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Inhibition of peroxisome fission, but not mitochondrial fission, increases yeast chronological lifespan

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Mitochondria are key players in aging and cell death. It has been suggested that mitochondrial fragmentation, mediated by the Dnm1/Fis1 organelle fission machinery, stimulates aging and cell death. This was based on the observation that *Saccharomyces cerevisiae* Δ*dnm1* and Δ*fis1* mutants show an enhanced lifespan and increased resistance to cell death inducers. However, the Dnm1/Fis1 fission machinery is also required for peroxisome division. Here we analyzed the significance of peroxisome fission in yeast chronological lifespan, using yeast strains in which fission of mitochondria was selectively blocked. Our data indicate that the lifespan extension caused by deletion of *FIS1* is mainly due to a defect in peroxisome fission and not caused by a block in mitochondrial fragmentation. These observations are underlined by our observation that deletion of *FIS1* does not lead to lifespan extension in yeast peroxisome deficient mutant cells.

Introduction

Cells continuously have to cope with factors that affect their viability. Important threats are reactive oxygen species (ROS), which can damage important macromolecules such as proteins, lipids and DNA. Intracellular accumulation of damaged components contributes to aging, a process that can be defined as the deterioration of cells in time, accompanied by a loss of viability. However, ROS can also have positive effects by acting as second messengers that activate pathways aimed at saving the cell from demise.[1]

Eukaryotic cells contain two types of organelles that produce ROS as byproduct of oxidative metabolism, namely mitochondria and peroxisomes. It is generally accepted that the mitochondrion is a key player in aging.[2,3] Recent data however indicate that peroxisomes play a role in this process as well.[4-7]

In respiratory active yeast cells mitochondria form a large network of tubular organelles. However, during aging this network fragments resulting in the formation of multiple spherical organelles.[8] In *Saccharomyces cerevisiae* this process depends on the organelle fission machinery consisting of the GTPase Dnm1, the tail-anchored mitochondrial outer membrane protein Fis1 and the accessory proteins Mdv1 and Caf4. It has been suggested that mitochondrial fragmentation contributes to aging, because deletion of genes encoding proteins of the mitochondrial fission machinery (*DNM1*, *FIS1* or *MDV1*) enhanced the cellular lifespan.[8,9]

However, the Dnm1/Fis1 machinery is not only responsible for mitochondrial fragmentation, but also involved in peroxisome fission.[10,11] So far, the effect of *DNM1*, *FIS1* or *MDV1* deletion on yeast aging were supposed to be the consequence of a defect in mitochondrial fission, but a possible role for peroxisomal fission in aging has not been investigated to date.

Here we reanalyzed the role of the Fis1/Dnm1 organelle fission machinery in *S. cerevisiae* chronological aging focusing on a possible contribution of peroxisome fission to the reported effects. Our data indicate that a defect in peroxisome fission is the major cause of yeast lifespan extension caused by the absence of the Fis1/Dnm1 machinery.

Results

Construction of strains specifically affected in mitochondrial fission

Because in *S. cerevisiae* two dynamin-like proteins, Vps1 and Dnm1, are involved in peroxisome fission,[11] this process is more severely blocked in a Δ*vps1*Δ*dnm1* double deletion strain relative to Δ*vps1* and Δ*dnm1* single deletion strains.[10] Dnm1 is only involved in mitochondrial fission, therefore deletion of *VPS1* does not affect this process (**Fig. 3A**, compare **Fig. S1B**). In order to be able to selectively assess the role of the Fis1/Dnm1-containing

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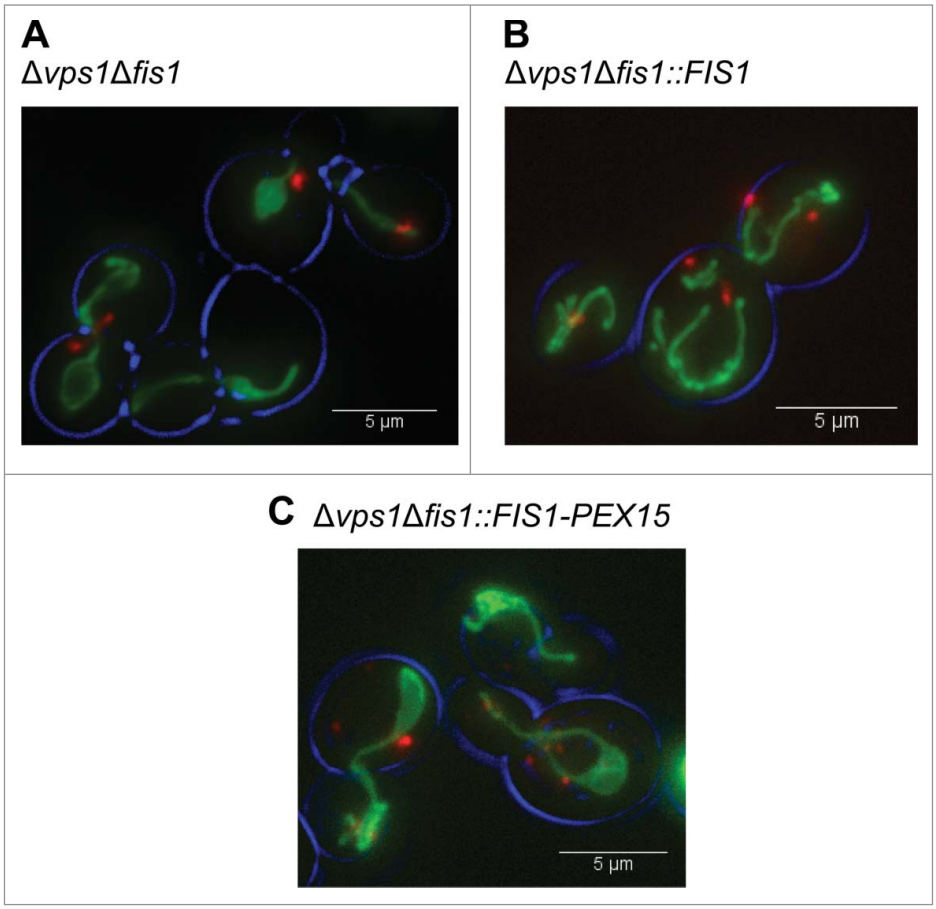

Figure 1. Peroxisome and mitochondrial fission defects in various yeast mutant strains. Fluorescence microscopy images showing mitochondrial and peroxisome morphology in Δ*vps1*Δ*fis1* (**A**), Δ*vps1*Δ*fis1*::*FIS1* (**B**) and Δ*vps1*Δ*fis1*::*FIS1-PEX15* (**C**) cells. Cells were grown until the mid-exponential growth phase on MM containing 2% glucose. Peroxisomes are marked by DsRED-SKL and mitochondria by mitoGFP.

fission machinery in yeast chronological aging, we performed all experiments in a Δ*vps1* background. Because Hughes and Gottschling recently showed that vacuolar defects may affect yeast mitochondrial function and lifespan,[12] we first examined the effect of deletion of *VPS1* on the chronological lifespan yeast. As shown in **Fig. S1A**, the chronological lifespan (CLS) of Δ*vps1* cells did not significantly differ from that of wild-type (WT) cells. Also, Δ*vps1* cells were capable to grow on the non-fermentable carbon source glycerol, indicating that mitochondrial function is not strongly compromised (data not shown).

Previous reports indicated that deletion of *FIS1* in *S. cerevisiae* can result in the acquisition of a secondary mutation in the stress-response gene *WHI2*.[13] To exclude the occurrence of such secondary mutations, all Δ*fis1* strains used in this study were checked for the absence of mutations in *WHI2*.

In line with our previous observations,[10] fluorescence microscopy analysis of Δ*vps1*Δ*fis1* cells revealed the presence of very low peroxisome numbers relative to Δ*vps1* control cells (**Fig. 1A**,**Table 1**). In addition, these cells harbor a collapsed mitochondrial network, which is characteristic for mutants defective in mitochondrial fission (**Fig. 1A**). As expected, upon reintroduction of *FIS1* in Δ*vps1*Δ*fis1* cells (strain Δ*vps1*Δ*fis1*::*FIS1*), the WT mitochondrial morphology was restored and the number of peroxisomes increased to those observed in Δ*vps1* cells (**Table 1**, **Fig. 1B**).

In order to assess the role of peroxisome fission in yeast chronological aging, we constructed a yeast strain that is selectively blocked in mitochondrial fission. Previously, Halbach et al. showed that a fusion protein consisting of the N-terminal, soluble domain of *S. cerevisiae* Fis1 and the C-terminal peroxisomal membrane anchor of Pex15 exclusively sorts to peroxisomes.[14] Moreover, Motley et al. showed that this Fis1-Pex15 fusion protein is able to recruit the Dnm1 fission machinery to yeast peroxisomes.[11] Using the identical construct we confirmed that upon introduction of this Fis1-Pex15 fusion protein in Δ*vps1*Δ*fis1* cells (produced under control of the *FIS1* promoter), the cells still showed collapsed mitochondria, indicative for a mitochondrial fission defect (**Fig. 1C**). However, the number of peroxisomes increased to an average of 3.3 per cell, indicating that peroxisome fission is not blocked anymore (**Fig. 1C**; **Table 1**). The enhanced peroxisome number in this strain relative to the Δ*vps1* control is in line with previous observations[11] and most likely is due to the fact that the entire cellular Fis1 pool is localized to peroxisomes instead of being distributed over both peroxisomes and mitochondria.

The lifespan extension observed in Δ*fis1* cells is restored upon sorting of Fis1 to peroxisomes

Next, we compared the CLS of strains in which fission of mitochondria and peroxisomes was blocked (Δ*vps1*Δ*fis1*), unaffected (Δ*vps1* and Δ*vps1*Δ*fis1*::*FIS1*) or in which only mitochondrial fission was defective (Δ*vps1*Δ*fis1*::*FIS1-PEX15*). CLS experiments revealed no significant differences between the mean lifespans of these 4 strains (**Fig. 2B**). However, deletion of *FIS1* in Δ*vps1* cells (Δ*vps1*Δ*fis1*) significantly increased the maximum lifespan relative to the Δ*vps1* control ($p = 3.10^{-4}$; **Fig. 2B**; **Table 2**). Upon *FIS1* reintroduction (Δ*vps1*Δ*fis1*::*FIS1*) this effect was abolished (**Fig. 2A**; **Table 2**).

Interestingly, no statistically different changes in mean or maximum lifespans were observed when mitochondrial fission was selectively blocked (Δ*vps1* versus Δ*vps1*Δ*fis1*::*FIS1-PEX15* cells) (mean CLS, $p = 0.829$; maximum CSL, $p = 0.064$), whereas the maximum CLS of Δ*vps1*Δ*fis1* was significantly higher than that of

Δ*vps1*Δ*fis1*::*FIS1-PEX15* cells (mean CLS, p = 0.208; maximum CLS, p = 0.001) (**Fig. 2**; **Table 2**). These data suggest that the extension of the maximum lifespan caused by *FIS1* deletion is mainly caused by a block in peroxisome fission (**Fig. 2B**; **Table 2**).

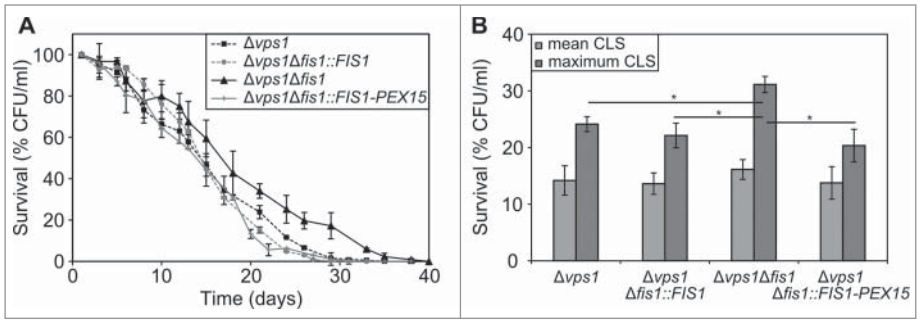

Figure 2. Exclusive sorting of Fis1 to peroxisomes does not result in lifespan extension. (**A**) Chronological lifespans of Δ*vps1*, Δ*vps1*Δ*fis1*, Δ*vps1*Δ*fis1*::*FIS1* and Δ*vps1*Δ*fis1*::*FIS1-PEX15* cells. Data represent mean ± SEM from at least 2 experiments. (**B**) Statistical analysis for mean and maximum lifespans of strains presented in panel A. *, p < 0.001.

Enhanced levels of Fis1 on the peroxisomal membrane do not negatively affect the CLS

The number of peroxisomes is enhanced in Δ*vps1*Δ*fis1*::*FIS1-PEX15* relative to Δ*vps1*Δ*fis1*::*FIS1* (**Fig. 1B**, **Table 1**),[11] most likely due to increased levels of Fis1 protein at the peroxisomal membrane. In order to test whether this influences the CLS, we expressed *FIS1-PEX15* in Δ*vps1* cells and compared the CLS with Δ*vps1* control cells. Indeed, peroxisome numbers increased (2.9 per cell in Δ*vps FIS1-PEX15* relative to 1.3 in Δ*vps1* cells; **Fig. 3A**; **Table 1**) to a value similar as observed in Δ*vps1*Δ*fis1*::*FIS1-PEX15* cells (3.3; **Table 1**). CLS analysis revealed no significant changes in mean or maximum lifespan (mean CLS, p = 0.129; maximum CLS, p = 0.215) (**Fig. 3B**; **Table 2**), indicating that enhanced peroxisomal Fis1 levels or fission do not affect yeast CLS.

Deletion of *FIS1* has no impact on lifespan in Δ*pex3* cells

To further substantiate that a block in mitochondrial fission does not result in an increased CLS, we deleted *FIS1* in Δ*pex3* cells. Pex3 is a peroxisomal membrane protein that is essential for peroxisome biogenesis. Δ*pex3* cells fully lack normal peroxisomal structures and all peroxisomal matrix proteins are mislocalized to the cytosol.[15]

Fluorescence microscopy analysis revealed that Δ*pex3* cells show normal branched mitochondria, which collapse upon deletion of *FIS1* (**Fig. 4A**; **Table 1**). As expected, the peroxisomal matrix marker DsRed-SKL is mislocalized to the cytosol in both Δ*pex3* and Δ*pex3*Δ*fis1* cells (**Fig. 4A**). Chronological aging experiments revealed that both strains show similar mean and maximum lifespans (**Fig 4B**; **Table 2**). This observation indicates that *FIS1* deletion only results in a decreased CLS when peroxisome are present, demonstrating that blocking mitochondrial fission does not increase the CLS in *S. cerevisiae*.

Table 1. Summary of the results of the fluorescence microscopy analyses.

Strain	mitochondria morphology	peroxisome number*
Δ*vps1*	Normal	1.30 ± 0.02
Δ*vps1*Δ*fis1*	Collapsed	0.85 ± 0.05
Δ*vps1*Δ*fis1*::*fis1*	Normal	1.27 ± 0.01
Δ*vps1*Δ*fis1*::*fis1-PEX15*	Collapsed	3.32 ± 0.18
Δ*vps1 fis1-PEX15*	Normal	2.90 ± 0.08
Δ*pex3*	Normal	ND
Δ*pex3*Δ*fis1*	Collapsed	ND

*Mean peroxisome numbers (± standard error of mean).

Discussion

Here we show that a block in peroxisome fission, but not a block in mitochondrial fission, results in an increased chronological lifespan of *S. cerevisiae*.

First, we showed that, like reported before for WT cells, deletion of *FIS1* in a Δ*vps1* background results in an increase in chronological lifespan.[9] This allowed us to specifically study the role

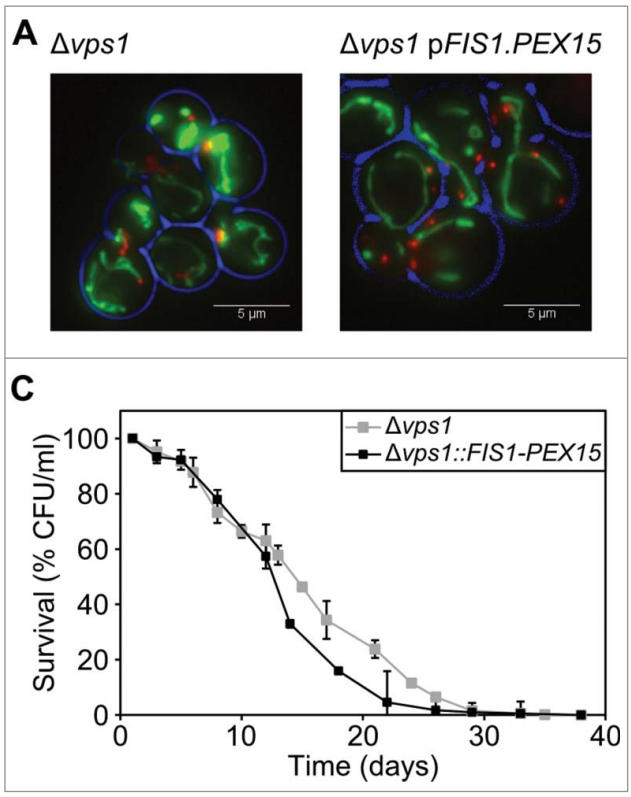

Figure 3. Increased peroxisome fission does not affect the CLS. (**A**) Mitochondrial and peroxisomal morphology in Δ*vps1* and Δ*vps1 FIS1-PEX15* cells. Peroxisomes were labeled with DsRED-SKL and mitochondria by mitoGFP. (**B**) Chronological lifespan analysis of Δ*vps1* and Δ*vps1 FIS1-PEX15* cells. Data represent mean ± SEM from at least 2 experiments.

Table 2. Mean and maximal lifespans.

Strain	Mean CLS (days)	Maximum CLS (days)
Δ*vps1*	14.2 ± 1.45	24.4 ± 1.06
Δ*vps1*Δ*fis1*	16.0 ± 1.22	31.1 ± 0.36
Δ*vps1*Δ*fis1::FIS1*	13.6 ± 1.34	22.1 ± 1.52
Δ*vps1*Δ*fis1::fis1-PEX15*	13.9 ± 1.35	20.3 ± 1.91
Δ*vps1 fis1-PEX15*	12.3 ± 0.35	23.0 ± 2.83
Δ*pex3*	9.9 ± 0.18	16.0 ± 0.00
Δ*pex3*Δ*fis1*	9.6 ± 0.88	16.0 ± 0.00

The mean chronological lifespan is defined as the time point where 50% of the cells are viable. The maximum lifespan is the time point where 10% of the cells are viable. Mean values (± standard error of mean) are presented of at least 2 independent cultures.

of Fis1/Dnm1 mediated peroxisome and mitochondrial fission in yeast aging. In Δ*fis1* Δ*vps1* cells both peroxisome and mitochondrial fission are blocked. By re-introducing a Fis1 variant that is selectively targeted to peroxisomes, we were able to create a strain that was selectively blocked in mitochondrial fragmentation, whereas peroxisomes still divided. Because in these cells the CLS was not extended anymore, we conclude that blocking peroxisomes fission, but not mitochondrial fission increases yeast CLS. Our observation that deletion of *FIS1* does not increase the lifespan of cells lacking peroxisomes (Δ*pex3*) supports the conclusion that the positive effect of *FIS1* deletion on yeast CLS is mainly related to peroxisomes and not to mitochondria.

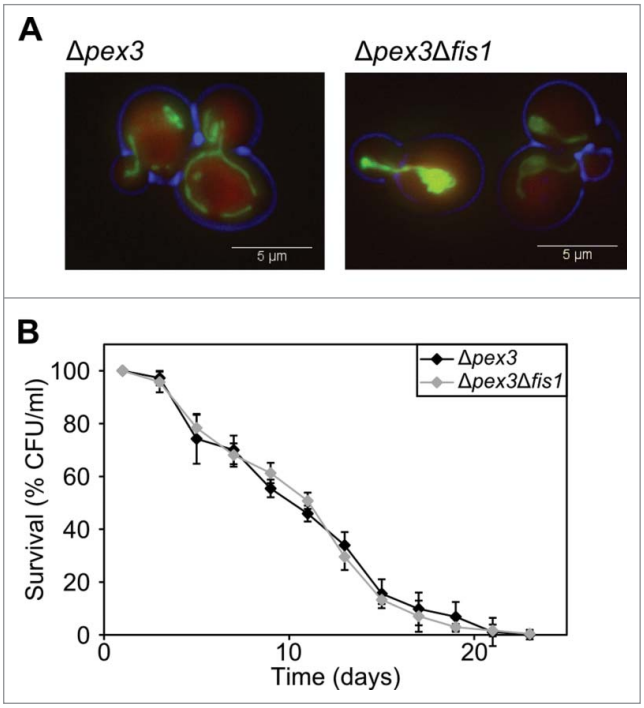

Figure 4. *FIS1* deletion in Δ*pex3* cells has no effect on the CLS. (**A**) Fluorescence microscopy of Δ*pex3* and Δ*pex3*Δ*fis1* cells producing DsRED-SKL or mitoGFP. (**B**). Chronological lifespan experiment of Δ*pex3* and Δ*pex3*Δ*fis1* cells. Data represent mean ± SEM from at least 2 experiments.

Our data reveal that only the maximum CLS and not the mean CLS is enhanced by blocking peroxisome fission. This suggests that peroxisome fission has a pro-aging effect mainly at later stages of chronological aging. We speculate that this may be related to the block in pexophagy which accompanies peroxisome fission defects.[16-18] We previously showed that peroxisomal β-oxidation is important for energy supply during yeast chronological aging,[15] because a reduction in β-oxidation caused by either the absence of an enzyme of this pathway or a defect in peroxisome biogenesis resulted in a decrease in chronological lifespan. Conversely a defect in pexophagy is likely to result in enhanced peroxisomal β-oxidation and thus a higher capacity of the cells to generate energy during chronological aging especially at the later stages.

An alternative explanation may be related to the effect of peroxisome fission on ROS homeostasis. Peroxisomes play an important role in ROS homeostasis and oxidative stress. This is illustrated by the observation that deletion of *S. cerevisiae PEX6*, a crucial gene for peroxisome biogenesis, resulted in enhanced ROS levels during chronological aging ultimately leading to necrotic cell death.[19] Also, the absence of the peroxisomal peroxiredoxin *PMP20* in the yeast *Hansenula polymorpha* caused increased oxidative stress and necrotic cell death.[20] These results suggest that intact peroxisomes containing sufficient levels of peroxisomal antioxidant enzymes may prevent necrosis. The block in pexophagy and/or the presence of enlarged peroxisomes in Δ*vps1*Δ*fis1* cells may result in altered ROS homeostasis, thereby reducing the induction of necrosis and an extension of the CLS in Δ*vps1*Δ*fis1* cells.

Mitochondrial fragmentation has been suggested to be important for selective mitochondrial autophagy (mitophagy). Studies in mammals suggested that fission, fusion and autophagic degradation of mitochondria are important processes to prevent accumulation of dysfunctional mitochondria.[21-24] According to this model mitochondrial fission results in uneven daughter mitochondria thereby separating dysfunctional and functional parts. Subsequently, the dysfunctional organelles are degraded by autophagy whereas the functional ones fuse to form healthy mitochondrial networks. If this model is correct, one would assume that in mammalian cells a block in mitochondrial fission (by *DNM1* or *FIS1* deletion) results in the accumulation of dysfunctional organelles leading to a decreased instead of an enhanced lifespan. In yeast this issue is still controversial, because data have been presented indicating that mitochondrial fission is important for autophagy,[25] whereas other studies suggest that fission is not required for mitophagy.[26]

Summarizing our data indicate that a block in peroxisome fission, but not in mitochondrial fission, enhances yeast chronological lifespan.

Materials and Methods

Strains and growth conditions

The *S. cerevisiae* strains, all derived from wild-type BY4742, are listed in **Table 3**. We sequenced the *WHI2* gene in all strains

Table 3. Yeast strains used in this study.

Strains	Genotype	Reference
BY4742 WT	MATα *his3A1 leu2A0 lys2A0 ura3A0*	Euroscarf
Δ*vps1*	*vps1::KanB*	10
Δ*vps1*Δ*fis1*	*vps1::loxP fis1::loxP*	10
Δ*vps1*Δ*fis1::FIS1*	*vps1::loxP fis1::FIS1*	this study
Δ*vps1*Δ*fis1::FIS1-PEX15*	*vps1::loxP fis1::FIS1-PEX15*	this study
Δ*vps1 FIS1-PEX15*	*vps1::KanB FIS1/FIS1-PEX15*	this study
Δ*pex3*	*pex3::KanMX4*	Euroscarf
Δ*pex3*Δ*fis1*	*pex3::KanMX4 fis1::NatMX4*	this study

Table 5 Primers used in this study.

Primer	Sequence (5′−3′)
FIS1.A	GCATACAGTTCATCCCAGTATTTTT
FIS1.D	CATGCGTAGTTAAACCTTGACTGTA
FIS1up	CACATAGAAGCACAGATCAGAGCACAGCCATACAACATAAGTAT GCTTAACTATGCGGCATCAGAG
FIS1dw	ATTCTTATGTATGTACGTATGTGCTGATTTTTTATGTGCTTGTTAGCG CCCAATACGCAAACC
FIS1.6	CCGGCTTCCAGTCACCACTA
FIS1.9	CGTCCGCGGTGGGTCTAAACCGTATGA
NATrev	GTAAGCCGTGTCGTCAAGAG
POT1.6	AATTCAACGCGTCTGTGAGG
WHI2.1	CGCAAGAAGACAACTCCTTCA
WHI2.2	ACCGTTTTGCCAGTTTCTTG
WHI2.3	AGGGGTCCAATTCTTCTTCAAAT
WHI2.4	TGTGTCTTTGGCCCGATCT

and found no mutations (data not shown). Cells were grown in batch cultures at 30°C, 200 rpm, on mineral media (MM)[27] containing 0.25% ammonium sulfate, 0.05% yeast extract and 2% or 0.5% glucose. MM was supplemented with the required amino acids to a final concentration of 20 μg/ml (histidine) or 30 μg/ml (leucine, lysine, and uracil). For growth on agar plates YPD medium (1% yeast extract, 1% peptone, 1% glucose) was supplemented with 2% agar. *Escherichia coli* DH5α was used for plasmid constructions and cultured at 37°C on LB medium (1% trypton, 0.5% yeast extract, 0.5% NaCl) supplemented with 100 μg/ml ampicillin, when required.

Construction of strains

Plasmids and primers used in this study are listed in **Tables 4 and 5**. Yeast were transformed according to the lithium acetate transformation protocol described by Ito et al. 1983[27] and modified by Hill et al. 1991 and Gietz et al. 1992.[28,29]

pEH107 (*FIS1* ORF) and pEH111.2 (*FIS1-PEX15* hybrid gene) are a gift from A. Motley.[11] Both are 2μ plasmids allowing expression of either full-length *FIS1* or the gene encoding the fusion protein Fis1-Pex15361–383 in which the C-terminal anchor domain of Fis1 is replaced by the one of Pex15.[14] Both genes are under control of the *FIS1* promoter. Because integrative plasmids are more accurate while investigating chronological aging, we subsequently cloned both constructs into an integrative plasmid containing an antibiotic resistance marker. p*FIS1* (pSL35) and p*FIS1-PEX15* (pSL36) were constructed by digestion of pEH107 and pEH111.2 with *Eco*RI/*Hind*III and cloned into pBluescript SK^+. The hygromycin resistance gene from pAG32[30] was then introduced using *Spe*I/*Bam*HI. pSL35 and pSL36 were then linearized using *Bst*BI and transformed into yeast cells allowing genomic integration into the *FIS1* promoter. Positive clones were selected on YPD plates containing 300 μg/ml hygromycin B (Invitrogen). Successful integration by homologous recombination was checked using FIS1.6 + FIS1.D and POT1.6 + FIS1.9 primers (**Table 5**).

FIS1 deletion in Δ*pex3* cells was obtained by replacement of the *FIS1* open reading frame by *NatMX4* gene from pAG25[31] using primers FIS1up and FIS1dw. For the selection of positive clones, YPD plates containing 100 μg/ml nourseothricin (Invitrogen, Carlsbad, CA, USA) was used. Correct integration was checked using Euroscarf primers FIS1.A and FIS1.D and the NATrev primer (Table 5).

Fluorescence microscopy

Cells from fresh plates were grown overnight on MM medium containing 2% glucose. Cultures were diluted at $OD_{600\ nm} = 0.1$ in fresh MM 2% glucose and grown for 4 hours before imaging. Fluorescence images were captured by an inverted microscope (Observer Z1; Carl Zeiss) using AxioVision software (Carl Zeiss) and a digital camera (CoolSNAP HQ^2; Photometrics). All fluorescence images were acquired using a 100x 1.30 NA Plan-Neofluar objective (Carl Zeiss). GFP signal was visualized with a 470/40-nm band pass excitation filter, a 495-nm dichromatic mirror, and a 525/50-nm band pass emission filter. DsRed fluorescence was visualized with a 546/12-nm bandpass excitation filter, a 560-nm dichromatic mirror, and a 575–640-nm bandpass

Table 4. Plasmids used in this study.

Plasmid	Description	Reference
mitoGFP*	pVT100U-mtGFP*, P_{ADH1}-*Su9(1–69)DsRed.T1/URA3*, 2μ	30
mitoDsRED*	pVT100U-mtDsRED, P_{ADH1}-*Su9(1–69)DsRed.T1/URA3*, 2μ	30
DsRED-SKL	pUG34-DsREDskl, P_{MET25}-*DsRED-T1.SKL/HIS3*, 2μ	10
pEH107**	P_{FIS1}-*FIS1*-T_{FIS1}/URA3, 2μ**	11
pEH111.2**	P_{FIS1}-*FIS1-PEX15*-T_{PEX15}/URA3, 2μ**	11
p*FIS1*	pSL35, P_{FIS1}-*FIS1*-T_{FIS1}/*HghMX4*, integrative	this study
p*FIS1.PEX15*	pSL36, P_{FIS1}-*FIS1-PEX15*-T_{PEX15}/ *HghMX4*, integrative	this study

*gift from Dr. Jodi Nunnari (University of California, Davis, USA).
**gift from Dr Alison M. Motley (University of Sheffield, Sheffield UK)

emission filter. Fluorescence image were analyzed using ImageJ software software (US National Institutes of Health, Bethesda, MD, USA). Pictures presented here are z-projection of stacks resulting in complete overview of organelles morphology/number. The number of peroxisomes per cell was calculated from 2 different clones grown at the same conditions and the results presented are mean values and standard error of mean.

Chronological lifespan measurements

Overnight cultures were grown in MM medium containing 0.5% glucose and diluted twice at $OD_{600\ nm} = 0.1$ in fresh MM and grown for 8 hours. After the last pre-cultivation step, cells were diluted in final fresh MM. Survival was assayed by determining the colony forming units (CFUs) after 2 d of incubation at 30°C on YPD agar plates. Twenty-four hours after the last dilution (D1) was considered as 100% of survival. The results shown are mean values and standard error of mean. At the end of each aging experiments, the *WHI2* gene was amplified using primers WHI2.1 and WHI2.4 and sequenced with primers WHI2.2 and WHI2.3 to check appearance of a random mutation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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