region to enable the integration into the H. polymorpha genome.

pEXP-PEX11-GFP was constructed using Gateway® Technology. The PEX11 coding sequence lacking a stop codon was amplified using the primers BB-JK-003 and BB-JK-004 and cloned into the vector pDONR 221. The resulting plasmid pENTR-221-PEX11 was recombined with vectors pDONR-221-AMO and pDONR-221-NAT and vector pDEST-R4-R3-NAT. The resulting expression vector pEX-P11-GFP was then linearized by NarI to enable integration into the H. polymorpha genome.

For the construction of plasmid pHIPvZpEX3-GFP the 2.2 kb BamHI–SmaI fragment from pHOR46 was inserted between the BamHI and PaelI (blunted) of pHIPvZ. The resulting plasmid was linearized with BsiW1 for stable integration into the genome of H. polymorpha.

Integration of pHIPvZ-GFP-SKL [32] and pHIPvZ-DsRed-SKL into the P_{AMO} region of the H. polymorpha genome was achieved by transforming Spbi-linearized plasmid DNA. Specific integration of pHIPvZ-GFP-SKL into the AMO locus was performed by the transformation of BsmI-linearized plasmid DNA.

Plasmid pSNA04 was constructed by replacing the URA3 marker of plasmid pRBG3 [33] with the dominant marker NATI that confers resistance towards nourseothricin. For this replacement, the 1.2 kb BglII–EcoRV fragment from pAG25 [34] was ligated with the 3.7 kb BglII–EcoRV fragment of pRBG3. Subsequently, the deletion cassette obtained by PCR using primers pex3-nat-fw and pex3-nat-rev was used to transform H. polymorpha dnm1.vps1 cells. Correct integration was confirmed by PCR and Southern blot analysis. The resulting strain was designated dnm1.vps1.pex3.

2.6. Morphological analysis

Wide field fluorescence images were made using a Zeiss Axioskop fluorescence microscope [35]. Confocal images and time lapse videos were made using a Zeiss LSM510 confocal laser scanning microscope (CLSM; Zeiss). For quantitative determination of the number of fluorescent spots per cell, images were prepared using wild field fluorescence microscopy or CLSM. Cells were fixed in 4% formaldehyde in 10 mM potassium phosphate buffer at pH 7.5 for 2 h on ice. The number of fluorescent spots was quantified by counting the spots in non-budding, single cells. In each experiment approximately 300 cells were counted (approx. 2 × 150 cells from 2 independent cultures) using ImageJ software (http://rsb.info.nih.gov/ij/download). Statistical differences in average numbers were determined using a Z-test. The kymogram was prepared using ImageJ.

3. Results

3.1. Peroxisomes may proliferate by fission in WT H. polymorpha

In continuation of the ground-breaking recent data that peroxisomes may form in yeast pex3 mutant cells from the endoplasmic reticulum [35–39], we aimed to investigate whether all organelles derive by this process or whether they also may originate by fission of pre-existing ones and, if so, to which extent the two machineries contribute to the total population of peroxisomes per cell. To analyze this, we integrated two
plasmids into the genome of WT *H. polymorpha* cells, containing P"AOX"DsRed-SKL and P"AMO"GFP-SKL, which allows marking peroxisomes red or green, depending on the cultivation conditions. Upon introduction of the P"AOX"DsRed-SKL expression cassette cells produce the red fluorescent protein DsRed containing the C-terminal peroxisomal targeting signal SKL under control of the alcohol oxidase promoter (P"AOX"). P"AMO"GFP-SKL is responsible for the production of peroxisomal green fluorescent protein (GFP-SKL) under control of the amine oxidase promoter (P"AMO").

To analyze peroxisome proliferation, cells were pre-grown on glucose/methylamine (to initiate production of GFP-SKL by the amine substrate). Cells from the early-exponential growth phase of this culture characteristically contained a single peroxisome, marked by GFP fluorescence but lacking DsRed fluorescence (Fig. 1A). These cells were harvested by centrifugation, washed and resuspended for 30 min in fresh mineral medium containing ammonium sulphate in the absence of any carbon source. At these conditions P"AMO" is fully repressed (by ammonium sulphate) and, as established previously, P"AMO"-induced mRNAs will be fully depleted in this time interval [40]. Then the cells were placed in fresh medium supplemented with methanol/ammonium sulphate to induce P"AOX" and thus, DsRed-SKL synthesis and peroxisome proliferation concurrent with full repression of GFP-SKL synthesis (by ammonium sulphate). Upon methanol induction, the cells were analyzed by live cell imaging techniques. Time lapse videos (Video 1; stills in Fig. 2A) of an early stage budding yeast cell demonstrated that the pre-existing GFP-marked organelle present in the cells divided to form an additional organelle. This newly formed, smaller organelle rapidly migrated into the developing daughter cell. In a time frame of 23 min, elongation of the peroxisome is observed prior to actual fission. Fission occurred concomitantly with the initiation of bud formation followed by migration of the smaller new organelle into the bud. The fission event is also evident in the kymogram derived from this video and reinforced by the
observation that in the kymogram the original organelle, after fission, is slightly reduced in size (Fig. 2B).

Quantifications were carried out to analyze the increase in peroxisome numbers in identical cultures during the initial hours after the shift of cells to methanol/ammonium sulphate. Prior to the shift bulk of the cells contained a single green fluorescent spot (Fig. 1). But after 4 h of cultivation on methanol/ammonium sulphate the number of cells containing two peroxisomes significantly increased concomitantly with a reduction of cells containing a single organelle (Fig. 1B). At this stage in virtually all organelles green fluorescence was observed (data not shown). Since most organelles showed green fluorescence upon growth at conditions that GFP-SKL synthesis is fully blocked, we conclude that the increase in number is due to fission of the pre-existing (green) ones (Fig. 1A). The matrix content of the original organelles is generally unevenly distributed over the resulting organelles upon fission, suggesting that peroxisome fission is an asymmetrical process (Fig. 1A; Video 1).

Fig. 3. Peroxisome morphology and numbers in methanol induced cells. Peroxisomes in non-budding WT (A), vps1 (B) and dnm1 (C) cells, marked with GFP-SKL. In WT and vps1 cells the number of peroxisome amounts was generally 3 whereas in dnm1 cells only a single organelle is observed of enhanced size. Upper row: fluorescence images, second row: merged images of fluorescence and phase contrast pictures. Images were taken by wide field fluorescence microscopy. D shows the quantification of peroxisome numbers in WT, vps1, dnm1 and dnm1.vps1 double mutant cells, grown on methanol. Organelle numbers were determined by counting from randomly taken CLSM images. For each sample peroxisomes were counted from 2 × 150 cells from 2 independent experiments. The frequency of cells containing the indicated number of peroxisomes is shown. The bar represents the Standard Error of Mean (SEM).

Table 4

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean±SEM</th>
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<tr>
<td>WT (HF246)</td>
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</tr>
<tr>
<td>vps1.GFP-SKL</td>
<td>2.25±0.05</td>
</tr>
<tr>
<td>dnm1.GFP-SKL</td>
<td>1.11±0.02</td>
</tr>
<tr>
<td>dnm1.vps1.GFP-SKL</td>
<td>1.19±0.03</td>
</tr>
</tbody>
</table>

Average numbers of peroxisomes per cell observed in various methanol-grown H. polymorpha strains are depicted as mean±SEM. Statistical analysis (Z-test) revealed that the differences in average number of peroxisomes in dnm1, dnm1.vps1 cells relative to WT controls were significant (P-values<0.005), whereas that of vps1 cells were not significant. Peroxisomes were marked by GFP-SKL.

3.2. Peroxisome abundance is reduced in dnm1, but not in vps1 cells

Like the S. cerevisiae genome, the genome of H. polymorpha [31] contains three genes encoding dynamin-like proteins, namely Vps1p, Dnm1p and Mgm1p. Dynamin-like proteins have been indicated to play a role in peroxisome proliferation in S. cerevisiae, in particular Dnm1p and Vps1p [8,9]. To analyze the machinery of peroxisome fission in H. polymorpha, we have analyzed the function of Dnm1p and Vps1p in an integrated approach.

Cells of constructed dnm1 and vps1 mutants and WT controls, all producing GFP-SKL to mark peroxisomes, were grown on peroxisome-inducing methanol media and analyzed by fluorescence microscopy. The data, depicted in Fig. 3, show that WT cells generally contained 3 peroxisomes per cell (average 2.40; Fig. 3A, Table 4). In vps1 cells, the morphology and average numbers of peroxisomes were largely similar to those of WT cells (average 2.25, Fig. 3B, Table 4). However, in dnm1 cells the number of peroxisomes was strongly reduced. Most of these cells contained a single enlarged organelle (average 1.11; Fig. 3C, Table 4). Similarly, in dnm1.vps1 cells generally only one peroxisome was present per cell (average 1.19; Table 4). These data convincingly demonstrates the shift in organelle numbers in dnm1 and dnm1.vps1 to predominantly a single organelle per cell relative to WT and vps1 cells (Fig. 3D).

3.3. Dnm1p is present in dynamic punctuate spots that localize to peroxisomes and mitochondria

Baker’s yeast Dnm1p [8] and its mammalian homologue Dip1p [41] have a dual location on mitochondria and peroxisomes. The location of Dnm1p in H. polymorpha was analyzed in cells in which the genomic DNM1 gene was replaced by DNM1-GFP and peroxisomes were labelled by DsRed-SKL. In this strain peroxisome numbers and mitochondrial morphology were like in WT control cells, indicating that the Dnm1p-GFP fusion protein is fully functional (data not shown). Confocal laser scanning microscopy (CLSM) analysis of these cells, in which mitochondria were visualized using Mitotracker Deep Red, revealed that Dnm1p-GFP is present in distinct spots, similar as previously observed in S. cerevisiae [15,16]. Bulk of the Dnm1p-GFP spots were localized to mitochondria; however, co-localization of Dnm1p-GFP spots...
with DsRed-SKL was also clearly evident indicating that *H. polymorpha* Dnm1p is located on both mitochondria and peroxisomes (Fig. 4A). Remarkably, live cell imaging of such cells strongly suggested that Dnm1p-GFP spots were not permanently localized to peroxisomes or mitochondria, but dynamically moved from peroxisomes to mitochondria and vice versa (Video 2; stills in Fig. 4B).

3.4. Peroxisomes in *dnm1* cells form protrusions during budding of cells

Methanol-induced cells of the *H. polymorpha* *dnm1* strain generally contain a single peroxisome per cell (Fig. 3C,D). Fluorescence analysis of *dnm1* cells revealed an aberrant organelle morphology and inheritance process relative to that of WT cells. During bud formation, the organelles in *dnm1* cells form a protrusion that extends into the bud (Fig. 5A). These protrusions may readily measure over 1 μm and stay intact during subsequent bud development. After bud maturation and subsequent cytokinesis the protrusion is divided resulting in the presence of a small peroxisome in the young bud (Fig. 5A; see also Video 3). We have never observed new-formation of additional organelles suggesting that the presence of peroxisomes in *dnm1* cells solely results from fission.

In budding cells, bulk of the peroxisomes (over 75%; data not shown) normally migrated to the neck between mother cell and bud, as in WT cells [35], prior to forming extensions, suggesting that Dnm1p is not essential for trafficking or positioning of the organelles during vegetative cell reproduction [42,43]. Infrequently, as in WT [35], the organelles remained at the anterior site of budding cells, and in these cases formed relatively long extensions (Fig. 5A,B).

To analyze the site of Dnm1p action, we constructed a *dnm1* strain producing Dnm1p-GFP under control of *P_{AMO}*. This strain, that also produced DsRed-SKL under control of *P_{AOX}*, was pre-grown on glucose/ammonium sulphate, conditions at which both the *AOX* and *AMO* promoters are repressed. A shift of such cells to media containing methanol/ammonium sulphate induces peroxisome formation and results in one enlarged peroxisome per cell, characterized by DsRed-SKL, similar as in *dnm1* cells. Methanol/ammonium sulphate-grown cells were subsequently placed in fresh methanol/methylamine media to induce the *AMO* promoter. Within 2 h of cultivation in this media, the first Dnm1p-GFP spots could be observed (Fig. 6A). This initially formed Dnm1p-GFP spot transiently and dynamically localized to the peroxisomal extensions prior to organelle fission. Within a few hours of prolonged cultivation the strain displayed a normal WT phenotype with multiple peroxisomes and Dnm1p-GFP spots per cell.

3.5. *Pex11* is important for the formation of protrusions at the onset of peroxisome fission

We previously cloned and analyzed the *H. polymorpha* *PEX11* gene and generated a *PEX11* disruption strain (Kuravi, K.; unpublished data). Like that observed for other species, in *H. polymorpha* the absence of Pex11p resulted in strong reduction of peroxisome numbers (generally a single enlarged peroxisome per cell; Fig. 6B). Localization studies in a *pex11* strain producing Pex11p-GFP revealed that this protein is exclusively localized to peroxisomes and generally equally distributed over the organellar surface (Fig. 6C).

To investigate the location of Pex11p in *dnm1* cells, we constructed a *dnm1* strain producing Pex11p-GFP. As shown in Fig. 6D, Pex11p-GFP fluorescence is present at the peroxisomal surface, but concentrates at the region where the protrusion emerges from the organelle.
To further establish a role of Pex11p in the formation of peroxisome extensions in dnm1 cells, we constructed a double deletion strain. Cells of this strain contain generally one peroxisome (Fig. 5D), similar as in pex11 or dnm1 cells (Fig. 5C). Interestingly, as demonstrated in this image (Fig. 5C,D), peroxisome extensions are never observed in these cells during budding.

3.6. Dnm1p and Vps1p are not required for the formation of peroxisomes from the ER

Various recent studies revealed that peroxisomes may be re-introduced in yeast pex3 (or mammalian pex16) cells from the ER after re-introduction of the deleted gene [37–39,44]. This raises the question whether Dnm1p is involved in this peroxisome re-introduction process. To analyze this, we constructed a double mutant dnm1.pex3. As expected, cells of this strain were unable to grow on methanol, because of the peroxisome deficiency due to the absence of Pex3p [33]. Subsequently, in these cells we re-introduced PEX3-GFP under control of PAMO and monitored the growth of cells on methanol and peroxisome formation.

After pre-cultivation of dnm1.pex3.Pex3-GFP cells in glucose–ammonium sulphate, thus repressing PAMO, peroxisomes were undetectable (not shown). After a shift of such cells to methanol/methylamine GFP fluorescence was detected after 1 h of cultivation in a single spot (Fig. 7A) that did not proliferate and remained one organelle per cell also after prolonged cultivation for 16 h. These peroxisomes showed the same elongation phenomena during cell division, indicating that they displayed a dnm1 phenotype (not shown). The kinetics of peroxisome re-introduction were similar to pex3.Pex3-GFP cells, but in these cells normal organelle proliferation was observed after 16 h of cultivation in the methanol/methylamine media (Fig. 7D; [38]). In identical experiments using vps1.pex3. Pex3-GFP or dnm1.vps1.pex3.Pex3-GFP cells peroxisomes were also formed (Fig. 7B,C).
From these data we conclude that Dnm1p and Vps1p are not essential for re-introduction of peroxisomes in *H. polymorpha* pex3 cells.

4. Discussion

This paper presents evidence for the role of the dynamin-like protein Dnm1p and Pex11p in the peroxisome fission machinery of *H. polymorpha*. Fission as a mode for peroxisome proliferation has been documented before in both yeast and mammals. The data obtained indicated that the peroxisomal and mitochondrial fission machineries share several proteins, among which Dnm1p/DLP1/DRP3A [8,11,45] and Fis1p [22]. Our present data shows that in *H. polymorpha* Dnm1p, but not Vps1p, is required for peroxisome fission. In *S. cerevisiae* however, Vps1p is the main player in peroxisome fission [9] in conjunction with a role for Dnm1p albeit mainly at peroxisome-inducing growth conditions [8]. This illustrates that the peroxisome fission machinery is not fully conserved in yeast.

In contrast to Pex11p, the location of Dnm1p on peroxisomes of *H. polymorpha* is not permanent and not distributed over the entire organelle, as was evident from fast live cell analysis. The observed fluctuation of Dnm1p-GFP spots between organelles of different types has not been demonstrated before. Organelles containing Dnm1p-GFP spots have been observed that lost the GFP fluorescence but regained it at later stages of cultivation. Dnm1p-GFP spots have also been observed in *S. cerevisiae* and most likely represent multimetric protein complexes [15,16].

It has been well documented that the membrane protein Fis1p is essential to recruit Dnm1p to its target organelle. In a recent study, Kobayashi et al. showed that in mammals Fis1p and Pex11p directly interact and, together with Dnm1p, function in peroxisome fission in mammals [23].

Our observation that Pex11p is enriched at the basis of the peroxisomal extensions in budding *dnm1* cells reinforce the data of Kobayashi et al. and suggest that the three proteins (Dnm1p, Fis1p and Pex11p) are indeed dominant players in the peroxisome fission machinery. The single organelles, present in *pex11* cells did not form protrusions and therefore may be unable to divide.

Recently, an alternative mode of peroxisome biogenesis has been elucidated that involves formation of the organelles from the ER [37,39] or, in *Y. lipolytica*, maturation of the ER-derived organelles via immature peroxisome vesicle fusion processes [46]. Also in *H. polymorpha* ER-derived peroxisome biogenesis has been described [35,38]. As yet, it remains unknown to which extent ER-mediated formation and fission contribute to the total peroxisome population per cell. In mammals, Kim et al., concluded from their excellent fluorescence studies that bulk of the organelles is derived from the ER [44].

However, our current data in *H. polymorpha* convincingly demonstrate that Dnm1p and Vps1p are not involved in ER-mediated peroxisome formation. This, together with the finding that in *dnm1* cells generally a single organelle is maintained, led us to conclude that the fission machinery is the main component in peroxisome proliferation in *H. polymorpha* at peroxisome-inducing conditions (methanol). This is in line with the recent observation that in WT *S. cerevisiae* cells peroxisomes also multiply by fission, and not by de novo formation [47]. In conclusion: the suggestion of Kim et al. that peroxisomes predominantly form from the ER may require reconsideration in that the rate of ER-derived biogenesis versus the increase in organelles via fission probably is to a large extent species dependent [44].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.10.018.

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