Spatial and temporal dynamics of budding yeast mitochondria lacking the division component Fis1p

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Summary

The mitochondrial compartment of budding yeast (*Saccharomyces cerevisiae*) is a highly dynamic net-like structure of tubules that constantly undergo fusion and fission. The outer membrane protein Fis1p plays a crucial role in mitochondrial fission. Here we report on the temporal and spatial dynamics of this organelle in wild-type cells and in *fis1Δ* mutants. Mitochondria of *fis1Δ* mutants adapt their mitochondrial network to a change in carbon source. We find that the frequencies of apparent matrix separation and fusion events decrease in both wild-type cells and in mutants lacking Fis1p upon glucose repression. Matrix separation could be caused by matrix constriction and does not necessarily require fission of the inner or outer membrane. Double-labelling experiments demonstrated that some of these matrix separations in *fis1* mutants are due to genuine tubule fissions, whereas others do not involve fission of the outer membrane. The rates of matrix separation in *fis1Δ* mutants almost approach those of the wildtype, demonstrating that, although apparently involved in outer membrane fission, Fis1p is not crucial for the separation of the mitochondrial matrix. In mutants lacking the GTPase Dnm1p no complete tubule fissions were recorded, although *dnm1Δ* mutants display matrix separations as well. The data suggest that different molecular machineries are responsible for the separation of the matrix and the fission of the outer membrane in budding yeast.

Key words: *Saccharomyces cerevisiae*, Fluorescence microscopy, Glucose repression, Membrane fission, Organelle morphology

Introduction

Mitochondria carry out numerous essential metabolic reactions and constitute the cellular centres for energy production via oxidative phosphorylation (Scheffler, 2001 and references therein). They are polymorphic structures, constantly adjusting their shape to the metabolic requirements of the cell. In the yeast *Saccharomyces cerevisiae* the mitochondrial compartment forms an extended tubular network located at the cell cortex (Egner et al., 2002; Hoffmann and Avers, 1973; Stevens, 1981; Stevens, 1977). The establishment and maintenance of this reticulate structure has been proposed to require a balanced frequency of mitochondrial fusion and fission events (Bleazard et al., 1999; Nunnari et al., 1997; Sesaki and Jensen, 1999).

The dynamin-related GTPase Dnm1p is a key component of the yeast mitochondrial fission machinery (Bleazard et al., 1999; Otsuga et al., 1998; Sesaki and Jensen, 1999). It interacts with the WD40-repeat protein Mdv1p (Cerveny et al., 2001; Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Tieu et al., 2002). Dnm1p colocalizes with Mdv1p in punctate structures on the mitochondrial outer membrane. These structures have been proposed to mediate mitochondrial membrane constriction and/or division. Assembly and proper distribution of Dnm-p/Mdv1p complexes on the mitochondrial surface depend on Fis1p, an integral component of the outer membrane (Mozdy et al., 2000; Tieu and Nunnari, 2000). Similar to *dnm1*and *mdv1* mutants (Mozdy et al., 2000; Otsuga et al., 1998; Tieu and Nunnari, 2000), *fis1* mutant cells frequently display fenestrated mitochondria that are often reminiscent of miniaturized fishing nets. It has been suggested that these fenestrated mitochondria are generated because mitochondrial fission is severely compromised in these mutants while fusion is still going on (Jensen et al., 2000; Shaw and Nunnari, 2002; Yaffe, 1999).

Two outer membrane proteins, Fzo1p and Ugo1p, are involved in the fusion of mitochondria (Hermann et al., 1998; Rapaport et al., 1998; Sesaki and Jensen, 2001). Yeast mutants lacking functional Fzo1p contain fragmented mitochondria, presumably because fusion is blocked while fission is going on. Remarkably, double mutants of *fzo1* and *fis1* display tubular or net-like mitochondria rather than fragmented organelles (Mozdy et al., 2000; Tieu and Nunnari, 2000). This finding led to the proposal that deletion of the *FIS1* gene prevents fragmentation of mitochondria in *fzo1* mutants by blocking the fission pathway, which further supports a central role for Fis1p in mitochondrial fission.

Mitochondria as double-membrane-bounded organelles have to fuse and divide their inner and outer membranes in a coordinated manner (Westermann, 2002). It has been proposed that the outer membrane fusion machinery is in contact with yet unknown factors in the inner membrane (Fritz et al., 2001). Recently, the inner membrane protein Mdm33p has been

suggested to be involved in inner membrane fission (Messerschmitt et al., 2003). To date, the functional characterization of components of the mitochondrial fission machinery in yeast has relied mostly on genetic and biochemical data and observations of morphology mutants under steady-state conditions. Owing to the dynamic nature of mitochondria, important aspects of mutant phenotypes have most probably been overlooked.

Here, we report on a detailed analysis of mitochondrial dynamics resolved in time and space, focusing on wild-type and *fis1*Δ mutant cells. Using confocal microscopy, the dynamical behaviour of mitochondria labelled with GFP targeted to the matrix has been followed over a time period of up to 5 hours. To follow mitochondrial changes over time, we employed the structural adaptations of mitochondria occurring upon the exchange of the non-fermentable carbon source glycerol by glucose in the growth medium. The three-dimensional confocal time-lapse data sets provide insight into the complex structural adaptations of mitochondria, enabling us to accurately count apparent matrix separation and fusion events. We provide evidence that some of these matrix separations in *fis1*Δ mutants are due to genuine tubule fissions, whereas others do not involve fission of the outer membrane. The analysis of mitochondrial dynamics using four-dimensional microscopy gives new insight into the function of proteins involved in mitochondrial fission that would have been missed otherwise.

Materials and Methods

Yeast strains and growth conditions

Growth and manipulation of yeast was carried out according to standard procedures (Sherman, 1991). Cells were routinely grown in yeast extract-peptone-glucose medium (YPD) (1% yeast extract, 2% peptone, 2% glucose), yeast extract-peptone-glycerol medium (YPGlycerol) (1% yeast extract, 2% peptone, 3% glycerol) or in peptone-minimal-galactose medium (PMGal) (1.7 g/l yeast nitrogen base (Difco, USA), 20 mg/l adenine sulfate, 5 g/l ammonium sulfate, 5 g/l peptone 140 (Gibco BRL, UK), 2% Galactose) at 30°C. All strains used in this study were isogenic to BY4743 (Brachmann et al., 1998). Strains used were as follows: BY4743 (wildtype): *MAT**a**/MATα; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; met15Δ0/MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0*. Y31458 (*fis1Δ*):*MAT**a**/MATα; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; fis1::kanMX4/fis1::kanMX4* (Giaever et al., 2002). Y31489 (*dnm1Δ*): *MAT**a**/MATα; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; dnm1::kanMX4/dnm1::kanMX4* (Giaever et al., 2002). Deletion strains were obtained from EUROSCARF (Frankfurt, Germany). Disruptions were confirmed by polymerase chain reaction (PCR).

Plasmid constructions

Standard cloning procedures were used (Sambrook et al., 1989). PCR was performed using Pfu polymerase (Stratagen, La Jolla, USA) according to the manufacturer's instructions. For labelling the matrix with GFP the plasmid pVT100U-mtGFP was employed. This plasmid, containing a DNA fragment encoding GFP fused to subunit 9 (aa1-69) of the F_0-ATPase of *Neurospora crassa* under control of the constitutive alcohol dehydrogenase promoter, is described elsewhere (Westermann and Neupert, 2000). The plasmid pHS12-DsRed.T4 encoding pCox4-DsRed fusion proteins is published elsewhere (Bevis and Glick, 2002). To label the outer membrane with GFP, pAS43 was constructed. This vector, a *2μ-URA3* plasmid that expresses OM45p-GFP (Cerveny et al., 2001), was constructed as follows. A 1223 base pair (bp) DNA fragment encoding the OM45 ORF and 41 bp of upstream sequences was amplified from yeast genomic DNA using the oligonucleotides 5′-GCGAAGCTTGGCCAGTAACGTTAATCA-3′ and 5′-GCGGGTACCGTCCTTTTTCGAGCTCCA-3′. The PCR fragment was digested with *Hin*dIII and *Kpn*I and inserted into pVT100U-mtGFP (Westermann and Neupert, 2000) replacing the Su9 presequence. To label the matrix with DsRed the plasmid pSJ55 was constructed. For this vector the coding sequence of DsRed.4 was amplified by PCR using pHS12-DsRed.T4 (Bevis and Glick, 2002) as a template. The PCR fragment was inserted into pYX142-mtGFP (Westermann and Neupert, 2000) replacing the DNA sequence encoding GFP, resulting in a vector encoding Su9(1-69)-DsRed.4 under control of the constitutive triosephosphate isomerase promoter (TPI). Finally the TPI- Su9(1-69)-DsRed.4 cassette was inserted into the *Sma*I restriction site of the *2μ*-based vector pRS323 (Sikorski and Hieter, 1989), resulting in pSJ55.

Beam-scanning confocal microscopy

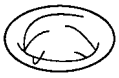
For Fig. 1A-C, Fig. 2A-C and Fig. 3A-C, cells were collected by centrifugation, resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) and immobilized in PBS with 1% low melting point agarose on a microscope slide. For Fig. 3D-J cells were grown in PMGal medium to mid-log phase and placed in a chamber as described for time-lapse confocal microscopy (see below). For image acquisition a beam-scanning microscope (Leica TCS SP2, Leica Lasertechnik, Heidelberg, Germany) equipped with a 1.4 numerical aperture oil immersion lens (Leica 100X, Planapo, Wetzlar, Germany) was employed. GFP- and DsRed-expressing cells were imaged as described previously (Jakobs et al., 2000). All imaging was performed at ambient temperature (~22°C).

Time-lapse confocal microscopy

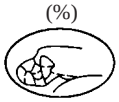
Cells expressing mtGFP were grown in YPGlycerol to mid-log phase and subsequently transferred to YPD. Following further incubation in YPD at 30°C (45 minutes, wildtype or 70 minutes, deletion mutants) cells were harvested. They were embedded in a solution of one part YPD with two parts PBS and 1% low melting point agarose and filled in a microscopic chamber. For the duration of the experiment, the chamber was constantly flushed with YPD (~100 μl/minute) and kept at ambient temperature. Images were acquired digitally with a multi-focal single-photon excitation confocal system (UltraView confocal scanner; PerkinElmer, Boston, USA) attached to an inverted Leica DMIRBE microscope. Three-dimensional image data of the mitochondrial compartment were acquired at each time point. The three-dimensionality of the data permitted the recognition of matrix separation and fusion events, some of which would have been missed otherwise (in case of separation) or spuriously included (in case of fusion). Data collection was carried out with a 100×/1.40 oil immersion lens (Leica, Planapo, Wetzlar, Germany) and a cooled CCD camera (Imager, LaVision, Göttingen, Germany). The shutters, stage motion and image acquisition were computer controlled. Three-dimensional stacks of mitochondria were acquired by recording focal plane images (xy) and moving the stage in 0.25 μm steps along the optic axis. Three dimensional datasets of mitochondria from *fis1*Δ and *dnm1*Δ cells were deconvolved by non-linear image restoration using a maximum likelihood algorithm (Lucy, 1974; Nagorni and Hell, 2001; Richardson, 1972). In addition to providing a slightly higher resolution this algorithm is particularly suitable for disproving disconnections in mitochondrial tubules with weak GFP fluorescence. For collecting the images of wild-type mitochondria we used an exposure time of 550 milliseconds; for the deletion mutants the exposure time was 1000 milliseconds. Quadratic focal plane pixel areas were 0.04 μm^2 and 0.01 μm^2 for wild-type and

Table 1. Mitochondrial morphology of wild type and *fis1*Δ cells

Wildtype

Growth medium	Highly branched (%) 	Intermediately branched (%) 	Simple network (%) 
YPGlycerol (*n*=340)	89	10	1
YPD (*n*=115)	0	5	95

***fis1*Δ**

Growth medium	Large fenestrated net (%) 	Small net (%) 	Tubular structure (%) 
YPGlycerol (*n*=1085)	23	28	49
YPD (*n*=495)	6	10	84

mutant cells, respectively. Individual stacks were recorded every 78 seconds (wild-type cells), 116 seconds (*fis1*Δ) or 60 seconds (*dnm1*Δ). For presentation, maximum intensity projections were generated, that is, optical planes were added along the optic axis. With the exception of contrast stretching, no further image processing was employed.

Results

Cells lacking the mitochondrial division component Fis1p adapt mitochondrial networks to the carbon source

Yeast wild-type cells adapt to the carbon source of the growth medium by substantial changes in the energy metabolism (Gancedo, 1998 and references therein) and by modifying the size and degree of ramification of their mitochondria (Egner et al., 2002; Pon and Schatz, 1991; Stevens, 1977). The formation and maintenance of the mitochondrial networks require a balanced frequency of fission and fusion (Nunnari et al., 1997). A change from glycerol to glucose in the growth medium is an easy way to induce modifications of the overall structure of the mitochondrial network. We assumed that these adaptations also require mitochondrial fission and fusion. Therefore, we investigated whether *fis1*Δ mutant cells, which are proposed to be deficient in mitochondrial fission, also adapt the morphology of their mitochondria to different carbon sources. *fis1*Δ cells expressing matrix-targeted GFP were grown to log phase in media containing either a non-fermentable (glycerol) or a fermentable (glucose) carbon source. Subsequently, organellar morphologies were compared. To quantify the different facets of *fis1*Δ mitochondria, we introduced the following arbitrary, but clearly distinguishable, morphological categories: large fenestrated mitochondrial nets without prominent emerging tubules, small fenestrated nets with large emerging tubules, and tubular mitochondrial structures with very small or no fenestrated nets. Sketches of these morphological categories are depicted in Table 1. On glycerol about half of the *fis1*Δ cells displayed recognizable (large or small) fenestrated mitochondria (Table 1). The share of fenestrated mitochondria was reduced to 16% when *fis1*Δ cells were grown on glucose. Cells displaying mitochondria with a tubular structure, the predominant morphology in glucose, were also abundant in the glycerol-grown population. Hence, *fis1*Δ cell populations grown in glycerol or glucose were heterogeneous with respect to their mitochondrial morphologies, although the two populations were clearly discernible. The structure of the mitochondria of *fis1*Δ cells is adapted to the carbon source.

90% of the mitochondria of wild-type cells grown on glycerol were highly branched, whereas on glucose more than 90% of the organelles were simple networks (Table 1). In contrast to *fis1*Δ cells, the structure of wild-type mitochondria was largely homogenous on the same carbon source.

Carbon-source-dependent simplification of mitochondria is delayed in *fis1*Δ cells

As *fis1*Δ mutants have been proposed to be deficient in mitochondrial fission, it was surprising to discover similar carbon-source-dependent morphology adaptation processes in *fis1*Δ and wild-type mitochondria. To identify a possibly subtle effect of a lack of Fis1p on the restructuring process of the organelle, the adaptation process of mitochondria was followed over time. Unsynchronised cultures of wild-type cells were grown to mid-log phase in glycerol-containing medium, transferred to glucose-containing medium and mitochondrial networks were examined by conventional fluorescence microscopy at various time points (Table 2). As shown above, in glycerol-containing medium, the vast majority of wild-type mitochondria could be attributed to the highly branched mitochondria type. After merely 1 hour in glucose-containing medium, only about one third of the cells contained a highly branched mitochondrial network. After three hours the share of highly branched networks was less than 10% (Table 2).

A corresponding analysis of the *fis1*Δ strain was performed (Table 2). For the analysis the same categories of *fis1*Δ

Table 2. Mitochondrial morphology of wild type and *fis1*Δ cells following exchange of carbon source

Wildtype

Growth condition	Highly branched (%)	Intermediately branched (%)	Simple network (%)
Log-phase (*n*=340) in YPGlycerol	89	10	1
1 hour in YPD (*n*=340)	31	54	15
2 hours in YPD (*n*=345)	20	61	19
3 hours in YPD (*n*=340)	8	57	35

***fis1*Δ**

Growth condition	Large fenestrated net (%)	Small net (%)	Tubular structure (%)
Log-phase (*n*=440) in YPGlycerol	22	30	48
1 hour in YPD (*n*=460)	22	30	48
2 hours in YPD (*n*=450)	19	31	50
3 hours in YPD (*n*=470)	14	30	56
4 hours in YPD (*n*=465)	9	28	63

mitochondria as above were used. In contrast to the wild-type situation the distribution of mitochondrial morphologies within the *fis1*Δ population remained almost unchanged for two hours after transfer to glucose. However, after four hours, more than half of the large fenestrated nets had been converted to more simple structures. Hence, structural adaptation of the mitochondrial compartment induced by a change of the carbon source is markedly delayed in cells lacking Fis1p.

Temporal dynamics of the restructuring process of the wild-type mitochondrial network

To obtain three-dimensional information on the dynamics of the mitochondrial compartments we employed confocal time-lapse microscopy. Wild-type cells harbouring mitochondrial matrix-targeted GFP were grown to log phase in glycerol-containing medium, transferred to glucose-containing medium and incubated for an additional 45 minutes. After this time, the majority of mitochondrial networks were expected in the initial phase of the restructuring process (compare Table 2). For all experiments described in this study the cells were kept in a microscope chamber at ambient temperature. The chamber was continuously flushed with medium to ensure a constant nutrient supply. Since we employed matrix-targeted GFP, our observations of the mitochondrial networks were restricted to the observation of the matrix compartment. In this context we use the term ‘separation’ to describe an apparent discontinuity of the GFP-labelled matrix compartment. Such a separation could be due to a local matrix constriction, a fission of the inner membrane or a fission of both inner and outer membranes. We use the term ‘tubule fission’ to describe complete fission of both membranes.

To follow the restructuring process we chose wild-type cells displaying highly ramified mitochondrial morphologies. We followed shape changes in the mitochondrial network in a total number of 15 wild-type cells. Nine of these cells budded during the imaging period. In each of these dividing cells we observed an unambiguous simplification of the mitochondrial network. The remaining six cells did not bud during the observation period. In spite of ongoing matrix separations and fusions, mitochondria of these cells did not simplify. The latter observation suggests a connection between organellar remodelling events and the cell cycle in wild-type cells.

The overall pattern of mitochondrial metamorphoses was similar in all examined budding wild-type cells. Representative cells imaged at various intermediate stages of the process are displayed in Fig. 1A-C. A newly formed daughter cell was always invaded by a mitochondrial tubule shortly after emergence of the bud. The invading tubule appeared to move without restrictions within the bud for about 10-15 minutes. This was followed by attachment to one site of the cortex of the daughter cell. During subsequent outgrowth of the bud the mitochondrion remained attached to this point, whereas the remaining mitochondrial tubules continued to move freely. Tubules connecting the mitochondrial compartments of mother and daughter cells frequently underwent fusion and fission (Fig. 1D-I, Movie 1and Movie 2, available at jcs.biologists.org/supplemental). The amount of mitochondrial tubules in the bud appeared to increase after each fission event, suggesting that mitochondrial tubules were efficiently transported from the mother into the daughter cell. Movement of mitochondria into the bud was paralleled by ongoing matrix separation and fusion in the mother cell. In addition, numerous tubule fissions that were followed by fusion at a different site were recorded (e.g. Fig. 1D-F, upper arrows). It is conceivable that the latter separation and fusion events were responsible for simplification of the network within the mother cell.

Remodelling of the fenestrated *fis1*Δ mitochondrial network is accompanied by mitochondrial tubule fusion and fission

To analyse whether tubule fission in mutants lacking Fis1p occurs upon glucose repression as seen in wild-type cells, we used the same experimental set-up as described above.

For time-lapse analysis we chose cells that displayed large fenestrated nets. We followed mitochondrial shape changes in a total number of 17 *fis1*Δ cells, some of which were observed for more than 3 hours. In this context we will use the term ‘simplification’ to describe a size reduction of the fenestrated *fis1*Δ net concomitant with an enlargement of long tubules connected to the net. Three of the observed cells budded during the imaging period and simultaneously simplified the mitochondrial compartment. Seven cells displayed a marked simplification of the mitochondrial network without any signs of budding. Hence in cells lacking Fis1p simplification appears to be partly disconnected from the cell cycle. In case of budding, invasion of mitochondria into the bud (Fig. 2A-C) followed a pattern similar to that of wild-type cells. An emerging bud was invaded by a mitochondrial tubule that remained connected to the fenestrated net of the mother cell. Later, the tubule was attached to one point at the cell cortex of the bud where it remained fixed during outgrowth of the daughter cell.

The simplification process upon glucose repression followed a similar pattern in all examined *fis1*Δ cells, irrespective of budding (Fig. 2D-I, Movie 3 and Movie 4, available at jcs.biologists.org/supplemental). Surprisingly, we observed

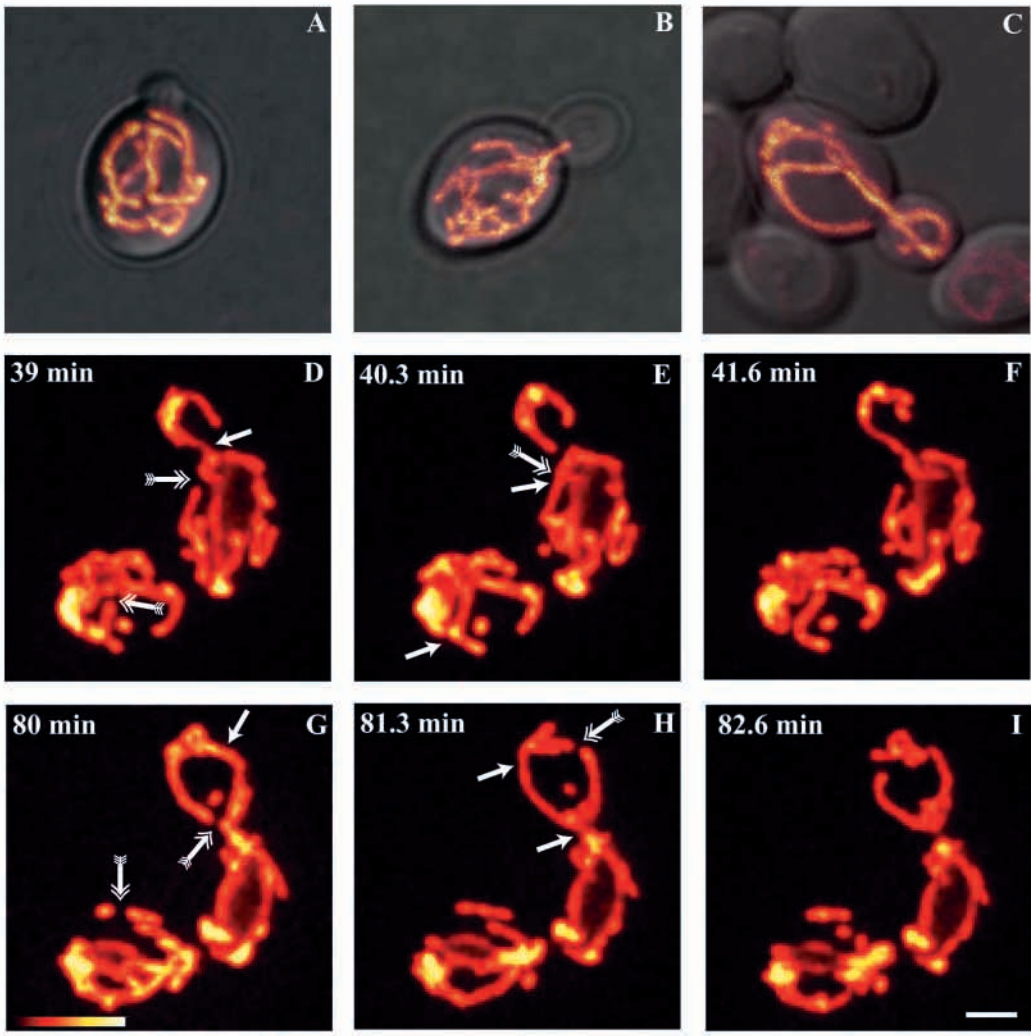


Fig. 1. Remodelling of mitochondria of wild-type cells upon glucose repression. Wild-type cells expressing matrix targeted GFP were grown in glycerol to log phase and transferred for 45 minutes into glucose-containing medium. (A-C) Beam-scanning confocal images of representative budding cells. Maximum intensity projections of several optical planes of the fluorescence signal overlaid with a bright field transmission image are displayed. (D-I) Confocal time-lapse microscopy recording the reorganization of the wild-type mitochondrial network upon glucose repression. Selected maximum intensity projections are displayed. The underlying data-stack comprises 165 three-dimensional stacks with ~4000 single optical sections. Solid arrows indicate sites of future matrix separation. Feathered arrows indicate future fusion sites. The mitochondrial network had a morphology characteristic of cells grown in glycerol at time point zero. Bar, 2 μm. The complete dataset is available in two movies (Movie 1 and Movie 2 at jcs.biologists.org/supplemental).

frequent separation and fusion events of the GFP-labelled matrix. In many cases matrix separations were followed by fusions occurring at the same site of the organelle (Fig. 2G, lower arrow). We also recorded some tubule fission events that were either followed by fusion events occurring at a different site (Fig. 2G-I, upper arrows) or that were not followed by re-fusion at all (Fig. 2D). Separation and fusion events were also detected within the core of the fenestrated nets (Fig. 2E).

Matrix separation can occur in the absence of outer membrane fission in *fis1*Δ mutants

We assumed that separations followed by re-fusion were due to a constriction of the matrix or fission of the inner membrane without involving fission of the outer membrane. To test this hypothesis we labelled the outer mitochondrial membrane with GFP fused to the C-terminus of the mitochondrial outer membrane protein OM45 (OM-GFP) (Cerveny et al., 2001) and simultaneously expressed DsRed fused to the presequence of subunit 9 of the F_0-ATPase of *Neurospora crassa*. This presequence targets DsRed to the mitochondrial matrix (data not shown). Mitochondria of cells grown in medium containing galactose as a carbon source were imaged. Generally we found a strict colocalization of the OM-GFP and the DsRed matrix label. However, occasionally we observed continuous tubules as proven by the OM-GFP label, which enclosed separated matrix compartments (Fig. 3A-C). This finding demonstrates that a separation of the matrix without fission of the outer membrane can occur in *fis1*Δ mutants. Hence, matrix separation and outer membrane fission appear to be separable processes. We note that the term matrix separation does not necessarily imply a fission of the inner membrane. A matrix separation could also be the result of a mere matrix constriction with a continuous inner membrane.

Complete tubule fission is not abolished in *fis1*Δ mutants

Tubule fissions that were not followed by fusion demonstrated that complete mitochondrial fissions occur in *fis1*Δ mutants (Fig. 2). To validate this finding we employed mitochondria labelled at the outer membrane with OM-GFP and at the inner membrane with DsRed fused to the presequence of Cox4p (Bevis and Glick, 2002). Biochemical analysis revealed that the latter fusion protein is directed to the inner membrane (data not shown). In time-lapse series of galactose-grown cells double-labelled with these constructs, we recorded simultaneous separations of the outer and the inner membrane (Fig. 3D-J).

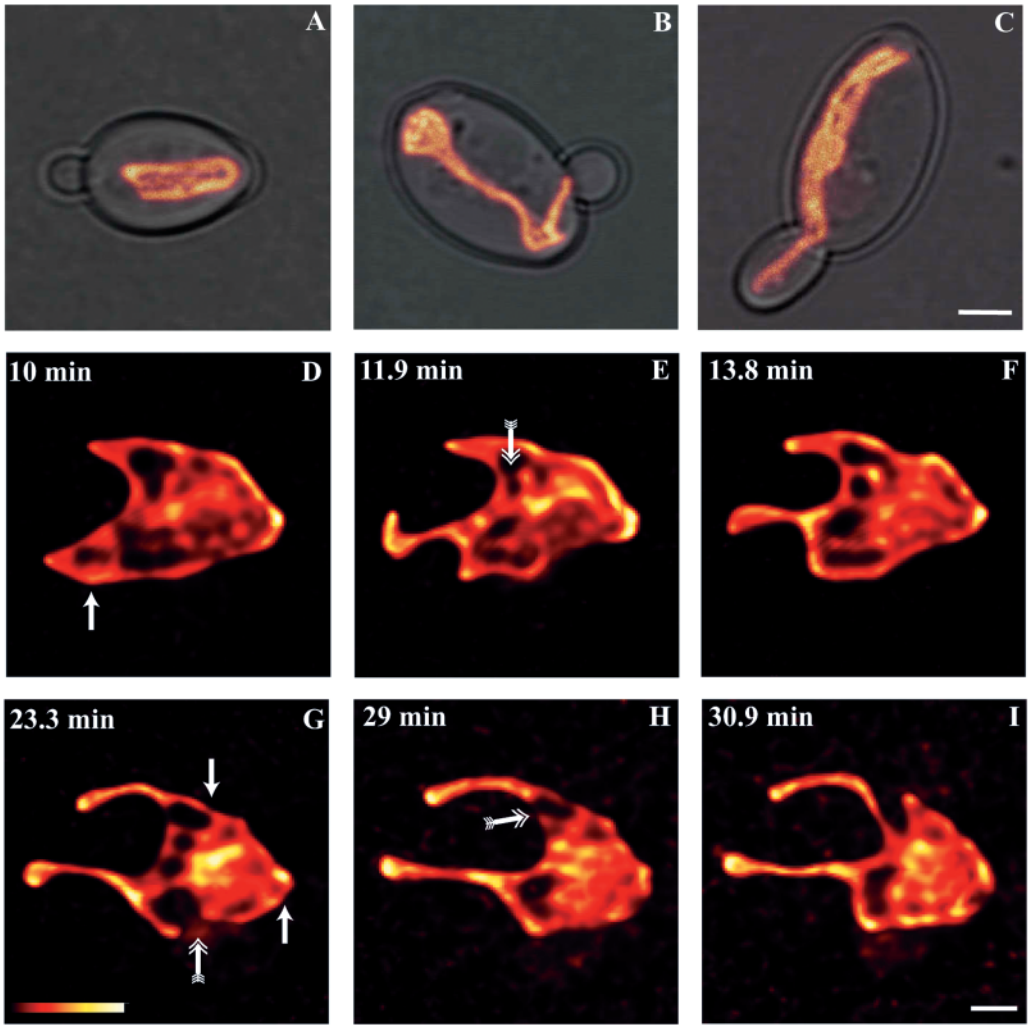


Fig. 2. Remodelling of mitochondria of *fis1Δ* cells upon glucose repression. *fis1Δ* mutants expressing matrix-targeted GFP were grown in glycerol to log phase and transferred for 70 minutes into glucose-containing medium. (A-C) Beam-scanning confocal images of representative budding *fis1Δ* cells as in Fig. 1A-C. (D-I) Confocal time-lapse microscopy recording the reorganization of the *fis1Δ* mitochondrial network upon glucose repression. Selected maximum intensity projections are displayed. The underlying data-stack comprises 99 three-dimensional stacks with ~2200 single optical sections. Solid arrows indicate sites of future matrix separation. Feathered arrows indicate future fusion sites. At time point zero, the mitochondrial compartment resembled a large fenestrated net. Bars, 2 μm (A-C) and 1 μm (D-I). The complete dataset is available in two movies (Movie 3 and Movie 4 at jcs.biologist.org/supplemental).

This demonstrates that in the absence of Fis1p complete tubule fissions involving both membranes can occur.

The frequency of matrix separation and fusion events is similar in *fis1*Δ and wild-type mitochondria

Next, we counted the number of separation and fusion events in single wild-type and *fis1*Δ cells during glucose-repression-induced remodelling of the organelle. We recorded three-dimensional stacks every 78 or 116 seconds (wild-type or *fis1*Δ cells, respectively). By scrutinizing each optical section within a three-dimensional stack consisting of about 20-25 single optical sections, we recorded all recognizable matrix separation and fusion incidents in the mother cells. As we might have overlooked some events, especially in the core of fenestrated *fis1*Δ mitochondria, we might have slightly underestimated the frequency of separation and fusion.

We performed a detailed analysis based on the data stacks of a wild-type cell and a *fis1*Δ cell depicted in Fig. 1D-I and Fig. 2D-I. For both cells, the ratio of matrix separation to fusion was balanced. We counted 104 separation and 103 fusion events in the wild-type cell, and 85 separation and 80 fusion events in the *fis1*Δ cell over a time frame of 105 minutes (Fig. 4A). In the wild-type cell the number of matrix separation and fusion events per minute decreased from 2.5 to 0.6 during remodelling. Similarly, the frequencies of events in the mitochondrial compartment of the cell lacking Fis1p decreased from 2.4 to 1.2 separation and fusion events per minute during simplification (Fig. 4A).

To verify whether the analysed cells were representative we counted separation and fusion events in three additional cells at characteristic time periods during glucose adaptation (Fig. 4B,C). Similar numbers of separation and fusion events were counted; the frequencies of separation and fusion decreased during simplification in the analysed wild type and *fis1*Δ cells upon change of the carbon source. This suggests that a decrease in the number of matrix separation and fusion events is a characteristic process during the simplification of mitochondrial networks. The frequency of separation and fusion remained constant at about 1.7 events per minute in mitochondria of cells that were continuously kept on glucose-containing medium (Fig. 4B).

Mitochondrial tubule fusion and fission is reduced in the *dnm1*Δ mitochondrial network

Our data demonstrate that the ability of mitochondria of *fis1*Δ cells to sever inner and outer mitochondrial membranes is not

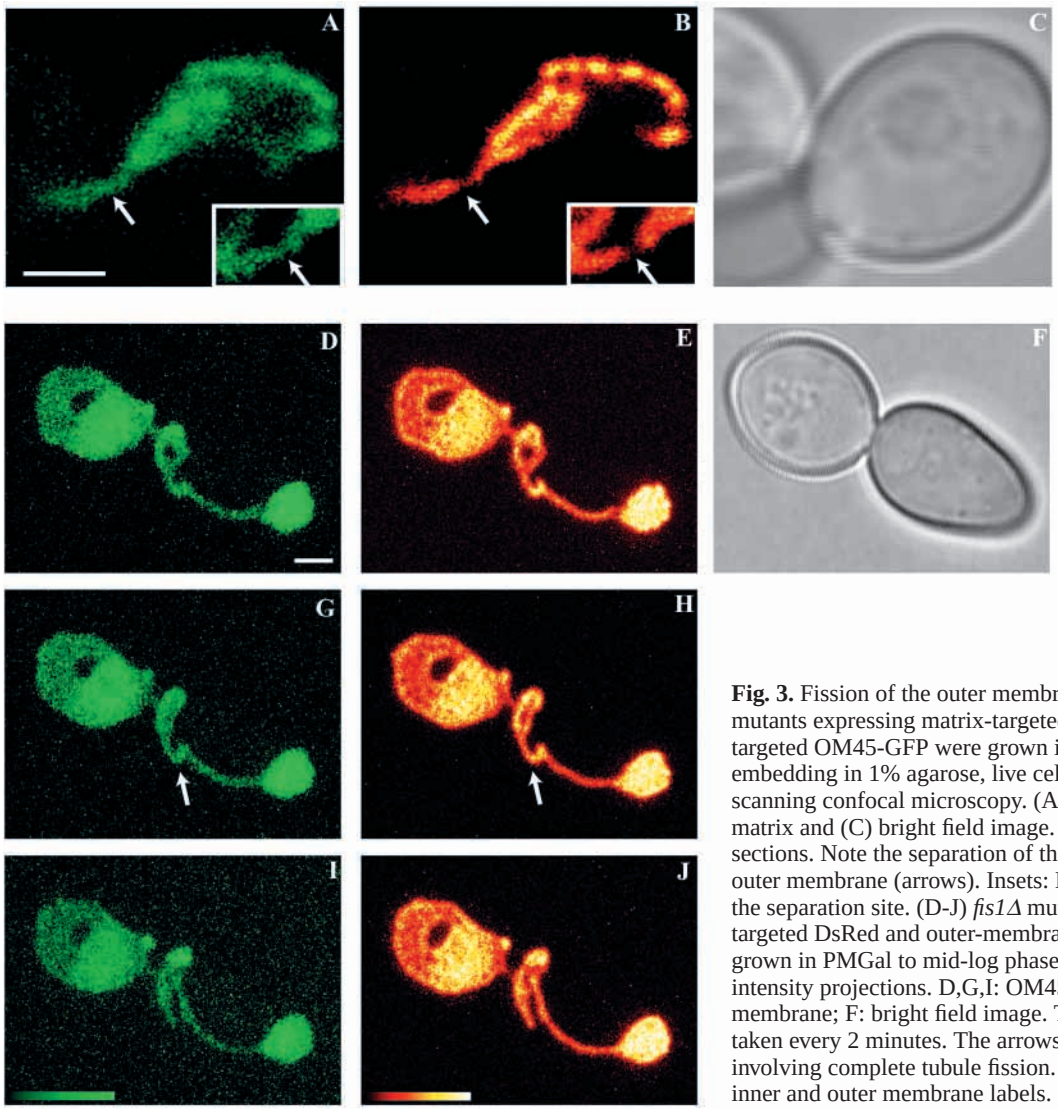


Fig. 3. Fission of the outer membrane in *fis1Δ* cells. (A-C) *fis1Δ* mutants expressing matrix-targeted DsRed and outer-membrane-targeted OM45-GFP were grown in PMGal to mid-log phase. After embedding in 1% agarose, live cells were imaged by beam-scanning confocal microscopy. (A) OM45-GFP, (B) DsRed in matrix and (C) bright field image. Displayed are single optical sections. Note the separation of the matrix without fission of the outer membrane (arrows). Insets: Maximum intensity projections of the separation site. (D-J) *fis1Δ* mutants expressing inner-membrane-targeted DsRed and outer-membrane-targeted OM45-GFP were grown in PMGal to mid-log phase. Displayed are maximum intensity projections. D,G,I: OM45-GFP; E,H,J: DsRed in inner membrane; F: bright field image. Time-lapse images (D-J) were taken every 2 minutes. The arrows point to a remodelling event involving complete tubule fission. Note the colocalization of the inner and outer membrane labels. Bars, 2 μm.

completely absent. The GTPase Dnm1p has been suggested to act as a key component of the mitochondrial fission machinery (Bleazard et al., 1999; Otsuga et al., 1998; Sesaki and Jensen, 1999). Dnm1p complexes present on mitochondrial tubules are reduced in *fis1*Δ cells but not completely absent (Mozdy et al., 2000; Tieu and Nunnari, 2000). Thus, it seemed possible that residual Dnm1p-containing complexes were responsible for the observed tubule fission activity during remodelling of *fis1*Δ mitochondria.

To verify this hypothesis, we analysed mitochondria of cells lacking Dnm1p for their ability to separate their matrix. The same experimental conditions were employed as for the analysis of mitochondria of wild-type and *fis1*Δ cells. Confocal time-lapse microscopy following glucose repression revealed that mitochondria of *dnm1*Δ cells perform matrix separations and fusions (Fig. 5A-C, Movie 5). In contrast to mitochondria of *fis1*Δ mutants, separation was apparently always followed by a fusion event at the same site in *dnm1*Δ mitochondria (see Movie 5). Intriguingly, we did not observe the generation of free mitochondrial tips by fission. Although we cannot rule out rare tubule fission events, complete mitochondrial division appears to be severely hampered in *dnm1*Δ cells. We conclude that the matrix can still be separated in *dnm1*Δ mutants; however, unlike Fis1p, the presence of Dnm1p is apparently essential for complete mitochondrial tubule fission.

Discussion

Our data reveal that cells lacking Fis1p are able to undergo mitochondrial restructuring upon glucose repression albeit with a temporal delay (a generalized view of mitochondrial simplification processes in wild-type and *fis1*Δ cells is presented in Fig. 6). During remodelling, numerous events of separation of GFP-labelled matrix compartments in *fis1*Δ cells were observed. Double-labelling of the matrix with DsRed and the outer membrane with GFP revealed that some of these matrix separations occur without fission of the outer membrane. In addition, we found that in mitochondria of *fis1*Δ mutants simplification is accompanied by occasional complete tubule fissions. Because genetic and morphological data strongly suggest an involvement of Fis1p in tubule fission (Mozdy et al., 2000; Tieu and Nunnari, 2000; Tieu et al., 2002), it is

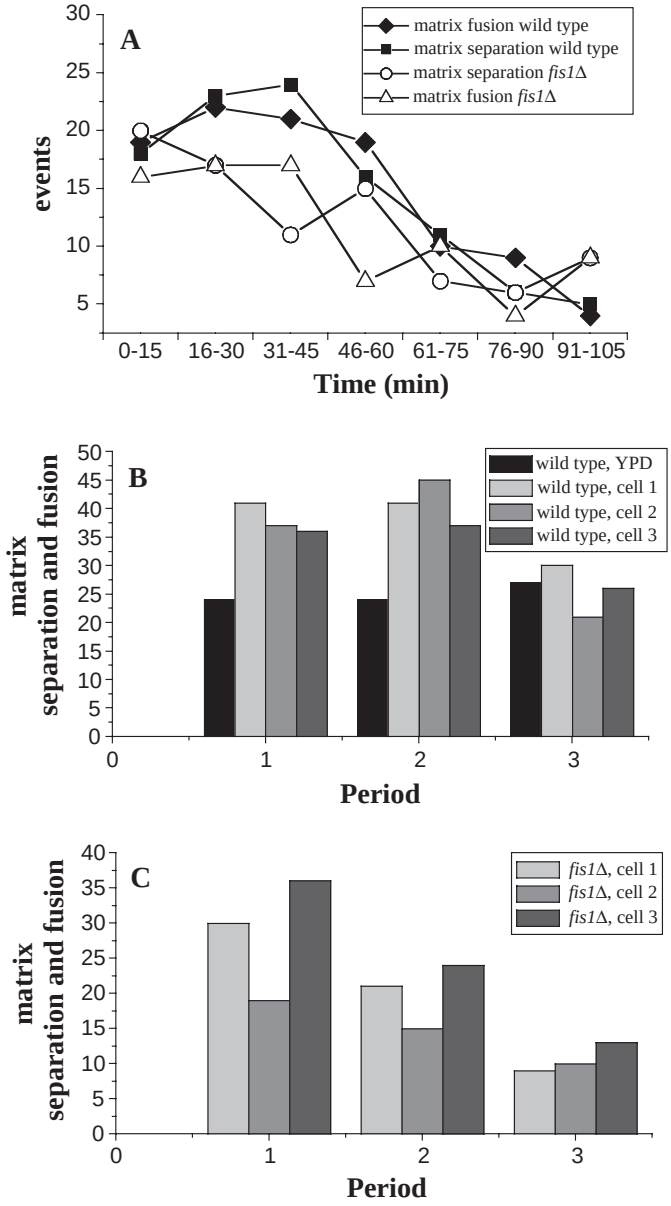


Fig. 4. Frequency of matrix separation and fusion events during remodelling upon glucose repression. (A) All detectable separation and fusion events of the GFP labelled matrix in a single wild-type cell and a single *fis1Δ* cell were counted over a time frame of 105 minutes. Only events in the mother cell were recorded. At time point zero, the mitochondrial morphologies were of a typical glycerol type. At the end of the analysis both networks were clearly simplified. (B,C) Separation and fusion frequencies at different time points following change of carbon source. (B) Wild-type cells and (C) *fis1Δ* cells. The number of matrix separations and fusions was counted at three 15 minute time frames, namely before recognizable simplification (period 1), after final separation of mother and daughter mitochondrial compartments (period 3) and in between (period 2). In the case of *fis1Δ*, period 3 was defined as the point of time when no further size reduction of the fenestrated net could be observed. Only events in the mother cells were recorded. Control: (B) Wild-type cell grown to log phase in YPD and transferred to fresh YPD medium.

conceivable that the rate of complete tubule fission is markedly reduced in *fis1*Δ mutants compared with the wildtype. This assumption is supported by the low rate of recognizable tubule fissions during remodelling of mitochondria of *fis1*Δ cells (on average less than one tubule fission was observed in 10 minutes). Deletion of the *FIS1* gene leads to a reduction in tubule fission activity but not to its complete halt. Hence, we assume that most matrix separations do not involve a fission of the outer membrane in cells lacking Fis1p. Matrix separation without a fission of the outer membrane was only observed when the matrix itself was labelled. We did not directly observe inner membrane fission without outer membrane fission. Thus it is tempting to assume that many of the observed matrix separations do not include fissions of mitochondrial membranes at all. Frequently the matrix might be continuous but severely thinned at the location of an apparent matrix separation. Such apparent matrix separations might arise from the development of very large cristae, but are more likely to be the result of a matrix constriction. We propose that such matrix constrictions are a prerequisite for complete tubule fission. A quantitative analysis of the absolute numbers of matrix separation events (not distinguishing between mere matrix constrictions and tubule fissions) revealed that the frequencies of matrix separation are only slightly reduced compared with the wildtype. For wild-type cells grown on glycerol we counted about 2.5 matrix fusion and fission events per minute, whereas in *fis1*Δ cells around 2.4 events per minute were detected. Moreover, in both *fis1*Δ and wild-type cells the number of matrix separations is reduced upon glucose repression. Therefore deletion of the *FIS1* gene apparently does not affect the regulatory pathways controlling separation of the matrix. The total length of the wild-type mitochondrial tubules is reduced under glucose-repressed conditions (data not shown) (Egner et al., 2002). Similarly, the fenestrated mitochondrial networks of *fis1*Δ cells are on average smaller on glucose containing medium. Hence, there appears to be a correlation between tubule length and the number of matrix separations.

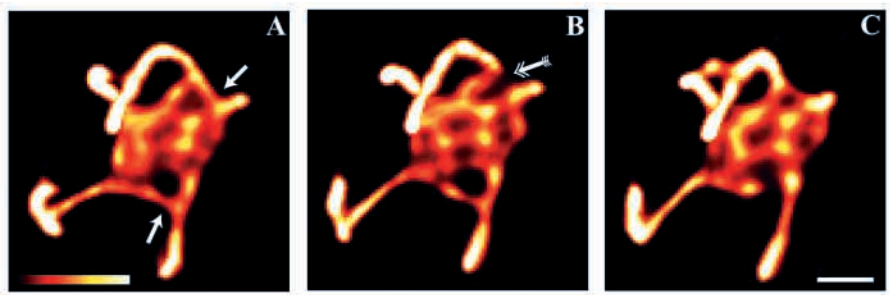


Fig. 5. Mitochondrial remodelling of *dnm1Δ* mutants upon glucose repression. Selected maximum intensity projections are displayed. The experimental set-up was similar as for Fig. 2D-I. Solid arrows indicate future matrix separation sites. Feathered arrows indicate matrix fusion sites. The underlying data-stack comprises 40 three-dimensional stacks. Stacks were recorded every 60 seconds for 40 minutes. Bar, 1 μm. The complete dataset is available as Movie 5 at jcs.biologists.org/supplemental.

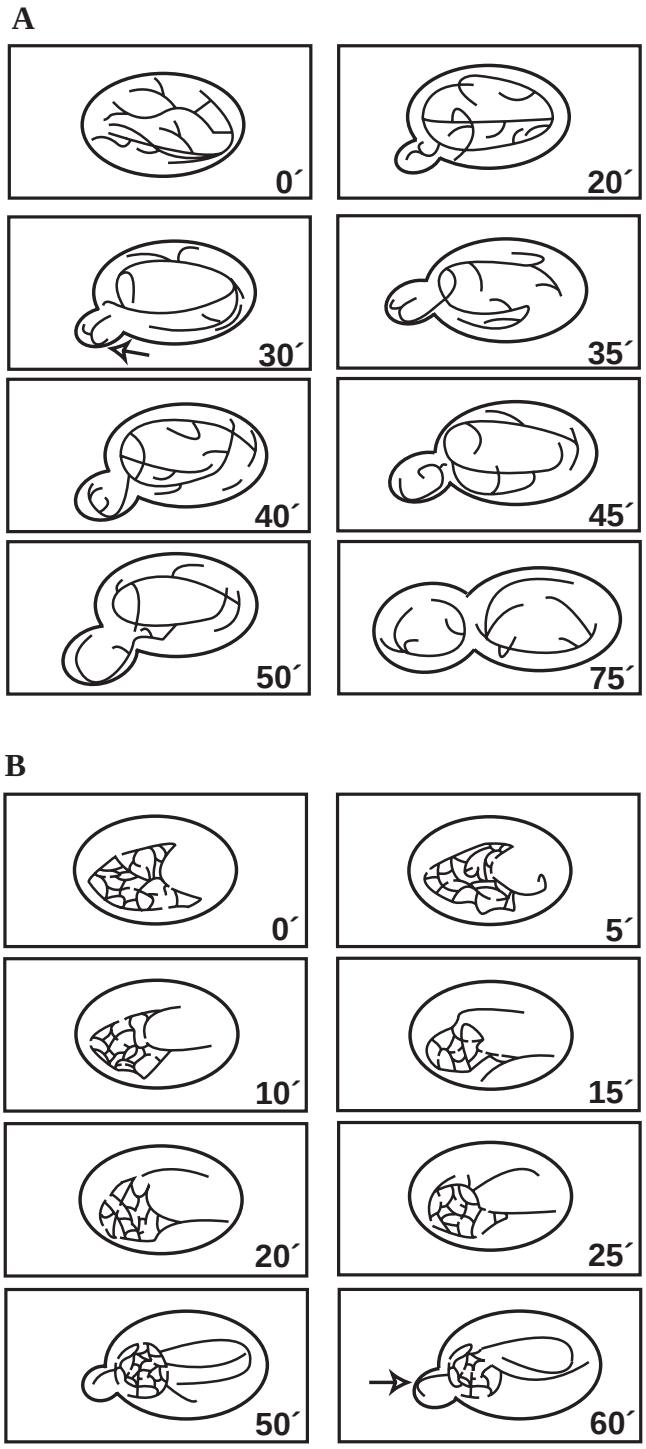


Fig. 6. Schematic representation of mitochondrial simplification upon glucose repression in wild-type and *fis1Δ* cells. Numbers indicate characteristic time points within the ongoing remodelling process. At time point zero, the mitochondrial networks exhibit morphologies typical for cells grown in medium containing glycerol. (A) In wild-type cells, the daughter cell is invaded by a single mitochondrial tubule shortly after budding. Subsequently the tubule attaches to the cell cortex within the emerging bud (arrow). During subsequent growth the mitochondrion remains attached at this site while the remaining part of the network is moving freely within the daughter cell. During budding, the connection between the mitochondrial compartments of mother and daughter cell is frequently disrupted. Concomitant with the transport of mitochondrial membranes into the daughter cell the network undergoes constantly fission and fusion. Most fusion events occur between two mitochondrial tips. It is conceivable that mitochondrial interactions with cytoskeletal elements are important for these processes (Boldogh et al., 2001). (B) The mitochondria of *fis1*Δ cells grown on glycerol are frequently large fenestrated nets located at one side of the cell cortex. In *fis1*Δ mitochondria simplification appears not to be directly correlated to budding. Upon glucose repression, single meshes of the fenestrated network are opened by fission. These fission events lead to an ongoing size reduction of the fenestrated network. Membrane fusions extend the (mostly one or two) tubules emerging from the net. The long tubules are frequently separated from the fenestrated net by fission. They subsequently refuse to the same or to a different site. Upon budding one of these single tubules penetrates into the emerging daughter cell. As observed in the wildtype, the tubule attaches with one site at the cortex of the daughter cell (arrow). The rest of the tubule moves freely within the bud. Simplifying a large fenestrated net to an almost tubular structure most probably requires several cell cycles.

Possibly there is a constant spacing between the molecular machineries responsible for inducing matrix separations. To date the molecular mechanisms controlling the rates of mitochondrial fusion and fission are elusive.

Upon glucose repression in wild-type cells the size reduction of the mitochondrial network occurs concomitantly with the budding of the cell. It is conceivable that an important part of the size reduction of the mitochondrial compartment is due to a partitioning of the mitochondrial tubules between mother cell and bud. Although this does not preclude additional mechanisms like autophagy from being involved in the size regulation of mitochondrial networks.

The dynamin-like GTPase Dnm1p is another key component of mitochondrial fission. It is conceivable that the tubule fission activity in *fis1*Δ mutants is due to Dnm1p-containing complexes, which are still able to assemble on mitochondria lacking Fis1p, albeit at a reduced number and with an altered distribution (Mozdy et al., 2000; Tieu and Nunnari, 2000; Tieu et al., 2002). Indeed, we were not able to identify unequivocally any tubule fission events in cells lacking Dnm1p. In contrast to cells lacking Fis1p, virtually all matrix separation events are followed by re-fusion in *dnm1*Δ mutant cells. This suggests that Dnm1p might be essential for outer membrane fission. However, as proven by the observed matrix separations in *dnm1*Δ mutants, constriction of the mitochondrial matrix – or fission of the inner membrane – occurs in mitochondria lacking Dnm1p. A similar observation has been made in *Caenorhabditis elegans* mutants, which expressed dominant interfering mutant versions of DRP-1, a homolog of yeast Dnm1p. These worms harboured mitochondria with separated matrix compartments that were still connected by the outer membrane, presumably because inner membrane fission – or matrix constriction – persisted in the absence of outer membrane fission (Labrousse et al., 1999). Our observations support the idea that constriction of the matrix space and/or fission of the inner membrane can occur in the absence of outer membrane fission activity. This suggests that in *S. cerevisiae* matrix constriction is a prerequisite for tubule fission and that these two processes are mediated by independent molecular machineries.

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