Aktinabhängige Bewegung und Vererbung von Mitochondrien in *Saccharomyces cerevisiae*

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INHALTSVERZEICHNIS

Abkürzungsverzeichnis

AM	Mitochondriale Außenmembran
AIF	Apoptose induzierender Faktor
CAD	Diablo/Smac und Caspase aktivierte DNAse
DIC	Differential interference contrast; Differentieller Interferenz Kontrast
DOX	Doxyzyklin
ER	Endoplasmatisches Retikulum
mtDNA	Mitochondriale DNA
IM	Mitochondriale Innenmembran
SAM	Sortierungs- und Assemblierungsmaschinerie
S. cerevisiae	*Saccharomyces cerevisiae*
SRP	Signal recognition particle; Signalerkennungspartikel
TIM	Translocase of the inner membrane; Proteintranslokase der mitochondrialen Innenmembran
TMS	Two membrane spanning structure; membrandurchspannender Komplex
TOM	Translocase of the outer membrane; Proteintranslokase der mitochondrialen Außenmembran

1. Summary

As mitochondria cannot be synthesized *de novo*, the coordinated inheritance of mitochondria and mitochondrial DNA (mtDNA) ensures the maintenance of functional organelles in the cells during cytokinesis. In the model organism *Saccharomyces cerevisiae* it is well established that the actin cytoskeleton is responsible for these mitochondrial transport events. Furthermore a two membrane spanning structure (TMS) is suggested to link mtDNA to the cytosolic segregation apparatus during cytokinesis. At present the mechanism and the components of actin-dependent mitochondrial movement as well as the overall structure of the TMS are poorly understood.

In this work a systematic screen for essential mitochondrial morphology and distribution components was performed. 119 strains of a yeast strain collection harbouring essential genes under control of a regulatable promoter showed aberrant mitochondria after promoter shut-off. This led to the identification of five cellular pathways that are important for maintenance of mitochondrial morphology: ergosterol biosynthesis, vesicular trafficking, mitochondrial protein import, ubiquitin/26S proteasome-dependent protein degradation and actin cytoskeleton-dependent transport. Two components of the latter group, the class V myosin Myo2 and its essential light chain Mlc1, were found to display an especially interesting phenotype. Fluorescence microscopy and electron microscopy revealed a ring-shaped mitochondrial morphology in mutant cells, and mitochondrial cristae structures were absent. These defects could be observed in cells with a normal actin cytoskeleton indicating a primary effect of Myo2 on the interaction between mitochondria and the actin cytoskeleton. This suggestion was corroborated by *in vitro* actin-binding assays demonstrating a severely impaired binding capacity for mitochondria isolated from strains lacking Myo2 or Mlc1 as well as from strains carrying specific point mutations in the cargo-binding domains of Myo2. Additionally, time resolved fluorescence microscopy of *myo2* point mutants revealed that Myo2 is responsible for the bud-directed anterograde movement of mitochondria. These results demonstrate for the first time that a myosin motor protein plays an important and direct role for mitochondrial motility and distribution in *S. cerevisiae*.

Furthermore Mdm31 and Mdm32, two functionally independent subunits of two complexes in the mitochondrial inner membrane (IM), have been established as components necessary for coordinated mtDNA inheritance. It could be shown that deletion of both genes resulted in a

loss of interaction between mtDNA and a known outer membrane protein of the TMS. This points to a function of Mdm31 and Mdm32 as inner membrane TMS components.

2. Zusammenfassung

Da Mitochondrien nicht *de novo* synthetisiert werden können, wird durch die koordinierte Vererbung von Mitochondrien und mitochondrialer DNA (mtDNA) sichergestellt, dass während der Zytokinese alle Zellen im Besitz funktioneller Organellen bleiben. Dabei ist bekannt, dass im Modellorganismus *Saccharomyces cerevisiae* das Aktinzytoskelett für diese mitochondrialen Transportereignisse verantwortlich ist. Weiterhin wird angenommen, dass über einen beide mitochondriale Membranen durchspannenden Komplex (TMS) die mtDNA während der Zytokinese an den Segregationsapparat im Zytosol gebunden wird. Allerdings sind der Mechanismus und die Komponenten der aktinabhängigen Bewegung genauso wie die Gesamtstruktur des TMS bisher nur wenig verstanden.

Im Rahmen dieser Arbeit wurde ein systematischer Screen nach essentiellen, mitochondrialen Morphologie- und Verteilungskomponenten durchgeführt. 119 Stämme einer Hefe-Bibliothek, in der die Genexpression über einen Fremd-Promotor reguliert wird, zeigten nach Abschalten des Promotors einen veränderten mitochondrialen Phänotyp. Dies ermöglichte die Identifizierung fünf zellulärer Prozesse, die für die mitochondriale Morphogenese von entscheidender Bedeutung sind: Ergosterol-Biosynthese, vesikulärer Transport, mitochondrialer Proteinimport, Ubiquitin/26S Proteasom-abhängiger Proteinabbau und aktinzytoskelettabhängiger Transport. Zwei Mitglieder der letzten Gruppe, das Klasse V Myosin Myo2 und seine leichte Kette Mlc1, wiesen dabei besonders interessante Phänotypen auf. Fluoreszenz- und elektronenmikroskopische Untersuchungen ergaben eine ringähnliche mitochondriale Morphologie in den mutanten Zellen. Cristaestrukturen fehlten vollständig. Da diese Defekte in Zellen mit einem normal ausgeprägten Aktinzytoskelett beobachtet werden konnten, übt Myo2 wahrscheinlich einen primären Effekt auf die Interaktion zwischen Mitochondrien und den Aktinkabeln aus. Diese Vermutung wurde durch *in vitro* Aktinbindungs-Assays bestärkt. Dabei zeigten isolierte Mitochondrien aus Stämmen ohne Myo2 und Mlc1 und aus Stämmen mit spezifischen Punktmutationen in den Cargo-Bindungsdomänen von Myo2 eine stark beeinträchtigte Bindungskapazität. Zusätzlich ergab zeitauflösende Fluoreszenzmikroskopie der *myo2*-Punktmutanten, dass Myo2 auch für die knospengerichtete anterograde Bewegung von Mitochondrien verantwortlich ist. Diese Ergebnisse belegen zum ersten Mal die wichtige und direkte Beteiligung eines Myosins an der mitochondrialen Bewegung und Vererbung in *S. cerevisiae*.

Darüber hinaus wurden Mdm31 und Mdm32, zwei funktionell unabhängige Untereinheiten zweier Komplexe der mitochondrialen Innenmembran (IM), als notwendige Komponenten der koordinierten Vererbung von mtDNA etabliert. In vorliegender Arbeit konnte dabei gezeigt werden, dass die Deletion beider Gene jeweils in dem Verlust der Interaktion zwischen mtDNA und Mmm1, einer Außenmembrankomponente des TMS, resultierte. Dies deutet auf eine Funktion von Mdm31 und Mdm32 als Innenmembrankomponenten des TMS hin.

3. Einleitung

Mitochondrien sind Zellorganellen, die in fast allen eukaryotischen Zellen vorkommen. Neben der Energiegewinnung erfüllen sie weitere wichtige Aufgaben, die in allen eukaryotischen Zellen für das Überleben essentiell sind. Mutationen, die Form, Funktionalität, Verteilung oder Vererbung von Mitochondrien betreffen, führen daher in den meisten Fällen zu beeinträchtigem Zellwachstum oder sogar zum Zelltod. Aus diesem Grund wird zur Erforschung mitochondrialer Prozesse häufig die Bäckerhefe *Saccharomyces cerevisiae* (*S. cerevisiae*) verwendet, die als fakultativ anaerober Organismus eine große Anzahl andernfalls tödlicher, mitochondrialer Funktionsdefekte tolerieren kann. Doch auch hier gilt: Hefezellen ohne Mitochondrien sind nicht überlebensfähig. Da Mitochondrien nicht *de novo* synthetisiert werden können, ist während der Zellteilung besonders die Sicherstellung der Vererbung des gesamten Organells sowie der mitochondrialen DNA (mtDNA) zwischen Mutter- und Tochterzelle von essentieller Bedeutung. Die dazu nötigen zellulären Transportprozesse sind allerdings bis heute kaum verstanden. Es gilt zwar als bewiesen, dass Mitochondrien in der Bäckerhefe aktinabhängig in der Zelle bewegt werden (Drubin et al., 1993; Lazzarino et al., 1994), noch fehlt aber die Kenntnis zugehöriger Mechanismen und Proteine. Die Vererbung mitochondrialen Erbguts wird dabei vermutlich durch die indirekte Bindung an die Segregationsmaschinerie über einen Komplex, der beide mitochondriale Membranen durchspannt (TMS), gewährleistet (Berger & Yaffe, 2000; Meeusen & Nunnari, 2003). Doch auch hier besteht über Zusammensetzung und das Funktionsprinzip dieser Maschinerie Unklarheit.

3.1 Ursprung, Funktion, Aufbau und Morphologie von Mitochondrien

Es wurden zahlreiche Szenarien postuliert, die den Ursprung der eukaryotischen Zelle durch Fusion oder Endosymbiose zwischen einem Archaebakterium oder einer primitiven eukaryotischen Zelle und einem respirationskompetenten α-Proteobakterium erklären. Mittlerweile gilt es als gesichert, dass alle modernen Eukaryoten von einem einzigen Vorgänger mit Mitochondrien abstammen (Embley et al., 2003; Tovar et al., 2003; van der Gietzen & Tovar, 2005; Embley & Martin, 2006), da es keine lebenden Eukaryoten gibt, die nicht zumindest degenerierte mitochondriale Strukturen wie Mitosomen oder Hydrogenosomen aufweisen (Andersson & Kurland, 1999; Embley et al., 2003; Leon-Avila

& Tovar, 2004). Unklar ist allerdings, ob andere eukaryoten-spezifische Strukturen wie der Zellkern, das ER und lineare Chromosomen zeitlich vor oder nach der Entstehung von Mitochondrien einzuordnen sind. Nach der Integration des „Urmitochondrions“ in die ursprüngliche Euzyte wurde im Verlauf der Evolution beinahe das vollständige Genom des Endosymbionten über Gentransfer in den Zellkern übertragen oder in Form von redundanten Genen ausgesondert (Gray, 1993; Martin & Herrmann, 1998; Gray et al., 1999). Nur wenige Gene, die für Komponenten metabolischer Aktivitäten und Faktoren zur Expression des mitochondrialen Genoms codieren, verblieben im mitochondrialen Genom selbst.

Funktionell sind die Mitochondrien an einer Vielzahl verschiedener Stoffwechselprozesse beteiligt. Obwohl sie hauptsächlich wegen ihrer zentralen Rolle bei der Versorgung der Zelle mit ATP bekannt sind, erfüllen die Organellen auch in anderen Bereichen für das Überleben der Zelle notwendige Funktionen. So findet in Mitochondrien die Assemblierung von Eisen-Schwefel-Clustern statt (Kispal et al., 2005; Lill & Mühlenhoff, 2005). Fe/S-Cofaktoren kommen u.a. in Proteinen vor, die für den Export von Ribosomenuntereinheiten aus dem Zellkern ins Zytoplasma benötigt werden und somit für die Synthese aller kerncodierten Proteine unverzichtbar sind (Kispal et al., 2005; Yarunin et al., 2005) Darüber hinaus sind Mitochondrien an wichtigen Prozessen wie dem Fettsäureabbau, dem Harnstoffzyklus und der Synthese des Hämmoleküls, der Nukleotide, der Pyrimidine und einiger Phospholipide beteiligt (Scheffler, 2001). In den letzten Jahren richtete sich der Focus der mitochondrialen Forschung besonders auf die Rolle der Organellen bei der Regulation des programmierten Zelltodes, der Apoptose (Bernardi, 1999; Bernardi et al., 2001). Die Mitochondrien schütten dabei verschiedene Substanzen (Cytochrom c, Apoptose induzierende Faktoren (AIFs), Diablo/Smac und Caspase aktivierte DNAse (CAD)) ins Zytosol aus und aktivieren so verschiedene Caspasen, die den Beginn der Apoptose einleiten. AIF und CAD werden in den Zellkern transportiert und führen dort zum Abbau von Chromatin (Wallace, 1999). Weiterhin stellen Mitochondrien einen zentralen Bestandteil des Zell-Alterungsprozesses dar. Durch Akkumulierung von Nebenprodukten der oxidativen Phosphorylierung, den so genannten ROS (reaktive Sauerstoffverbindungen), werden Mutationen in der mtDNA begünstigt. Dies resultiert in einer Herabsetzung der mitochondrialen Lebensdauer (Wallace, 1999) und somit in der Minderversorgung der entsprechenden Zellen mit ATP, was schlussendlich zum Altern und Tod der Zelle führt.

Der Aufbau der Mitochondrien konnte bereits in den 50er Jahren durch

elektronenmikroskopische Betrachtungen aufgeklärt werden: Zwei Membranen, die mitochondriale Innen- und Außenmembran, begrenzen die Matrix und trennen den Intermembranraum vom Cytosol ab. Dabei ist die Struktur der inneren Membran, deren Oberfläche durch Membraneinstülpungen (Cristae) vergrößert wird, sehr variabel und hängt von der Art und metabolischen Aktivität des jeweiligen Zelltyps ab (Munn, 1974; Smith & Ord, 1983, Perkins et al., 2003). Eine weitere Besonderheit neben der Doppelmembran stellt die Existenz eines eigenen Genoms dar. In *S. cerevisiae* liegt die mtDNA in Form von ca. 20 Nukleoiden pro Zelle vor. Nukleoide sind große membranassoziierte Komplexe (Williamson & Fennell, 1979), bestehend aus mehreren DNA-Molekülen, der mtDNA-Polymerase Mip1 (Foury, 1989), dem DNA-bindenden Verpackungsprotein Abf2 (Diffley & Stillman, 1991; Diffley & Stillman, 1992) und einem weiteren DNA-bindenden Protein Mgm101, das für die Reparatur oxidativer mtDNA-Schäden verantwortlich ist (Chen et al., 1993; Meeusen et al., 1999). Das meist zirkuläre DNA-Molekül codiert, abhängig vom jeweiligen Organismus, allerdings nur für wenige Faktoren, darunter tRNAs, rRNAs, Untereinheiten der mitochondrialen Ribosomen und einige Proteine der Atmungskette. Der Großteil der mitochondrial benötigten Proteine wird hingegen vom Kern-Genom codiert, im Zytoplasma synthetisiert und posttranslational über Proteinkomplexe der mitochondrialen Außen- (TOM) und Innenmembran (TIM) in die Organellen importiert (Neupert, 1997; Neupert & Brunner, 2002; Pfanner et al., 2004).

Das äußere Erscheinungsbild der Mitochondrien ist sehr vielfältig und hängt stark vom Organismus und Zelltyp ab. Die typisch nierenförmige Gestalt, die in vielen Lehrbüchern propagiert wird, ist eine höchst vereinfachte Darstellung der tatsächlichen Organellengestalt, denn die mitochondriale Morphologie stellt sich in den meisten Organismen und Zelltypen als dynamisch agierendes verzweigtes Netzwerk dar, das gleichermaßen komplex wie anpassungsfähig ist (Hoffmann & Avers, 1973; Bereiter-Hahn, 1990; Bereiter-Hahn & Voth, 1994; Warren & Wickner, 1996; Griparic & van der Bliek, 2001). Vier übergeordnete Prozesse sind für die Aufrechterhaltung dieser mitochondrialen Struktur zuständig: Fusion, Teilung, Tubulation und Transport (Nunnari et al., 1997; Hermann & Shaw, 1998; Yaffe, 1999). Dabei wirken Fusion und Teilung als antagonistische Prozesse, die sich gegenseitig regulieren und so das mitochondriale Retikulum aufbauen und erhalten (Nunnari et al., 1997; Sesaki & Jensen, 1999; Shaw & Nunnari, 2002). Fällt eine der beiden Maschinerien aus, entstehen schwere morphologische Verformungen (Abbildung 3-1): eng geknüpfte „Fischernetze“ im Falle einer Teilungsstörung und fragmentierte Mitochondrien bei Vorliegen

eines Fusiondefektes (Hermann & Shaw, 1998; Rapaport et al., 1998; Bleazard et al., 1999; Sesaki & Jensen, 1999).

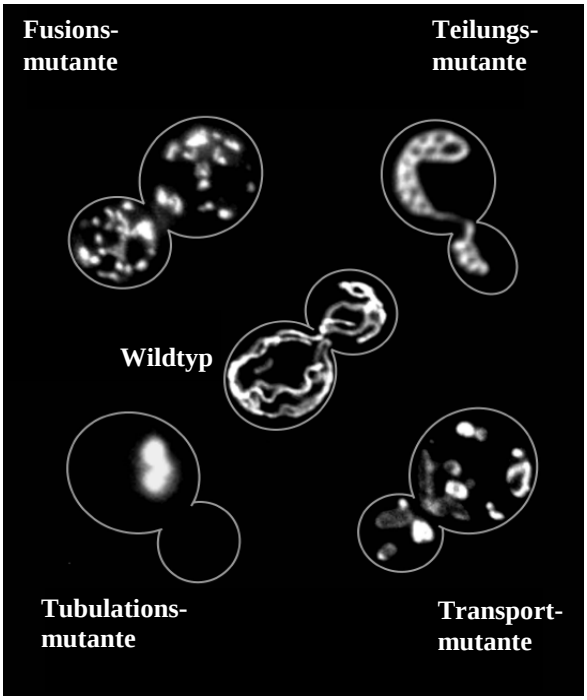

Abbildung 3-1: Mitochondriale Morphologie in der Bäckerhefe *S. cerevisiae*. Dargestellt sind der Wildtyp und charakteristische Mutanten, die Defekte bei Fusion (*Δugo1*), Teilung (*Δmdv1*), Tubulation (*Δmdm10*) und Transport (*myo2*) aufweisen (nach Merz et al., 2007*).

Für eine intakte mitochondriale Morphologie werden neben Fusion und Teilung auch die Komponenten der Tubulationsmaschinerie benötigt. Vermutlich bilden Tubulationsproteine Ankerpunkte für die Verbindung des Organells mit Bestandteilen des Zytoskelettes oder intramitochondrialen Strukturen, die wiederum maßgeblich an der Aufrechterhaltung der tubulären Morphologie beteiligt sind (Merz et al., 2007*). Die Deletion von Tubulationsgenen resultiert in der Ausbildung großer, unbeweglicher, sphärischer Mitochondrien (Abbildung 3-1), Störungen in der mitochondrialen Vererbung von Mutter- auf Tochterzelle und einer fehlerhaften Organisation und Vererbung von mtDNA (Burgess et al., 1994; Sogo & Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998; Aiken Hobbs et al., 2001; Dimmer et al., 2002; Boldogh et al., 2003; Youngman et al., 2004; Dimmer et al., 2005*). Neben Fusion, Teilung und Tubulation existiert ein vierter Prozess, der einerseits für die Aufrechterhaltung mitochondrialer Morphologie, andererseits für die Verteilung der Organellen von Mutter- auf Tochterzelle während der Zellteilung von Bedeutung ist: der Transport entlang des Zytoskelettes. Bewegungsdefekte ziehen ausgeprägte Organisationsstörungen der mitochondrialen Morphologie und Vererbung nach sich (Abbildung3-1). Die Mitochondrien

* Der Stern weist auf eigene Publikationen hin, die im Anhang dieser Arbeit zu finden sind.

bilden ringförmige Aggregate, die oft auf der zur Knospe distal gelegenen Seite der Mutterzelle lokalisiert sind (Boldogh et al., 2004). Während die Grundlagen von Fusion und Teilung zum großen Teil entschlüsselt sind, ist der Ablauf des aktinabhängigen Transports von Mitochondrien großteils nicht bekannt.

3.2 Identifizierung mitochondrialer Morphologie- und Vererbungskomponenten in *S. cerevisiae*

Um die basalen Prozesse der mitochondrialen Funktion und Vererbung zu entschlüsseln, ist die Identifizierung und Charakterisierung aller beteiligten molekularen Komponenten unumgänglich. Daher wurde in den vergangenen Jahrzehnten versucht, zunächst einen Großteil der für das respiratorische Wachstum (Tzagoloff & Dieckmann, 1990; Contamine & Picard, 2000) und den Aufbau und die Aufrechterhaltung der mitochondrialen Struktur benötigten Proteine (Hermann & Shaw, 1998; Jensen et al., 2000; Boldogh et al., 2001b) aufzuklären. Zur Identifizierung mitochondrialer Morphologie- und Transportkomponenten erwiesen sich dabei morphologische Screens zufallsgesteuert mutagenisierter Hefestämme, deren Mitochondrien durch spezifische Fluoreszenzmarker angefärbt wurden, als besonders geeignet (McConnell et al., 1990; Burgess et al., 1994; Hermann et al., 1997; Sesaki & Jensen, 1999). Danach ermöglichte die postgenomische Ära die Untersuchung vollständiger Gensätze hinsichtlich ihrer spezifischen Funktion durch systematische, genomweite Screens (Winzeler et al., 1999; Vidan & Snyder, 2001). Im Jahre 2002 wurde schließlich anhand eines systematischen Screens einer Deletions-Bibliothek von nicht-essentiellen Hefegenen ein Großteil aller Gene bestimmt, die an der mitochondrialen Strukturgebung und Funktionalität beteiligt sind (Dimmer et al., 2002). Die identifizierten Proteinkomponenten ermöglichten einerseits die Erweiterung bereits bestehender Modelle von Fusion, Teilung, Tubulation und Transport (Dimmer et al., 2005[*]), andererseits die Entdeckung gänzlich neuer Komplexe, wie der Teilungsmaschinerie der mitochondrialen Innenmembran (Messerschmitt et al., 2003).

Obwohl eine überwiegende Mehrheit der mitochondrialen Morphologie- und Vererbungsvorgänge durch nicht essentielle Genprodukte gesteuert wird, scheint zumindest die intakte Vererbung der Organellen für Hefezellen ein essentieller Prozess zu sein (Dimmer et al., 2005[*]). Somit besteht die hohe Wahrscheinlichkeit, dass neben den nicht essentiellen auch essentielle Proteine für die Vererbung und damit auch für den Transport von Mitochondrien von Bedeutung sein könnten.

3.3 Vererbung von Mitochondrien und mitochondrialer DNA in *S. cerevisiae*

Mitochondrien sind Organellen, deren Funktionen für die Zelle unabdingbar sind. Daher ist es notwendig, während der Zellteilung sicherzustellen, dass Mutter- und Tochterzelle im Besitz funktionsfähiger Mitochondrien bleiben. Dies geschieht, indem sich früh im Zellzyklus die Mitochondrien zunächst an die spätere Knospungsstelle annähern. Während der S- und G_2-Phase werden die Organellen dann in die wachsende Knospe transportiert und an der Spitze der Tochterzelle immobilisiert (Simon et al., 1997). Gleichzeitig werden am distal gelegenen Ende der Mutterzelle ebenfalls Mitochondrien fixiert (Yang et al., 1999). Nach Abschluss der Zytokinese werden die Mitochondrien aus den Verankerungen gelöst und können sich nun neu verteilen (Boldogh et al., 2001b). Dieser Prozess stellt sicher, dass jede Zelle mit Mitochondrien versorgt ist. Die Vererbung des Organells an sich gewährleistet allerdings noch nicht die Aufrechterhaltung der mitochondrialen Funktion, die wesentlich vom Vorhandensein der mtDNA bestimmt wird. Also ist von entscheidender Bedeutung, dass während des Segregationsprozesses Mitochondrien mit mindestens einer intakten Kopie mtDNA in die Tochterzelle gelangen bzw. in der Mutterzelle verbleiben.

Die koordinierte Verteilung der mtDNA auf Mutter- und Tochterzelle erfolgt dabei nicht passiv, sondern in einem aktiven, kontrollierten Prozess (Azpiroz & Butow, 1993; Okamoto et al., 1998; Berger und Yaffe, 2000; Garrido et al., 2003; Meeusen & Nunnari, 2003). Dabei stellt die Ausbildung des TMS (Berger und Yaffe, 2000; Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen & Nunnari, 2003) sicher, dass über die mitochondriale Doppelmembran hinweg die Nukleoide genau an der Stelle an die Innenmembran angelagert werden, an der die Außenmembran mit dem Aktinzytoskelett interagiert (Abbildung 3-2). Da das Zytoskelett Mediator für mitochondriale Bewegungen und Vererbung ist, kann durch die indirekte Verknüpfung der mtDNA mit den Aktinkabeln die Bewegung des Organells mit der Verteilung der mtDNA synchronisiert werden (Berger & Yaffe, 2000).

Der postulierte Aufbau des TMS stellt sich dabei folgendermaßen dar: drei Proteine, Mmm1, Mdm10 und Mdm12, die als Komponenten der Tubulationsmaschinerie identifiziert wurden, bilden einen Proteinkomplex (Mmm1-Komplex) in der äußeren Membran, der entweder direkt oder indirekt an das Aktinzytoskelett bindet (Burgess et al., 1994; Sogo & Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998; Aiken Hobbs et al., 2001; Boldogh et al., 2003;

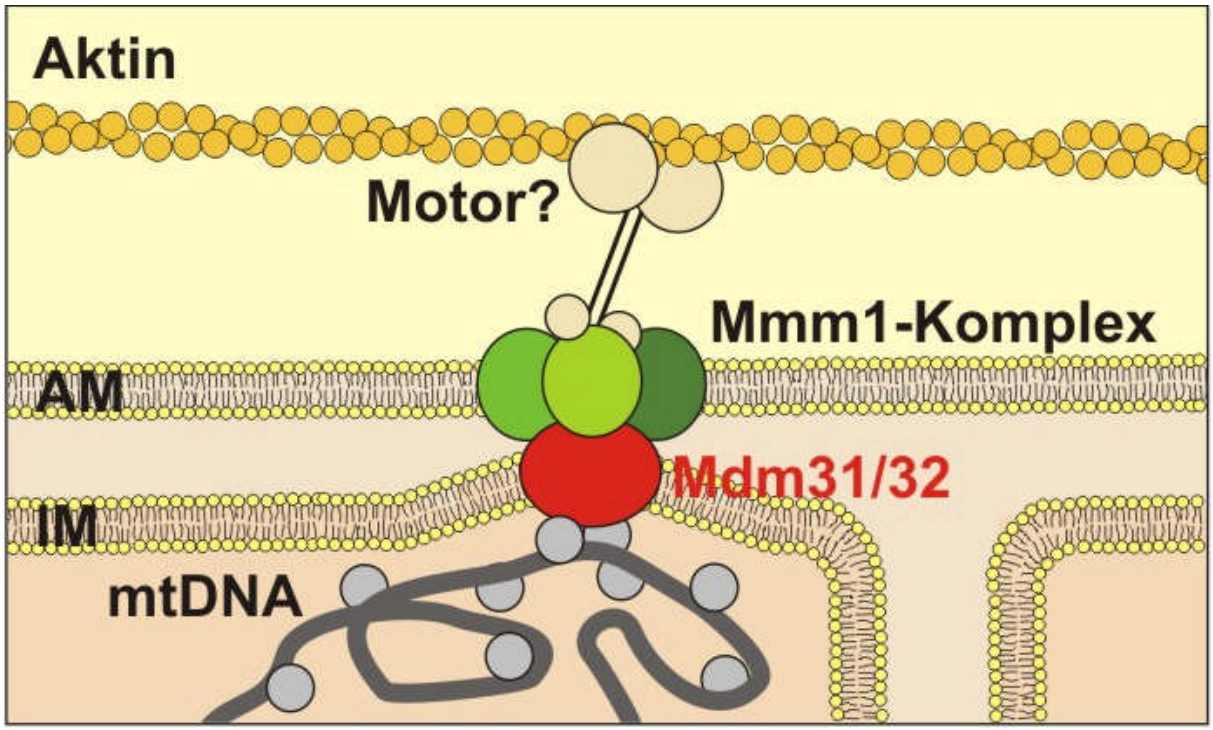

Abbildung 3-2: Hypothetische Struktur des membrandurchspannenden Komplexes zur Vererbung von mtDNA in *S. cerevisiae* (verändert nach B. Westermann, unveröffentlicht)
Der Mmm1-Komplex, bestehend aus den Proteinen Mdm10, Mdm12, und Mmm1 (grün), interagiert direkt oder indirekt mit aktinbindenden Proteinen des Zytoskeletts. Über mögliche Interaktionen mit zwei Proteinen der Innenmembran, Mdm31 und Mdm32 (rot), könnte eine Verknüpfung der mtDNA mit der Segregationsmaschinerie erzielt und damit die Vererbung der mtDNA mit der Vererbung der Mitochondrien synchronisiert werden. AM=Außenmembran, IM=Innenmembran.

Youngman et al., 2004). Fluoreszenzmikroskopische Untersuchungen zeigen, dass alle Proteine des Komplexes in der Nachbarschaft von mtDNA-Nukleoiden vorliegen (Aiken Hobbs et al., 2001; Boldogh et al., 2003), während Deletionen der Gene in einer Fehlorganisation von mtDNA resultieren. Beide Erkenntnisse weisen darauf hin, dass ein membrandurchspannender Komplex die Nukleoide mit der Segregationsmaschinerie im Zytosol verbindet. Da Mmm1 in Hefe beide mitochondrialen Membranen durchspannt (Kondo-Okamoto et al., 2003), wäre es möglich, dass die mtDNA direkt an den Proteinkomplex der äußeren Membran gebunden vorliegt. Dagegen sprechen Daten aus *Neurospora crassa*, die zeigen, dass hier das homologe Protein MMM1 weder die Innenmembran durchspannt, noch für den Erhalt der mtDNA benötigt wird (Prokisch et al., 2000). Daher erscheint es plausibler, dass der Mmm1-Komplex über Proteine der inneren Membran mit der mtDNA verbunden ist (Dimmer et al., 2005[*]). Ein weiteres Protein der äußeren Membran, Mmm2, das ebenfalls für die Aufrechterhaltung der tubulären mitochondrialen Gestalt benötigt wird, scheint zwar nicht stabil mit dem ternären Komplex der äußeren Membran assoziiert zu sein, kooperiert aber möglicherweise auf dynamische Art

mit Mmm1 und wirkt somit auf den Erhalt der mtDNA ein (Youngman et al., 2004).

3.4 Transport von Mitochondrien in *S. cerevisiae*

Mitochondrien werden in der Zelle entlang zytoskelettaler Strukturen transportiert. Organismusabhängig können sowohl Mikrofilamente als auch Mikrotubuli als Transportgeleise verwendet werden. Die Vererbung und die Aufrechterhaltung der mitochondrialen Morphologie resultiert allerdings immer aus Interaktionen zwischen Proteinen der mitochondrialen Außenmembran und der entsprechenden Zytoskelettklasse (Nangaku et al., 1994; Morris & Hollbeck, 1995; Langford, 1995; Pereira et al., 1997; Ligon & Steward, 2000). Die wiederholten und möglicherweise kurzlebigen Bindungen führen dabei einerseits zu Transportereignissen im herkömmlichen Sinn, können andererseits aber auch auf die Gesamtstruktur und Verteilung des mitochondrialen Retikulums innerhalb der Zelle regulierend einwirken (Yaffe, 1999). Da in *S. cerevisiae* Mitochondrien ausschließlich über das Aktinzytoskelett bewegt werden (Drubin et al., 1993; Lazzarino et al., 1994; Hermann & Shaw, 1998) ist die Bäckerhefe ein geeigneter Modellorganismus für Untersuchungen zum aktinabhängigen mitochondrialen Transport.

3.5 Aufbau und Funktion des Aktinzytoskelettes

Das Aktinzytoskelett besteht hauptsächlich aus dem Protein Aktin, dessen Interaktion an einer Vielzahl bindender und assoziierter Proteine die Zusammensetzung und Struktur der Mikrofilamente bestimmt. In *S. cerevisiae* wird das Protein von einem einzelnen essentiellen Gen, *ACT1*, codiert, das speziesübergreifend stark konserviert ist. In der Zelle liegt Aktin entweder als 43 kDa großes Monomer (G-Aktin) (Kabsch et al., 1990; Schutt et al., 1993; Otterbein et al., 2001) oder als Polymer, dem Filament (F-Aktin) vor (Holmes et al., 1990; Milligan et al., 1990). Das F-Aktin weist anhand von unterschiedlich schnell wachsenden Enden intrinsische Polarität auf. Die funktionellen Aktinfilamente des Zytoskeletts werden schließlich von zwei helixartig umeinander gewundenen Ketten F-Aktin gebildet. Durch ständige Polymerisierungs- und Depolymerisierungsereignisse wird eine hohe Dynamik des Aktinzytoskeletts erreicht (Pollard & Cooper, 1986). Neben der Unterscheidung zwischen monomerem G-Aktin und assembliertem Mikrofilament lässt sich in *S. cerevisiae* das F-Aktin selbst in zwei strukturell divergente Gruppen unterteilen: Zytoplasmatische Kabel in der

Mutterzelle, die aus longitudinal verlaufenden Bündeln von Aktinfilamenten bestehen (Adams & Pringle, 1984), und kortikale Patches, die als Filament-Knäuel charakterisiert sind und in der Knospe an Stellen des polarisierten Wachstums lokalisiert sind (Kilmartin & Adams, 1984; Ford & Pringle, 1991; Mulholland et al., 1994; Pruyne et al., 1998) (Abbildung 3-3).

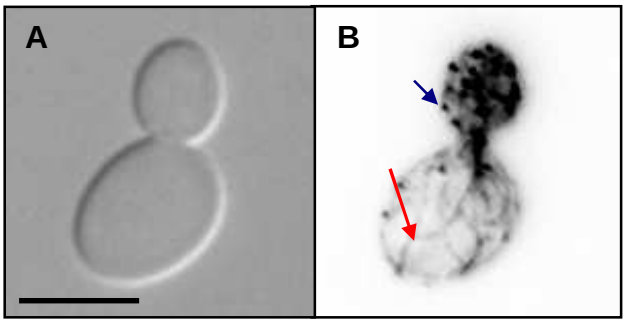

Abbildung 3-3: Polymerisierte Strukturen des Aktinzytoskelettes:
A: Differentielle Interferenz Kontrast (DIC) Aufnahme einer knospenden Hefezelle. Der Balken entspricht einer Länge von 5µm. B: Inverse Darstellung eines mit Rhodamin-Phalloidin gefärbten Aktinzytoskeletts. Der rote Pfeil markiert ein zytoplasmatisches Kabel, der blaue Pfeil die kortikalen Patches. Die Anzucht der Zellen, die Färbung der Aktinfilamente und die Aufnahme der Bilder erfolgte wie in Teilarbeit A beschrieben.

Der eigentliche Assemblierungsprozess von monomerem Aktin zu Mikrofilamenten und deren polarisierte Ausrichtung in der Zelle wird durch eine Vielzahl so genannter aktin-bindender Proteine (ABPs) reguliert, die sowohl an die monomere als auch an die polymere Aktinform binden können (Pollard, 1993). Zu dieser Gruppe zählen neben den essentiellen Proteinen Profilin (Haarer et al., 1990; Magdolen et al., 1988) und Cofilin (Iida et al., 1993; Mirzayan et al., 1992) auch Myosine und myosinähnliche Proteine. Weiterhin existieren Proteine, die einen aktinähnlichen strukturellen Aufbau besitzen, allerdings klar von der Familie der klassischen Aktine getrennt werden: die aktin-verwandten Proteine (actin-related proteins, ARPs) (Schwob & Martin, 1992). Zu den ARPs gehört auch das essentielle Protein Arp2, das zusammen mit Arp3 als Arp2/3 Komplex (Machesky et al., 1994) für Verzweigungen an oder nahe den Enden bereits existierender Filamente verantwortlich ist (Amann & Pollard, 2000; Blanchoin et al., 2000; Robinson et al., 2001).

Ebenso vielfältig wie die Proteine, die am Aufbau des Aktinzytoskelettes beteiligt sind, stellen sich auch die Funktionen der Mikrofilamente in der Zelle dar. Untersuchungen an temperatursensitiven Mutanten zeigen einerseits die Beteiligung des Aktinzytoskeletts an der Aufrechterhaltung der Zellstruktur und am polarisierten Wachstum (Pruyne et al., 2004),

andererseits agiert Aktin als „Gleis“ für die Beförderung von Membranvesikeln und Organellen, wie Vakuolen, Peroxisomen oder Mitochondrien (Wertman et al., 1992; Drubin et al., 1993; Bretscher, 2003; Pruyne et al., 2004).

3.6 Potentielle Motoren für den aktinabhängigen mitochondrialen Transport

Während in *S. cerevisiae* die „Gleis-Funktion“ des Aktinzytoskeletts für den mitochondrialen Transport als gesichert gilt (Drubin et al., 1993; Simon et al., 1995; Simon et al., 1997; Hermann & Shaw, 1998; Fehrenbacher et al., 2004), bleibt die Frage nach dem zugehörigen Motor, also dem eigentlichen Bewegungsantrieb, nach wie vor ungeklärt. Forschungsergebnisse der letzten Jahre zeigen allerdings, dass sowohl ein Motorprotein aus der MyosinV-Familie (Itoh et al., 2002; Boldogh et al., 2004), als auch der Arp2/3 Komplex (Boldogh et al., 2001a) potentiell für die Rolle als aktinassoziierte Motoren geeignet sind.

Hinweise auf den Transport von Mitochondrien unter Beteiligung des Motorproteins Myo2 aus der Klasse V der Myosine

Molekulare Motorproteine stellen den gebräuchlichsten Antriebsmechanismus für den intrazellularen Transport von Zellorganellen dar. Sie bewegen ihre „Fracht“ entlang zytoskelettaler Bahnen, wobei für verschiedene Zytoskelettstrukturen unterschiedliche Sätze von Motorproteinen benötigt werden: Kinesine und Dyneine für den mikrotubuliassoziierten Transport, Myosine für Bewegungen entlang des Aktinzytoskelettes.

Viele Mitglieder der Myosin-Familie sind dabei nach ähnlichem Muster aufgebaut. Die konservierte N-terminale Domäne (Myosin-Kopf) besitzt eine ATPase Funktion, die sowohl zur Antriebserzeugung als auch für die Bindung an das Aktinfilament notwendig ist (Korn & Hammer, 1988). Darauf folgt eine ebenfalls konservierte „Neck“-Region, die durch die Bindung von EF-Hand Proteinen an ihre regulatorische Domäne die Aktivierung der ATPase ermöglicht (Johnston et al., 1991). Die globuläre Tail-Struktur ist innerhalb der Myosine deutlich variabler, was auf eine Verwendung für unterschiedliche zelluläre Funktionen hindeutet (Kiehart, 1990; Cheney et al., 1993; Wright & Jackson, 1996). Weiterhin besitzen alle Myosine mindestens eine leichte Kette, die über ein IQ-Motiv an die schwere Kette bindet (Cheney & Mooseker, 1992; Rayment et al., 1993; Xie et al., 1994).

In der Bäckerhefe wurden insgesamt 5 Proteine aus unterschiedlichen Klassen der Myosin-Familie identifiziert, darunter auch das Motorprotein Myo2, ein unkonventionelles Myosin der Klasse V, das, ebenso wie seine assoziierte leichte Kette Mlc1, für das Wachstum von *S. cerevisiae* essentiell ist (Stevens & Davis, 1998). Proteine dieser Myosin-Klasse sind im Allgemeinen als multifunktionelle Cargo-Transporter charakterisiert und an einer großen Bandbreite von Bewegungen in der Zelle beteiligt (Reck-Peterson et al., 2000). Myo2, ein Myosin der Klasse V stellt in *S. cerevisiae* den Mediator für den aktinabhängigen sekretorischen Vesikeltransport dar (Govindan et al., 1995; Johnston et al., 1991; Schott et al., 1999). Darüber hinaus ist dieses Myosin als Motor für den vakuolären und peroxisomalen Transport beschrieben (Catlett & Weisman, 1998; Fagarasanu et al., 2006a; Fagarasanu et al., 2006b) und am Prozess des polarisierten Wachstums beteiligt (Brockerhoff et al., 1992; Brockerhoff et al., 1994). Die Koordination dieser unterschiedlichen Cargo-Bindungen an den Myo2-Transporter erfolgt durch spezielle Subdomänen des C-Terminus. Die Mutagenese der Cargo-Bindungsdomäne zeigt, dass für den Transport von sekretorischen Vesikeln und der Vakuole zwei verschiedene, cargo-spezifische Regionen in der globulären C-terminalen Myo2 Domäne benötigt werden (Catlett et al., 2000) (Abbildung 3-4).

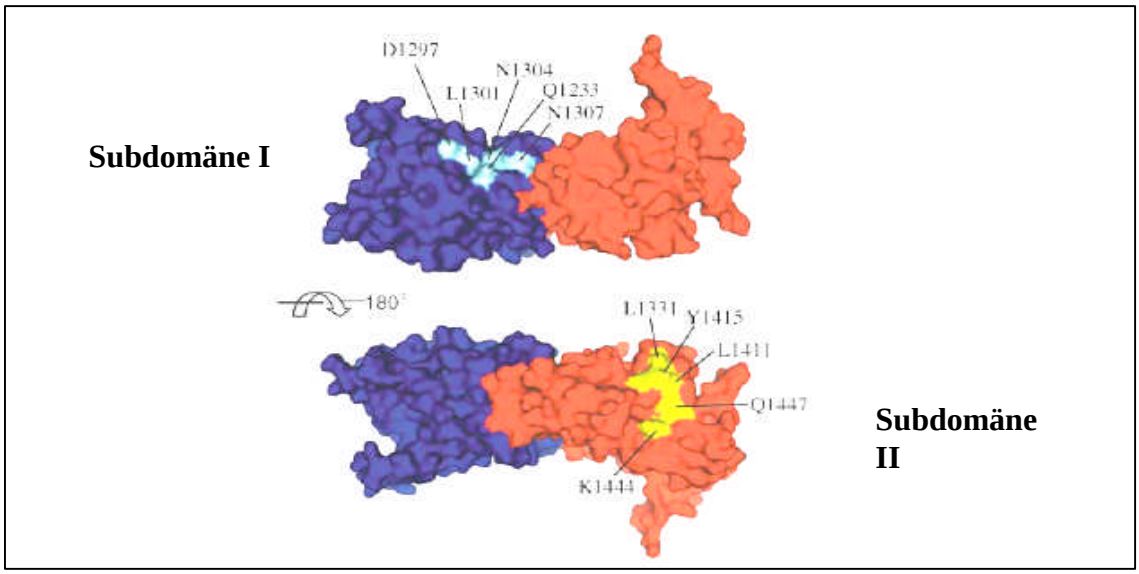

Abbildung 3-4: Molekulare Darstellung der globulären Tail-Domäne des Myosin V Proteins Myo2 aus *S. cerevisiae*.
Die Bindungsstellen für sekretorische Vesikel und die Vakuole sind innerhalb des C-Terminus strukturell voneinander getrennt. Oberflächenreste der Subdomäne I sind blau, die der Subdomäne II rot dargestellt. Die Vakuolenbindestelle ist hellblau markiert, die Bindestelle für sekretorische Vesikel ist gelb hervorgehoben. Vakuolen- und vesikelspezifische Aminosäurereste sind für die jeweiligen Bindedomänen angegeben (nach Pashkova et al., 2006).

Dabei dient die eine Subdomäne der Bindung von Vakuolen, die andere der Bindung von sekretorischen Vesikeln. Die Regionen liegen auf entgegen gesetzten Seiten der länglichen Cargo-Bindungsdomäne. Beide Bereiche weisen an der Oberfläche eine Anzahl hochkonservierter Aminosäurereste auf, die als Bindungsstellen für cargo-spezifische Rezeptoren fungieren (Pashkova et al., 2006).

Während mit Myo2 also ein Myosin als Motorprotein für fast alle Zellorganellen agiert, konnte eine Beteiligung von Myosinen im Allgemeinen und Myo2 im Speziellen am aktinabhängigen Transport von Mitochondrien in *S. cerevisiae* bisher nicht belegt werden. Allerdings gibt es bereits indirekte Hinweise, die auf eine Beteiligung von Myo2 auch am mitochondrialen Transport schließen lassen. Zum einen weist eine temperatursensitive Mutante, *myo2-573,* starke Beeinträchtigungen der mitochondrialen Morphologie und Verteilung auf (Itoh et al., 2002). Wesentlich dabei ist, dass der veränderte Phänotyp in Gegenwart eines normal ausgeprägten Aktinzytoskeletts vorliegt. Somit kann ein sekundärer Effekt durch eine Myo2-bedingte Störung der zytoskelettalen Struktur ausgeschlossen werden. Zum anderen zeigen verschiedene Beispiele aus der Literatur, dass mit den Myosinen verwandte Proteine in anderen Organismen durchaus am Transport von Mitochondrien beteiligt sind. So konnte ein unkonventionelles Myosin auf Mitochondrien in Photorezeptoren von Heuschrecken nachgewiesen werden (Stürmer & Baumann, 1998), während in *Caenorhabditis elegans* Mutanten der Myosin Klasse VI die Verteilung von Mitochondrien während der Spermatogenese gestört ist (Kelleher et al., 2000). Ein pflanzenspezifisches Myosin der Klasse XI kolokalisiert in Maiszellen sowohl mit Mitochondrien als auch mit Chloroplasten (Wang & Pescreta, 2004). Zusätzlich liegen in Säuger-Melanomzellen Mitochondrien mit einem Myosin Klasse V assoziiert vor, das von dem *dilute* Gen codiert wird (Nascimento et al., 1997).

Mitochondrialer Transport durch Arp2/3-Komplex vermittelte Aktinpolymerisation

Der Arp2/3 Komplex ist ein evolutionär hoch konservierter Proteinkomplex, dessen Hauptfunktion in der Initiation der Aktinpolymerisation liegt (Mullins & Pollard, 1999). Zu den Untereinheiten zählen neben den beiden Hauptkomponenten Arp2 und Arp3 noch fünf weitere Proteine (Gournier et al., 2001; Robinson et al., 2001). Nach Aktivierung des Komplexes durch Proteine der WASp/Scar Familie (Welch et al., 1998; Machesky et al.,

1999) bilden Arp2 und Arp3 ein Heterodimer aus, das strukturell dem Aktin-Dimer stark ähnelt. Somit kann der Arp2/3-Komplex freie Aktin-Enden imitieren, die als Nukleationskeim für neue Aktinfilamente in der Zelle verwendet werden (Blanchoin et al., 2000; Robinson et al., 2001).

Diese Funktion des Arp2/3 Systems wird in der Natur auch für Bewegungen von intrazellulären Organismen „zweckentfremdet“. Bakterielle Pathogene wie *Listeria monocytogenes* (*L. monocytogenes*) aktivieren den Komplex durch ein bakterielles Protein ActA und rekrutieren den Arp2/3 Komplex an ihr hinteres Zellende. Dort wird dann die Polymerisierung von Aktinfilamenten initiiert, die letztendlich zu einer vorwärtsgerichteten Bewegung des Bakteriums innerhalb der Wirtszelle führt (Welch et al., 1997; Welch et al., 1998; Stevens et al., 2006). Beobachtungen am Lichtmikroskop zeigen dabei die wachsenden Aktinfilamente als Struktur, die einem Kometenschweif ähnelt.

Jüngere Forschungsergebnisse postulieren, dass in *S. cerevisiae* dieser Bewegungsmechanismus auch zum mitochondrialen Transport entlang des Aktinzytoskelettes eingesetzt wird (Boldogh et al, 2001a). Hinweise darauf geben Experimente, die Untereinheiten des Arp2/3-Komplexes an der mitochondrialen Oberfläche lokalisieren. Zusätzlich weisen Zellen, die eine Mutation im *ARP2* Gen tragen, verminderte Transportgeschwindigkeit und den Verlust linearer, anterograder Bewegung auf (Boldogh et al., 2001a). Weiterhin zeigen lichtmikroskopische Beobachtungen so genannte Aktin-Wolken in der direkten Nähe von Mitochondrien, die auch bei *L. monocytogenes* als Intermediate bei der Bildung von Aktinschweifen auftreten (Welch et al., 1997; Welch et al., 1998). Allerdings unterscheidet sich die aktinabhängige Bewegung von Mitochondrien in einem wesentlichen Punkt von anderen polymerisierungsabhängigen Bewegungsvorgängen: Mitochondriale Bewegung erfolgt an bereits bestehenden Aktinkabeln (Fehrenbacher et al., 2004), während bakterielle Pathogene und internalisierte Endosomen durch das Wachstum eines neu erzeugten kometenähnlichen Aktinendes vorangetrieben werden (Stevens et al., 2006).

4. Fragestellung und Zielsetzung der Arbeit

Obwohl bereits seit über einem Jahrzehnt bekannt ist, dass der mitochondriale Transport in der Bäckerhefe aktinabhängig erfolgt, ist der eigentliche Ablauf der Bewegungsereignisse kaum verstanden und viele Zusammenhänge bleiben lückenhaft oder ungeklärt. Bereits die Frage nach dem eigentlichen Bewegungsmotor und dessen Rezeptoren auf der mitochondrialen Außenmembran wird kontrovers diskutiert. Stellt ein Mitglied der Myosinfamilie den Motor für den Transport dar oder treibt Aktinpolymerisation die Bewegung voran? Welche mitochondrialen Außenmembranproteine kommen als Rezeptoren für die Bindung an das Aktinzytoskelett in Frage? Welche weiteren Proteine sind an der Regulation von Bindung und Bewegung beteiligt? Darüber hinaus bleibt in diesem Zusammenhang zu klären, wie die Segregation von Mitochondrien während Transport- und Vererbungsereignissen an die Vererbung der mtDNA gekoppelt wird. Wie ist der verantwortliche membrandurchspannende Komplex aufgebaut? Erfüllen die beiden Innenmembranproteine Mdm31 und Mdm32 tatsächlich eine Rolle als Komponenten dieses Komplexes?

Ein Ziel der vorliegenden Arbeit war es, eine Hefestamm-Sammlung, die 2/3 aller essentiellen Gene unter Kontrolle eines reprimierbaren Promotors beinhaltet (Mnaimneh et al., 2004), systematisch nach bisher unbekannten Mutanten mit mitochondrialen Defekten zu durchmustern. Es sollten dabei essentielle Proteine und zelluläre Prozesse erfasst werden, die an der mitochondrialen Morphogenese und Verteilung beteiligt sein könnten. Darauf aufbauend sollten anhand des Screens identifizierte Komponenten der mitochondrialen Morphogenese funktionell charakterisiert werden. Insbesondere sollte anhand von molekularbiologischen und zytologischen Methoden die Frage geklärt werden, ob eines der ermittelten essentiellen Proteine als Mediator der Interaktion zwischen Mitochondrien und dem Aktinzytoskelett und/oder als Motor für den mitochondrialen Transport in Frage kommt.

Zusätzlich sollte geklärt werden, welchen Anteil die beiden mitochondrialen Innenmembran-Proteine Mdm31 und Mdm32 an der zytoskelettabhängigen, koordinierten Vererbung von mtDNA besitzen. Dazu sollte untersucht werden, ob in Mdm31-, bzw. Mdm32-Deletionsmutanten die räumliche Nähe von mtDNA-Nukleoiden und mitochondrialen Außenmembranproteinen des TMS aufgehoben wird.

5. Ergebnisse und Diskussion

5.1 Durchmusterung einer Hefe-Bibliothek essentieller Gene nach Mutanten mit Morphologie- und Verteilungsdefekten

Für die Identifizierung essentieller mitochondrialer Morphologie- und Vererbungskomponenten wurde eine Hefe-Bibliothek essentieller Gene systematisch durchgemustert. Die Expression jedes dieser Gene kann durch Zugabe von Doxyzyklin (DOX) über einen spezifischen regulierbaren Promotor ($TetO_7$) ausgeschaltet werden (Mnaimneh et al., 2004). Zur Durchführung des Screens wurden alle $TetO_7$-Stämme mit und ohne Zugabe von DOX angezogen und hinsichtlich ihres mitochondrialen Phänotyps untersucht. Um die Morphologie fluoreszenzmikroskopisch erfassen zu können, wurden die Organellen dazu mit spezifischen Farbstoffen angefärbt.

Nach Aussonderung aller morphologisch unauffälligen Stämme blieben 119 Mutanten übrig, die schwere mitochondriale Defekte aufwiesen (Altmann & Westermann, 2005*, Supplemental Material). Die jeweils verantwortlichen Gene und deren Produkte wurden nach Angaben der Saccharomyces Genome Database (Christie et al., 2004) und der Comprehensive Yeast Genome Database (Güldener et al., 2005) funktionellen Gruppen zugeordnet. Fünf dieser Kategorien enthielten einen signifikant hohen Prozentsatz an Mutanten mit veränderter mitochondrialer Morphologie (Altmann & Westermann, 2005*, Abbildung 1): „Ergosterol Biosynthese“, „Mitochondrialer Proteinimport“, „Vesikulärer Transport und Sekretion“, „Ubiquitin/26S Proteasom-abhängiger Proteinabbau“, „Aktinzytoskelettabhängiger Transport“. Die Prozesse, die diesen fünf Kategorien zu Grunde liegen, spielen folglich für die Aufrechterhaltung der mitochondrialen Morphologie eine wesentliche Rolle. Ob der Einfluss der identifizierten Mutanten auf die Strukturgebung allerdings primärer oder sekundärer Natur ist und welches dieser essentiellen Proteine als mögliche mitochondriale Transportkomponente in Frage kommen könnte, wird nachfolgend diskutiert.

Die Beteiligung der Ergosterol-Biosynthese an der mitochondrialen Strukturgebung

Alle elf in der Bibliothek enthaltenen Stämme mit promotorregulierten Genen, die für Komponenten des Ergosterol-Biosyntheseweges codieren, zeigten nach DOX-Zugabe aggregierte, geschwollene Mitochondrien (Altmann & Westermann, 2005*, Abbildung 2). Das

in Pilzen vorliegende Membranlipid, das im Wesentlichen dem Cholesterin von Tieren entspricht, ist also wesentlich an der Aufrechterhaltung der mitochondrialen Struktur beteiligt. Aktuelle Veröffentlichungen, die die Ergosterole als Komponenten der homotypischen Vakuolenfusion in Hefe beschreiben, weisen dabei auf eine direkte Beteiligung der Lipide an der mitochondrialen Morphogenese hin (Kato & Wickner, 2001; Fratti et al., 2004). Aufgrund des niedrigen Gehalts von Ergosterolen in der vakuolären Membran wird postuliert, dass sich die Aufgabe der Lipide während der Vakuolenfusion nicht auf die Modulation physikalischer Eigenschaften der Membranen beschränkt. Vielmehr erstreckt sie sich spezifisch auf die Aktivierung und/oder Anordnung von Fusionsproteinen. Eine ähnliche Funktion der Ergosterole als Komponenten der Fusionsmaschinerie wäre auch im Falle der Mitochondrien vorstellbar.

Die Rolle von Proteinimport- bzw. Proteinassemblierung bei der Aufrechterhaltung der mitochondrialen Morphologie

Auch ein Großteil der untersuchten mitochondrialen Proteinimport- und Proteinassemblierungsmutanten zeigten eine veränderte Morphologie (Altmann & Westermann, 2005[*], Abbildung 3), die u.a. durch das Fehlen essentieller Untereinheiten des TOM-Komplexes, des TIM23-Komplexes und des SAM-Komplexes (Sortierungs- und Assemblierungsmaschinerie) verursacht wird. Daher liegt die Schlussfolgerung nahe, dass der Proteinimport und die Proteinassemblierung für den Erhalt der mitochondrialen Struktur benötigt werden. Analoge Befunde für Tom7, einer nicht-essentiellen Untereinheit des TOM-Komplexes, stützen diese These (Dimmer et al., 2002). Die postulierte Beteiligung des SAM-Komplexes an der mitochondrialen Morphogenese wird durch Daten gestärkt, die für temperatursensitive Mutanten der Komplexuntereinheit Sam50 ähnliche morphologische Defekte beschreiben (Meisinger et al., 2004). Dabei wird angenommen, dass Sam50 zusammen mit weiteren Außenmembranproteinen für den Aufbau eines funktionalen TOM-Komplexes verantwortlich ist. Allerdings scheint die Beteiligung der Import- und Assemblierungsmaschinerie an der mitochondrialen Morphogenese in erster Linie ein sekundärer Effekt zu sein, da viele der bekannten strukturbildenden mitochondrialen Proteine in die Mitochondrien importiert und in eine der beiden Membranen eingebaut werden. Daher resultiert eine Störung des Imports oder der Sortierung von primären

Morphologiekomponenten in die Subkompartimente zwangsläufig in der Ausprägung von Mitochondrien mit gestörter Morphologie.

Die Verbindung zwischen vesikulärem Transport und der mitochondrialen Morphologie

Ungefähr die Hälfte der promotorregulierten Gene, die für vesikuläre Transportproteine codieren, zeigten nach Inkubation mit DOX fragmentiert-aggregierte Mitochondrien (Altmann & Westermann, 2005*, Abbildung 5). Erwähnenswert sind aus dieser Gruppe die Proteine Sec17 und Sec18, Homologe der Säugerproteine alpha-SNAP und NSF, die dort für die Regulation der Disassemblierung von SNARE-Komplexen verantwortlich sind, sowie das Translokon SEC61 und Srp101, die Alpha-Untereinheit des SRP-Rezeptors (Signal Recognition Particle). Unter Berücksichtigung bereits publizierter Fakten müssen diese Ergebnisse des Screens allerdings unterschiedlich bewertet werden. Während temperatursensitive *sec18* Mutanten keinerlei Beeinträchtigung der mitochondrialen Morphologie aufweisen (Nunnari et al., 1997) zeigten temperatursensitive *srp101* Mutanten ebenfalls fragmentierte Mitochondrien (Prinz et al., 2002). Eine Beteiligung des ER und des gesamten Sekretionsprozesses an der mitochondrialen Morphogenese bleibt auf Grund des hohen prozentualen Anteils von Morphologiemutanten trotzdem sehr wahrscheinlich. Dieses Ergebnis eröffnet einen vollständig neuen Weg, um den Lipidaustausch zwischen dem ER und den Mitochondrien zu untersuchen.

Einfluss des Ubiquitin/26S Proteasom-Systems auf die mitochondriale Morphogenese

Ungefähr zwei Drittel aller Stämme mit promotorregulierten Genen, die für essentielle Untereinheiten des Proteasoms codieren, weisen nach Abschalten des Promotors starke Störungen des mitochondrialen Reticulums in Form von fragmentiert-aggregierten Mitochondrien auf (Altmann & Westermann, 2005*, Abbildung 6). Hingegen scheinen Stämme, denen zellzyklus-spezifische Faktoren für den Proteinabbau fehlen, in der mitochondrialen Morphologie nicht beeinträchtigt. Frühere Beobachtungen zeigten bereits, dass Ubiquitin (Fisk & Yaffe, 1999), das 26S-Proteasom (Rinaldi et al., 1998) und der SCF-abhängige Proteinabbau (Fritz et al., 2003) an der Aufrechterhaltung der mitochondrialen

Morphologie beteiligt sind. Dabei kontrolliert das Ubiquitin/26S-Proteasomsystem Fusions- und Spaltungsereignisse vermutlich sekundär durch den Abbau von regulierenden Faktoren.

Beteiligung von Komponenten des Aktinzytoskelettes an der mitochondrialen Struktur

Mitochondrien bewegen sich in allen Organismen entlang zytoskelettaler Strukturen. In der Bäckerhefe übernehmen speziell die Aktinfilamente die Rolle dieser Transportgleise (Hermann & Shaw, 1998; Jensen et al., 2000; Boldogh et al., 2001b). Fehler in der Anordnung der Filamente oder die Depletion von strukturgebenden Aktinkomponenten resultieren also zwangsläufig in der Ausbildung von mitochondrialen Transport- und Vererbungsdefekten. Zwei Drittel der Stämme mit essentiellen Genen, die für aktinassoziierte oder aktinbindende Protein codieren, zeigten nach Ausschalten des Promotors aggregierte oder ringförmige Mitochondrien (Altmann & Westermann, 2005*, Abbildung 4). Nach Doppelfärbung von Aktin und Mitochondrien konnten anhand von fluoreszenzmikroskopischen Untersuchungen diese Stämme in zwei Kategorien aufgeteilt werden: Klasse A (Veränderte Mitochondrien und fehlorganisiertes Aktinzytoskelett) und Klasse B (Veränderte Mitochondrien und intaktes Aktinzytoskelett). Zu den Mitgliedern der Klasse A gehörten neben Komponenten des ARP2/3 Komplexes auch Untereinheiten des CCT Chaperons, das für die Aktin- und Tubulinfaltung verantwortlich ist. Weiterhin zählten auch das Motorprotein Myo2 und weitere Faktoren der Aktinorganisation zu dieser Gruppe. Die Klasse B enthielt lediglich Mlc1, eine leichte Kette, die in *S. cerevisiae* mit den Motorproteinen Myo2 und Myo1 assoziiert vorliegt.

Während einerseits durch den prozentual hohen Anteil an Mutanten in der Gruppe der Aktinkomponenten die wichtige Rolle des Aktinzytoskeletts für die mitochondriale Morphogenese unterstrichen wird, bleibt andererseits allerdings die Frage offen, ob die Einwirkung der Mutanten auf die Gestaltbildung primärer oder sekundärer Natur ist. Es scheint plausibel, dass die Senkung des Expressionslevels sämtlicher Proteine der Aktinassemblierung bzw. der Aktinorganisation in der Fehlorganisation des Aktinzytoskeletts resultiert. Daher ist der Einfluss dieser Komponenten auf die Morphogenese sicherlich sekundärer Natur.

Dagegen wurde bereits in anderen Publikationen ein möglicher primärer Einfluss des Arp2/3 Komplexes (Boldogh et al., 2001a; Fehrenbacher et al., 2004) und des Motorproteins Myo2 (Itoh et al., 2002; Boldogh et al., 2004; Itoh et al., 2004) auf den aktinabhängigen, mitochondrialen Transport und damit auch auf die Morphologie postuliert. Welcher der beiden Komplexe nun tatsächlich als Motor in Frage kommt ist umstritten (Boldogh et al., 2001a; Boldogh et al., 2004; Itoh et al., 2004). Die Ergebnisse aus der Durchmusterung der Bibliothek lassen allerdings vermuten, dass Myo2 und seine assoziierte leichte Kette Mlc1 eine weitaus bedeutendere Rolle am mitochondrialen Transport spielen könnten als bisher angenommen wurde. Denn für die Zellen der Stämme *$TetO_7$-mlc1* und *$TetO_7$-myo2* wurden auch ohne Inkubation mit DOX mitochondriale Defekte in Gegenwart eines intakten Aktinzytoskeletts beobachtet (Altmann & Westermann, 2005*, Abbildung 4). Dies spricht dafür, dass unter nicht-reprimierenden Bedingungen die Expression über den DOX-regulierbaren Promotor nicht dem physiologischen Expressionslevel des endogenen Promotors gleicht. Somit liegen also auch ohne Induktion beide Proteine in nicht-physiologischen Konzentrationen, vermutlich überexprimiert, in der Zelle vor. Dieser Proteinüberschuss könnte im Stamm *$TetO_7$-myo2* aufgrund der limitierenden Menge der essentiellen leichten Kette Mlc1 zu den toxischen Effekten führen (Stevens & Davis, 1998), die in der Ausprägung der ringähnlichen Mitochondrien resultieren. Da gleichzeitig mit den veränderten Phänotypen in den Stämmen *$TetO_7$-mlc1* und *$TetO_7$-myo2* ein normal ausgeprägtes Aktinzytoskelett zu beobachten war, handelt es sich bei dem Einfluss der Mutationen auf die mitochondriale Morphogenese mit hoher Wahrscheinlichkeit nicht um einen sekundären Effekt. Vielmehr kann vermutet werden, dass Myo2 und Mlc1 eine primäre Rolle zumindest bei der Bindung von Mitochondrien an die Aktinfilamente spielen. Damit sind beide Proteine Kandidaten für Komponenten des mitochondrialen Transportes und werden nachfolgend (Altmann et al., 2007*) als solche näher charakterisiert.

5.2 Beteiligung des Motorproteins Myo2 und seiner leichten Kette Mlc1 am Transport und der Vererbung von Mitochondrien in *S. cerevisiae*

Der Ablauf des aktinabhängigen Transports von Mitochondrien ist bis heute ungeklärt. Aktuelle Hypothesen stützen sich entweder auf Myosine als mögliche Bewegungsmotoren (Itoh et al., 2002; Itoh et al., 2004) oder favorisieren die Arp2/3-Komplex-vermittelte Aktinpolymerisation als Antrieb für den Transport der Organellen (Boldogh et al., 2001b; Fehrenbacher et al., 2004). Ein wichtiges Resultat des Screens war die Identifizierung zweier

Proteine, des Myosin-Motorproteins Myo2 und seiner leichte Kette Mlc1, deren Einfluss auf die mitochondriale Morphologie auf eine mögliche direkte Beteiligung beider Proteine am Transport der Organellen schließen ließ. Durch nachfolgend beschriebene Untersuchungen sollten tiefere Einblicke in die Rolle von Myo2 am mitochondrialen Transport gewonnen werden.

Depletion von Myo2 und Mlc1 verändert die mitochondriale Gestalt und Funktion

Zunächst war es notwendig, zu belegen, dass der veränderte mitochondriale Phänotyp in den *$TetO_7$-myo2* und *$TetO_7$-mlc1*-Stämmen nicht das Resultat einer Fehlorganisation des Aktinzytoskeletts ist, sondern einen primären Effekt darstellt, der ausschließlich auf die Abwesenheit beider Proteine zurückzuführen ist. Dazu wurden Zellen beider Stämme für unterschiedliche Zeitspannen in Gegenwart von DOX inkubiert, um die Struktur von Mitochondrien und Aktin bei unterschiedlichen Myo2-Konzentrationen beobachten zu können. Untersuchungen am Fluoreszenzmikroskop zeigten dabei, dass im Zeitraum 0-15 h nach DOX-Zugabe mit 30-50% eine signifikante Anzahl von Zellen ringförmige Mitochondrien in Gegenwart eines intakten Zytoskeletts aufwiesen. (Altmann et al., 2007*, Abbildung 1A). Dies verdeutlicht, dass in den *$TetO_7$-myo2* und *$TetO_7$-mlc1*-Stämmen die gestörte Morphogenese eindeutig primäre Konsequenz der Myo2- bzw. Mlc1-Depletion ist und nicht durch Veränderungen des Zytoskeletts erklärt werden kann.

Nach den fluoreszenzmikroskopischen Beobachtungen sollten elektronenmikroskopische Untersuchungen genaueren Aufschluss über die Ultrastruktur der Mitochondrien aus den *$TetO_7$-myo2* und *$TetO_7$-mlc1*-Stämmen geben. Auch hier wiesen die Organellen eine ringförmig-aggregierte Gestalt auf (Altmann et al., 2007*, Abbildung 1B). Das Fehlen von Cristaestrukturen legte die Vermutung nahe, dass beide Mutanten, ähnlich wie andere Stämme mit Morphologiedefekten (Berger & Yaffe, 2000), das mitochondriale Genom verloren haben könnten. Tatsächlich wiesen Zellen der *$TetO_7$-myo2* und *$TetO_7$-mlc1*-Stämme, in denen durch einen interkalierenden Farbstoff die mtDNA angefärbt wurde, keine Nukleoidstrukturen auf (Altmann et al., 2007*, Abbildung 1C). Dies verdeutlicht, dass physiologische Konzentrationen von Myo2 und Mlc1 für die Vererbung funktioneller Mitochondrien wichtig sind.

Spezifische Mutationen in der globulären Tail-Domäne von Myo2 zeigen einen Einfluss auf die mitochondriale Morphogenese

Nachdem anhand der Stämme *TetO$_7$-myo2* und *TetO$_7$-mlc1* der direkte Einfluss von Myo2 auf die mitochondriale Morphogenese und Funktionsfähigkeit gezeigt werden konnte, stellte sich nun die Frage, welche Domäne von Myo2 für die Aufrechterhaltung der mitochondrialen Struktur verantwortlich sein könnte. Dabei ist aus der Literatur bereits bekannt, dass die globuläre Tail-Domäne von Myo2 die Bindung des Motorproteins an verschiedene Cargo-Membranen wie Vakuolen oder sekretorische Vesikel vermittelt (Catlett & Weisman, 1998; Schott et al., 1999; Pashkova et al., 2006). Folglich liegt die Vermutung nahe, dass diese Domäne auch für die Anbindung von Myo2 an die Mitochondrien von Bedeutung sein könnte. Die dadurch erzeugte Interaktion zwischen den Organellen und dem Aktinzytoskelett wäre dann maßgeblich für eine wildtypische mitochondriale Morphologie.

Um diese Hypothese zu überprüfen, wurden temperatursensitive *myo2*-Stämme mit Punktmutationen in vakuolen- und vesikelspezifischen Subdomänen des C-Terminus (Catlett & Weisman, 1998; Catlett et al., 2000; Pashkova et al., 2006) hinsichtlich Störungen der mitochondrialen Verteilung und Morphologie fluoreszenzmikroskopisch untersucht. Dabei zeigten alle Punktmutanten mit Aminosäureaustauschen in der vesikelspezifischen Domäne wildtypische mitochondriale Tubuli in Mutter- und Tochterzelle. Hingegen wiesen sechs von acht vakuolenspezifischen Mutanten aggregierte Mitochondrien in Gegenwart eines intakten Aktinzytoskeletts auf. Die Verteilung der Organellen auf die Tochterzellen war stark gestört; der Großteil aller Knospen wies keine Mitochondrien auf (Altmann et al., 2007*, Abbildung 3A, 3C und 5B). Die Tatsache, dass zwei Aminosäureaustausche in dieser Domäne keinen Effekt zeigen, gibt einen Hinweis darauf, dass der Proteinabschnitt zur Bindung von Mitochondrien zwar mit der Bindungsstelle für Vakuolen überlappen könnte, nicht aber mit dieser identisch ist. Dieses Ergebnis macht deutlich, dass die für die Bindung der Vakuolen verantwortliche C-terminale Subdomäne von Myo2 auch einen Anteil an der Aufrechterhaltung der mitochondrialen Morphologie und Verteilung innehat.

Myo2 und Mlc1 sind notwendig für die Interaktion zwischen Mitochondrien und Aktinfilamenten in vitro

Um die Beteiligung von Myo2 und Mlc1 an der Interaktion zwischen Mitochondrien und Aktinfilamenten zu untermauern, wurde auf den etablierten *in vitro* Sedimentations-Assay zurückgegriffen (Lazzarino et al., 1994). In diesem Versuch werden isolierte Mitochondrien und polymerisierte Aktinfilamente in An- bzw. Abwesenheit von ATP miteinander inkubiert, über ein Saccharosekissen zentrifugiert und die Menge an gebundenem Aktin im Mitochondrien-Pellet per Westernblot-Analyse quantifiziert. Während ohne ATP eine irreversible Bindung eintritt, findet nach Zugabe von ATP keine stabile Interaktion zwischen den Komponenten statt. Auch nach Salzextraktion der peripheren Membranproteine der Mitochondrien ist eine Bindung zwischen den Organellen und Aktin nicht möglich. Die Zugabe von peripheren Membranproteinen (Salzextrakt) zu den extrahierten Mitochondrien stellt die Bindungskapazität vollständig wieder her (Altmann et al., 2007*, Abbildung 2A), was darauf hinweist, dass die ATP-sensitive Bindung der Organellen an das Zytoskelett durch periphere Membranproteine vermittelt wird.

Für nachfolgend beschriebenen Sedimentations-Assay wurden Mitochondrien aus den Stämmen *TetO$_7$-myo2* bzw. *TetO$_7$-mlc1* sowie aus zwei temperatursensitiven Punktmutanten mit Aminosäureaustauschen in der vakuolenspezifischen Bindungsdomäne isoliert. Das Experiment ergab in Abwesenheit von ATP für die Mitochondrien aller Stämme eine Verringerung der Aktin-Bindungskapazität auf ungefähr 50% gegenüber der Bindungskapazität von Wildtypmitochondrien (Altmann et al., 2007*, Abbildung 2B und 4A). Die Inkubation von *TetO$_7$-myo2* bzw. *TetO$_7$-mlc1*-Mitochondrien mit Salzextrakt aus Wildtyp-Mitochondrien vor der Zugabe der Organellen zu den Aktinfilamenten stellte die Bindungskapazität fast gänzlich wieder her (Altmann et al., 2007*, Abbildung 2C). Zusätzlich inhibiert die Zugabe von Antikörpern gegen Myo2 zu Wildtyp-Mitochondrien die ATP-sensitive Aktinbindung vollständig. (Altmann et al., 2007*, Abbildung 2D).

Um die beschriebenen Beobachtungen auch fluoreszenzmikroskopisch visualisieren zu können, wurde der *in vitro* Assay modifiziert. Mitochondrien wurden von mtGFP exprimierenden Wildtyp-, *TetO$_7$-myo2*- und *TetO$_7$-mlc1*-Zellen sowie den beiden Punktmutanten isoliert. Die gereinigten Organellen wurden mit Alexa Fluor 568-markierten Aktinfilamenten in Abwesenheit von ATP inkubiert und danach direkt

fluoreszenzmikroskopisch untersucht. (Altmann et al., 2007*, Abbildungen 2E, 2F und 4B). Dabei stimmten die Ergebnisse mit den Resultaten des Sedimentationsassays gut überein.

Diese Ergebnisse zeigen, dass mitochondrial gebundenes Myo2 und seine leichte Kette Mlc1 die Interaktion zwischen Aktin und den Mitochondrien vermitteln. Dabei scheint besonders die intakte Struktur der C-terminalen, vakuolenspezifischen Bindungsdomäne von Myo2 wesentlich für die Anbindung der Organellen an die Filamente *in vitro* zu sein. Allerdings genügt eine Restexpression der Proteine in den *TetO$_7$-myo2* bzw. *TetO$_7$-mlc1* Stämmen offensichtlich für die immer noch vorhandene, wenn auch eingeschränkte Möglichkeit der Mitochondrien an Aktinfilamente zu binden. Dagegen blockiert die Zugabe des Myo2 gerichteten Antikörpers die Funktion des Proteins komplett. In den temperatursensitiven Stämmen scheint auch unter restriktiven Bedingungen noch genügend funktionsfähiges Myo2 vorhanden zu sein, um ein geringes Maß an Interaktion zwischen Organellen und Filamenten zu ermöglichen.

Myo2 ist ein aktiver Motor für den anterograden Transport von Mitochondrien in die Knospe

In temperatursensitiven *myo2*-Stämmen mit Punktmutationen in der vakuolenspezifischen Bindungsdomäne war zu beobachten, dass die Vererbung der Organellen auf die Tochterzellen nur stark eingeschränkt erfolgte. Da dies Resultat eines mitochondrialen Transportsdefekts darstellen könnte, wurde mittels zeitaufgelöster 3D-Mikroskopie lebender Zellen versucht, die Bewegungsereignisse von Mitochondrien in den Zellen einer Punktmutante nachzuvollziehen.

Dabei war zu beobachten, dass sich die Mitochondrien über einen Zeitraum von 30 min nur langsam und über geringe Distanzen bewegten. Häufig wurden Knospen verschiedenster Größe gänzlich ohne Mitochondrien vorgefunden (Altmann et al., 2007*, Abbildungen 5A). Eine detaillierte Betrachtung der Aufnahmen ergab, dass, verglichen mit Wildtypzellen, nur ein geringer prozentualer Bruchteil von Mitochondrien in der Mutante noch in der Lage war, die Knospungsstelle zu passieren.

Dieses Resultat zeigt, dass der knospengerichtete Transport in Punktmutanten mit Aminosäureaustauschen in der vakuolenspezifischen Bindungs-Domäne am Myo2 C-

Terminus fast vollständig blockiert ist. Dieses Ergebnis impliziert eine direkte Beteiligung des Myosins am anterograden Transport von Mitochondrien. Damit ist erstmalig bewiesen, dass ein Motorprotein aus der Familie der Myosine in *S. cerevisiae* direkt in die koordinierte, aktive Bewegung von Mitochondrien involviert ist.

5.3 Die Beteiligung der beiden mitochondrialen Innenmembranproteine Mdm31 und Mdm32 an der Vererbung von mtDNA in *S. cerevisiae*

Die mtDNA liegt in der mitochondrialen Matrix in Form von Proteinkomplexen vor, den so genannten Nukleoiden. Von ihr codierte Proteine sind für die Funktion der Mitochondrien von wichtiger Bedeutung. Dies impliziert, dass neben der Vererbung des gesamten Organells auch die koordinierte Weitergabe von mtDNA in die Tochterzelle einen wesentlichen Prozess darstellt. Dabei wird die Verteilung wahrscheinlich in Zusammenarbeit mit einer zytoskelettabhängigen Segregationsmaschinerie im Zytosol erreicht (Aiken Hobbs et al., 2001; Berger & Yaffe, 2000; Boldogh et al., 2003; Meeusen & Nunnari, 2003). Ein Komplex (TMS), der beide mitochondrialen Membranen umschließt, dient dabei als Linker zwischen dem Segregationsapparat und den Nukleoiden in der mitochondrialen Matrix. Der TMS scheint dabei als eine Art Gerüststruktur zu agieren. Er bietet Ankerpunkte, über die die Mitochondrien an das Aktinzytoskelett gebunden werden (Boldogh et al., 1998) und somit in der Lage sind, ihre tubuläre Struktur aufrechtzuerhalten. Gleichzeitig kann durch die Anbindung von Nukleoiden die Vererbung von mtDNA in morphologisch intakten Mitochondrien sichergestellt werden. Während bereits einige Proteine (Mdm10, Mdm12, Mmm1) identifiziert wurden, die als außenmembranständige Komponenten des TMS in Frage kommen (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen & Nunnari, 2003), besteht über die Identität der Innenmembran-Komponenten noch Unklarheit. Im Rahmen der Durchmusterung einer Deletions-Bibliothek nicht-essentieller Hefegene wurden allerdings zwei Gene entdeckt, *MDM31* und *MDM32*, deren Deletion große, sphärische Mitochondrien erzeugt, wie sie auch in den Mutanten Δ*mdm10*, Δ*mdm12* und Δ*mmm1* zu beobachten waren (Dimmer et al., 2002). Dies gab einen ersten Hinweis darauf, dass die Proteine Mdm10, Mdm12 und Mmm1 am selben Prozess, nämlich der Vererbung von mtDNA, beteiligt sein könnten.

Mdm31 und Mdm32 wurden daraufhin hinsichtlich einer möglichen Rolle als Intermembran-Komponenten des membranumspannenden Komplexes untersucht (Dimmer et al., 2005[*]). Kai

Stefan Dimmer lokalisierte über Zellfraktionierungs-Experimente beide Proteine in der mitochondrialen Innenmembran und untermauerte mit dem Nachweis der synthetischen Letalität von Δ*mdm31*- und Δ*mdm32*-Stämmen mit Δ*mmm1*-, Δ*mdm10*- und Δ*mdm12*-Mutanten die Hypothese, dass diese Proteine an derselben zellulären Funktion beteiligt sein könnten. Außerdem zeigte er durch fluoreszenzmikroskopische Analysen für beide Deletionsmutanten starke Störungen in der mitochondrialen Verteilung, Bewegung und Fusion, die nicht auf einer inhibierten Interaktion der Mitochondrien mit dem Zytoskelett beruhen. Eine wahrscheinlichere Erklärung für die stark ausgeprägten Morphologiedefekte liegt in der elektronenmikroskopisch erkennbaren, stark veränderten Ultrastruktur der Mitochondrien aus den Δ*mdm31*- und Δ*mdm32*-Stämmen. Ein ähnlicher Phänotyp ließ sich bereits für Mitochondrien aus Δ*mmm1*-Stämmen beobachten, der mit dem Fehlen interner Gerüststrukturen und dem daraus resultierenden Verlust von mtDNA in Zusammenhang gebracht wurde (Aiken Hobbs et al., 2001). Dies lässt vermuten, dass auch Mdm31 und Mdm32, zusammen mit Mmm1, an der Aufrechterhaltung der Struktur beteiligt sind und gleichzeitig Ankerpunkte für mtDNA-Nukleoide darstellen könnten.

In meinem Teil dieser Arbeit beschäftigte ich mich mit der fluoreszenzmikroskopischen Bestätigung dieser Hypothese. Falls Mdm31 und Mdm32 tatsächlich zusammen mit Mmm1 Komponenten derselben mtDNA-bindenden Gerüststruktur sind, dann sollte nach Deletion beider Gene die in Wildtypzellen vorhandene räumliche Nähe zwischen Mmm1 in der äußeren Membran und den Nukleoiden in der Matrix (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen & Nunnari, 2003) aufgehoben sein. Für den experimentellen Nachweis wurde in Δ*mdm31*- und Δ*mdm32*-Stämmen Mmm1 mit einem Fluoreszenzmarker versehen. Gleichzeitig wurde eine Komponente des Nukleoidkomplexes (Abf2) mit einem GFP-Tag markiert, bzw. die mtDNA selbst angefärbt. In beiden Deletionsmutanten war daraufhin zu beobachten, dass zwischen den Mmm1-Foci und den Nukleoiden große räumliche Abstände lagen. Die mtDNA-Strukturen waren diffus organisiert (Dimmer et al., 2005[*]).

Das Experiment zeigt, dass Mdm31 und Mdm32 als Bindeglieder zwischen der mtDNA und dem Mmm1-Komplex in der äußeren Membran agieren. Dies belegt zusammen mit den Ergebnissen von Kai Stefan Dimmer eine Beteiligung beider Innenmembranproteine am TMS und beweist die Bedeutung der Proteine für die koordinierte Anbindung der mtDNA an den Segregationsapparat.

5.4 Schlussfolgerungen und Ausblick

Die Resultate der vorliegenden Arbeit belegen die Rolle von Myo2 und seiner assoziierten leichten Kette Mlc1 als Mediatoren der Interaktion zwischen Mitochondrien und Aktinfilamenten. Dies geht mit Beobachtungen anderer Arbeiten konform, die zeigen, dass Mutationen in den Myo2 Bindungsstellen auf den Aktinkabeln bzw. die Sättigung dieser Stellen mit funktionslosen Myo2-Kopfdomänen in einem Verlust der Aktin-Bindungskapazität von Mitochondrien resultieren (Drubin et al., 1993; Lazzarino et al., 1994). In der vorliegenden Arbeit konnte zusätzlich belegt werden, dass das Myosin als Motor für den anterograden Transport der Organellen benötigt wird. Beide Beobachtung stehen in direktem Widerspruch zu dem Modell, das eine direkte Rolle von Myo2 am mitochondrialen Transport ablehnt und den Arp2/3-Komplex als Motor und Mediator der Interaktion zwischen Mitochondrien und dem Aktinzytoskelett favorisiert (Boldogh et al., 2004). Als Hauptargument gegen Myo2 als Motorprotein für den mitochondrialen Transport wird dabei angeführt, dass in bestimmten *myo2*-Mutanten keine negative Beeinflussung der Bewegungsgeschwindigkeit von Mitochondrien festzustellen ist (Boldogh et al., 2004). Dieses Argument ist allerdings nicht stichhaltig, da die Geschwindigkeit von Myo2 für die Bewegungsgeschwindigkeit der Mitochondrien nicht zwangsläufig ein limitierender Faktor sein muss. Denn es ist davon auszugehen, dass vermutlich mehrere Motoren gleichzeitig für den Transport großer Organellen wie den Mitochondrien verantwortlich sind. In diesem Fall würde die Geschwindigkeit eines einzelnen, sich prozessiv bewegenden Motorproteins keine wesentliche Auswirkung auf die Geschwindigkeit des Gesamtprozesses zeigen. Damit konform gehen auch Beobachtungen, die für Mitochondrien aus Wildtypzellen große Schwankungen in der Bewegungsgeschwindigkeit aufzeigen (Fehrenbacher et al., 2004). Die Hypothese vom Arp2/3-Komplex vermittelten mitochondrialen Transport sieht für Myo2 zwar auch eine Funktion bei der mitochondrialen Vererbung, diese beschränkt sich allerdings auf die Rolle als Transporter von Faktoren, die in die Knospe für die Retention von Mitochondrien während der Zytokinese benötigt werden (Boldogh et al., 2004). Dagegen sprechen einerseits die *in-vitro* Assays, die für Myo2 und Mlc1 eine essentielle Bedeutung bei der Interaktion zwischen Aktinfilamenten und den Mitochondrien zeigen, andererseits die Defekte in der anterograden Bewegung, die durch das Fehlen von Retentionsfaktoren in der Knospe nicht erklärt werden können. Somit lassen die vorliegenden Ergebnisse den Schluss zu, dass Myo2 als Mediator der Interaktion zwischen Mitochondrien und dem

Aktinzytoskelett bzw. als Motor für die anterograde Bewegung den Hauptmechanismus für den mitochondrialen Transport in Hefe darstellt, während der Arp2/3 Komplex nur einen geringen Anteil an den Bewegungsereignissen innezuhaben scheint.

Für die Etablierung der Myo2-vermittelten Bewegung von Mitochondrien wird in der Zukunft zunächst die Identifizierung von Myo2 Rezeptoren an der mitochondrialen Oberfläche von entscheidender Bedeutung sein. Zwei kürzlich identifizierte Proteine, Ypt11, eine kleine rab-ähnliche GTPase, und Mmr1, ein in der Knospe mitochondrial gebundenes Protein, wären theoretisch als Interaktionspartner für Myo2 geeignet. Während die Doppelmutante Δ*ypt11*Δ*mmr1* tatsächlich schwere Defekte in der korrekten Verteilung der Mitochondrien auf Mutter- und Tochterzellen zeigt (Itoh et al., 2002; Itoh et al., 2004), weisen allerdings die Einzelmutanten kaum Störungen in der mitochondrialen Morphologie oder Verteilung auf (Dimmer et al., 2002; Itoh et al., 2002; Itoh et al., 2004). Somit kommt sicherlich keines der Proteine für sich als mitochondrialer Myo2 Rezeptor in Frage. Daher ist davon auszugehen, dass zusätzlich mindestens noch ein oder mehrere, bisher uncharakterisierte Proteine, für die Verankerung von Myo2 an den Mitochondrien verantwortlich sind.

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Anhang

Teilarbeit A: Altmann & Westermann, 2005

Teilarbeit B: Altmann et al., 2008

Teilarbeit C: Dimmer et al., 2005

Teilarbeit D: Altmann et al., 2007. Übersichtsartikel

Teilarbeit E: Merz et al., 2007. Übersichtsartikel

Teilarbeit A

Katrin Altmann und Benedikt Westermann (2005)

Role of essential genes in mitochondrial morphogenesis in *Saccharomyces cerevisiae*

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Darstellung des Eigenanteils

Alle Experimente wurden von mir durchgeführt und sämtliche Abbildungen sind Resultate meiner Arbeit.

Verfasst wurde diese Teilarbeit von Benedikt Westermann.

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Role of Essential Genes in Mitochondrial Morphogenesis in *Saccharomyces cerevisiae*[D]

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Mitochondria are essential organelles of eukaryotic cells. Inheritance and maintenance of mitochondrial structure depend on cytoskeleton-mediated organelle transport and continuous membrane fusion and fission events. However, in *Saccharomyces cerevisiae* most of the known components involved in these processes are encoded by genes that are not essential for viability. Here we asked which essential genes are required for mitochondrial distribution and morphology. To address this question, we performed a systematic screen of a yeast strain collection harboring essential genes under control of a regulatable promoter. This library contains 768 yeast mutants and covers approximately two thirds of all essential yeast genes. A total of 119 essential genes were found to be required for maintenance of mitochondrial morphology. Among these, genes were highly enriched that encode proteins involved in ergosterol biosynthesis, mitochondrial protein import, actin-dependent transport processes, vesicular trafficking, and ubiquitin/26S proteasome-dependent protein degradation. We conclude that these cellular pathways play an important role in mitochondrial morphogenesis and inheritance.

INTRODUCTION

Mitochondria supply the cell with ATP generated by oxidative phosphorylation (Saraste, 1999), they are involved in the biosynthesis of many cellular metabolites (Scheffler, 2000), they play a key role in the assembly of iron/sulfur clusters (Lill and Mühlenhoff, 2005), and they are central regulators of programmed cell death (Desagher and Martinou, 2000). Budding yeast *Saccharomyces cerevisiae* is an excellent model organism to study mitochondrial biogenesis and function. Because *S. cerevisiae* is a facultative anaerobic yeast capable of satisfying its energy requirements with ATP generated by fermentation, only few mitochondrial proteins are essential for viability in this organism. Among these are several factors required for biogenesis of iron/sulfur clusters that serve as cofactors for a number of mitochondrial and extramitochondrial enzymes (Lill and Mühlenhoff, 2005). Other essential mitochondrial proteins are subunits of the molecular machines that mediate import, folding, and assembly of nuclear-encoded mitochondrial proteins (Neupert and Brunner, 2002; Pfanner *et al.*, 2004; Rehling *et al.*, 2004). Recently, it was reported that a soluble ATP-binding cassette protein, Rli1, carries iron/sulfur clusters. Rli1 is a factor required for nuclear export of newly assembled subunits of cytosolic ribosomes and thus is essential for cell viability. As the biogenesis of iron/sulfur clusters requires the mitochondrial assembly machinery, the dependence of Rli1 on iron/sulfur clusters is sufficient to explain the essential nature of mitochondria in yeast (Kispal *et al.*, 2005; Yarunin *et al.*, 2005).

Because mitochondria cannot be generated de novo, they must be inherited during cell division (Warren and Wickner, 1996). Inheritance of the organelles is mediated by cytoskeleton-mediated transport, and maintenance of mitochondrial structure and function depends on continuous fusion and fission events (Bereiter-Hahn and Vöth, 1994; Yaffe, 1999). In recent years, an increasing number of proteins essential for mitochondrial inheritance and structure have been identified in yeast. These include factors of the mitochondrial membrane fusion and division machineries as well as proteins involved in structural maintenance of the organelle and cytoskeleton-dependent motility (Hermann and Shaw, 1998; Jensen *et al.*, 2000; Boldogh *et al.*, 2001b; Dimmer *et al.*, 2002; Shaw and Nunnari, 2002; Scott *et al.*, 2003). Surprisingly, all components specifically required for mitochondrial dynamics are encoded by nonessential genes. However, some synthetic lethal relationships have been revealed. Deletion of the genes encoding the mitochondrial inheritance components Mdm10, Mdm12, and Mmm1 is synthetically lethal with deletion of the genes encoding prohibitin family members (Berger and Yaffe, 1998); and deletion of the *MDM10*, *MDM12*, *MMM1*, and *MMM2* genes is synthetically lethal with deletion of the *MDM31* and *MDM32* genes (Dimmer *et al.*, 2005). Furthermore, overexpression of Mdm33, an inner membrane protein required for mitochondrial morphogenesis, is lethal (Messerschmitt *et al.*, 2003). These genetic findings underscore the fact that inheritance of mitochondria is an essential process.

To identify essential genes required for mitochondrial distribution and morphology in yeast, we screened a library containing mutants harboring essential genes that have been placed under control of a regulatable promoter (Mnaimneh *et al.*, 2004). Assignment of mutants with aberrant mitochondrial morphology to functional classes identified the essen-

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Abbreviations used: Dox, doxycycline; YPD, yeast extract/peptone/glucose.

tial cellular pathways that are important for mitochondrial morphogenesis.

MATERIALS AND METHODS

Yeast Genetic Methods

S. cerevisiae was cultivated and manipulated according to standard procedures (Sherman, 1991). Yeast Tet regulated ORF clone collection and update, the strain collections used for expression of essential genes from a regulatable promoter (Gari *et al.*, 1997; Mnaimneh *et al.*, 2004), were obtained from BioCat (Heidelberg, Germany). Repression of the $TetO_7$ promoter was achieved by the addition of 10 μg/ml doxycycline to the medium and incubation over night or for up to 3 d.

Screening for Essential Genes Involved in Mitochondrial Morphogenesis

After thawing 96-well plates containing frozen stocks of the yeast strains, cells were transferred to yeast extract/peptone/glucose (YPD) plates without or with 10 μg/ml doxycycline using a sterile pinning tool and incubated at 30°C over night. Media lacking doxycycline allowed expression of essential genes from the $TetO_7$ promoter, and these cultures served as negative controls. Media containing 10 μg/ml doxycycline (YPD/Dox) efficiently repress the $TetO_7$ promoter (Gari *et al.*, 1997; Mnaimneh *et al.*, 2004). About one third of the strains failed to grow on the YPD/Dox plate. Strains that did grow were subjected to two additional rounds of replica plating on fresh YPD/Dox plates using a pinning tool. About half of the strains were still able to grow on the third YPD/Dox plate. For these strains, growth could be observed even after seven rounds of replica plating.

For screening for mitochondrial morphology mutants, strains were inoculated in 0.5 ml YPD or YPD/Dox and grown over night at 30°C. YPD cultures were inoculated from YPD plates. YPD/Dox cultures were inoculated from YPD plates for strains that failed to grow on the first YPD/Dox plate, or from the first YPD/Dox plate for strains that failed to grow on the second YPD/Dox plate, or from the second YPD/Dox plate for strains that failed to grow on the third YPD/Dox plate, or from the third YPD/Dox plate for all remaining strains. The next morning, 3 ml fresh medium was added, and incubation was continued for at least 3 h. Mitochondria were stained by the addition of 0.1 μM rhodamine B hexyl ester (Molecular Probes, Eugene, OR) and inspected by fluorescence microscopy. Strains (n = 183) that showed aberrant mitochondrial morphology on YPD/Dox media, or that did not stain well, were transformed with pYX142-mtGFP expressing mitochondria-targeted GFP (Westermann and Neupert, 2000). These transformants were subjected to another round of screening to identify clones that reproducibly do not exhibit wild-type-like mitochondria under promoter shut off conditions. All rounds of screening were performed without prior reference to strain identity. To exclude the possibility that mitochondrial phenotypes were indirect consequences of cell death in the presence of doxycycline, cultures were stained with FUN 1 (LIVE/DEAD yeast viability kit, Molecular Probes) to score for viability of cells (Millard *et al.*, 1997). With very few exceptions (as indicated in Supplementary Table 1) 70–100% of the cells grown in YPD/Dox media were alive and metabolically active under screening conditions.

Staining of Cellular Structures and Microscopy

Mitochondria were stained in living cells with 0.1 μM rhodamine B hexyl ester (Molecular Probes) or mitochondria-targeted GFP expressed from pYX142-mtGFP (Westermann and Neupert, 2000; Dimmer *et al.*, 2002). The actin cytoskeleton was stained with rhodamine-phalloidin (Molecular Probes) as described (Amberg, 1998). Differential interference contrast (DIC) and epifluorescence microscopy was performed using an Axioplan 2 microscope equipped with a Plan-Neofluar 100×/1.30 Ph3 oil objective (Carl Zeiss Jena GmbH, Oberkochen, Germany). Images were recorded with an Evolution VF Mono Cooled monochrome camera (Intas, Göttingen, Germany) and processed with Image ProPlus 5.0 and ScopePro 4.5 software (Media Cybernetics, Silver Spring, MD).

RESULTS AND DISCUSSION

Identification of Essential Cellular Functions Required for Mitochondrial Morphogenesis

To identify essential genes required for establishment and maintenance of normal mitochondrial distribution and morphology, we systematically screened a promoter shut off yeast strain collection that covers approximately two thirds of all essential yeast genes (Mnaimneh *et al.*, 2004). The 768 strains contained in this collection were incubated under permissive and restrictive conditions, stained with mitochondria-specific probes and screened for mutants that showed aberrant mitochondrial morphology upon depletion of essential gene products. A total number of 119 mutants (corresponding to 15% of the strains present in the collection) were identified that reproducibly showed severe defects in the organization of their mitochondria (see Supplementary Table 1 for a complete list).

Based on information obtained from the Saccharomyces Genome Database (Christie *et al.*, 2004) and the Comprehensive Yeast Genome Database (Güldener *et al.*, 2005) and manual annotation, the strains present in the collection were grouped into functional categories (see Supplementary Table 1). The following five functional categories were identified that contained a high proportion of mutants with aberrant mitochondria: (1) ergosterol biosynthesis, with 100% of the screened mutants being affected; (2) mitochondrial protein import, 70% affected; (3) actin cytoskeleton-associated proteins, 67% affected; (4) vesicular transport and secretion, 61% affected; and (5) ubiquitin/26S proteasome-dependent protein degradation, 32% affected. In contrast, only 7% of the mutants lacking essential proteins of other functions showed aberrant mitochondria. A similar number, 8%, was obtained

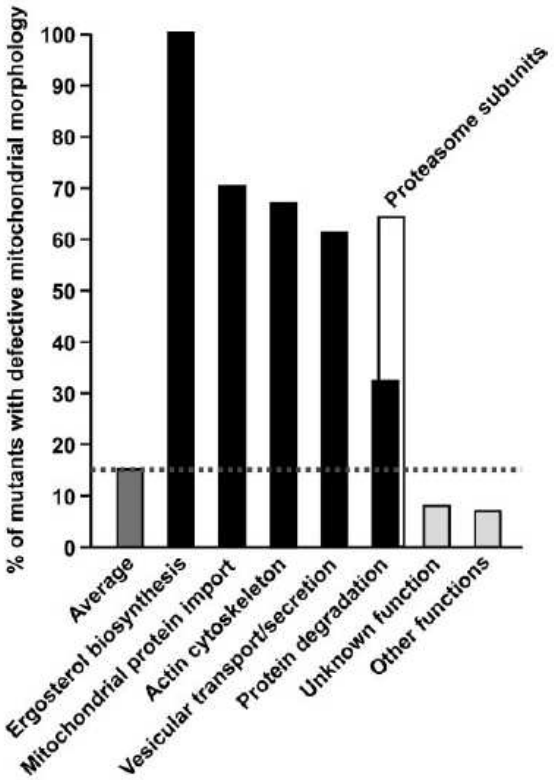

Figure 1. Essential cellular processes required for maintenance of mitochondrial morphology. Promoter shutoff strains (n = 768) lacking essential gene products were screened for mutants with aberrant mitochondrial morphology. Of these strains, 15% showed strong defects in mitochondrial morphology and distribution (average, indicated by dotted line). Black columns indicate functional classes that contained a high percentage of mutants with disorganized mitochondria. In case of protein degradation factors, genes encoding proteasome subunits were counted separately (white column). Light gray columns represent genes of unknown function, and genes of known functions other than that included in the black columns. A complete list of screened yeast strains can be found in Supplementary Table 1.

Table 1. Essential cellular pathways, protein complexes, and proteins required for mitochondrial morphogenesis in yeast

Function	Complex	Protein
Ergosterol biosynthesis		Erg1, Erg7, Erg8, Erg10, Erg12, Erg13, Erg25, Erg26, Erg27, Mvd1, Ncp1
Mitochondrial protein import and assembly	TOM complex	Mim1, Tom22
	SAM complex	Sam35, Sam50
	Tim23 complex	Mge1, Pam18, Zim17
Vesicular trafficking/protein secretion		Bfr2, Dsl1, Ret2, Sec2, Sec3, Sec4, Sec5, Sec8, Sec10, Sec13, Sec15, Sec14, Sec17, Sec18, Sec20, Sec21, Sec26, Sec27, Sec31, Sec53, Sec61, Sec63, Sec65, Sed5, Sly1, Srp14, Srp21, Srp68, Srp72, Srp101, Srp102, Trs20, Trs120, Use1, Yip1
Actin cytoskeleton-dependent transport	ARP2/3 complex	Arc35, Arc40, Arp2
	CCT complex	Cct4, Cct6
	Myosins	Mlc1, Myo2
	Other	Cof1, Iqg1, Pfy1
Ubiquitin/26S proteasome-dependent protein degradation	Proteasome	Pre1, Pre3, Pre5, Pre6, Rpn8, Rpt2, Rpt4
	SCF ubiquitin ligase	Cdc34, Cdc53
	Other	Uba1, Ufd1

Only standard names of proteins are indicated. A complete list including systematic gene names can be found in Supplementary Table 1.

for mutants lacking proteins of unknown function. These results are summarized in Figure 1, Table 1, and Supplementary Table 1. We conclude that mitochondrial morphogenesis and inheritance rely on the presence of a complete complement of ergosterol biosynthetic enzymes and intact machineries of mitochondrial protein import, actin cytoskeleton-dependent motility, vesicular transport, and proteasome-dependent protein degradation.

Role of Ergosterol Biosynthesis in Maintenance of Mitochondrial Morphology

Mutants lacking enzymes required for the biosynthesis of ergosterol showed clumped and swollen mitochondria (Table 1 and Supplementary Table 1). This is exemplified for *erg7* and *erg8* (Figure 2), whereas the other mutants looked very similar. When the strains were grown in the absence of doxycycline, i.e., under permissive conditions, they exhibited the characteristic mitochondrial tubular network of wild-type cells (Figure 2). Addition of doxycycline to the growth medium did not affect mitochondrial morphology of wild-type cells, indicating that the observed defects were consequences of depletion of essential gene products (Figure 2).

The fact that all strains with down-regulated ergosterol biosynthetic enzymes showed severe mitochondrial morphology defects points to an essential role of this membrane lipid for maintenance of the structure of membrane-bounded organelles. Interestingly, ergosterol has been reported to be required for the priming step of homotypic vacuole fusion in yeast (Kato and Wickner, 2001). Because vacuolar membranes have relatively low ergosterol content, the role of this lipid in membrane fusion presumably is not the modulation of the physical properties of the membrane. Rather, it has been suggested to specifically activate and/or rearrange membrane proteins required for the vacuolar fusion reaction (Kato and Wickner, 2001; Fratti *et al.*, 2004). The ergosterol content of mitochondrial membranes (6 μg ergosterol per mg of organellar protein in the outer membrane and 25 μg/mg in the inner membrane) is even lower than that of vacuolar membranes (49 μg/mg; Zinser *et al.*, 1993). Thus, it is conceivable that also in the case of mitochondria, ergosterol may have a modulatory function on the machinery of membrane fusion and/or fission.

Link between the Mitochondrial Protein Import/Assembly Machinery and Mitochondrial Morphology

Many mutants lacking proteins involved in mitochondrial protein translocation, sorting, and assembly showed severe

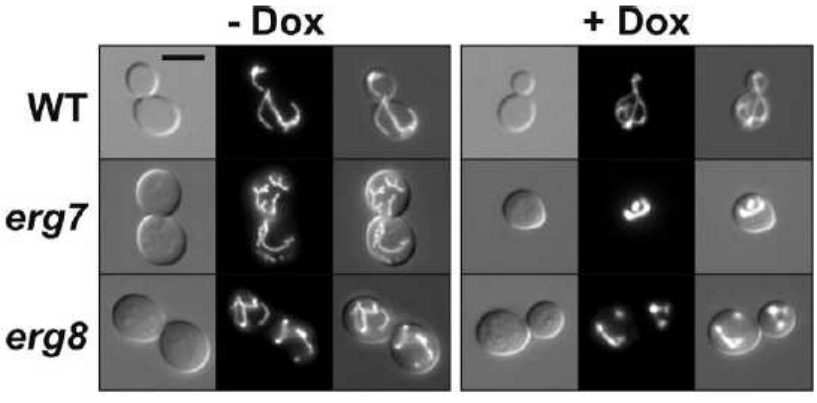

Figure 2. Yeast cells lacking ergosterol biosynthetic enzymes harbor aberrant mitochondria. Strains expressing mitochondria-targeted GFP were grown in the absence (− Dox) or presence (+ Dox) of doxycycline in YPD medium to logarithmic growth phase and analyzed by fluorescence microscopy. Left, differential interference contrast (DIC) image; middle, mitochondrial morphology of a representative cell; right, merged image. WT, wild type. Bar, 5 μm.

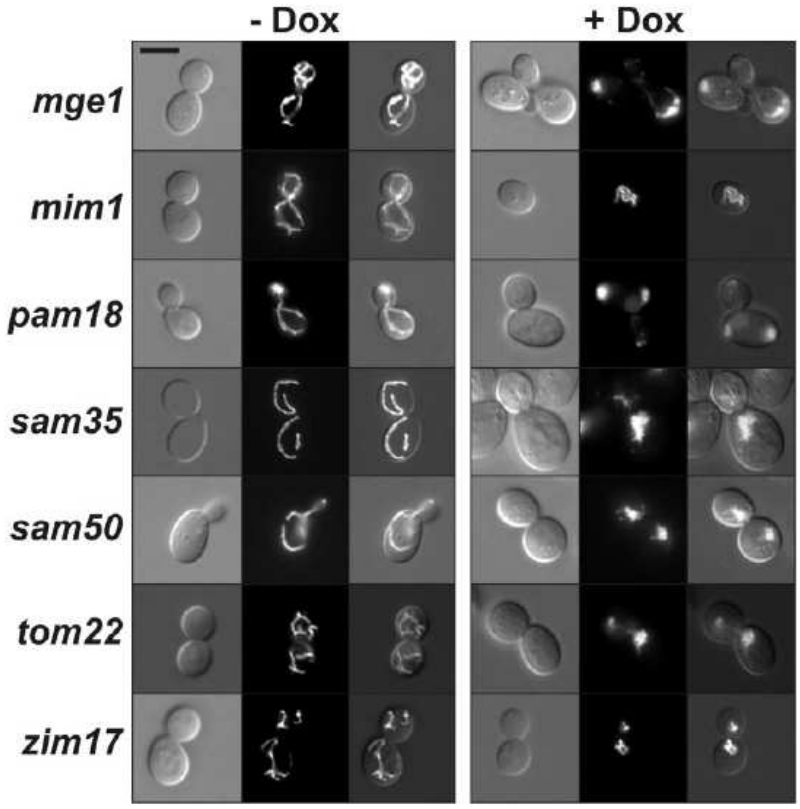

Figure 3. Yeast cells lacking components of the mitochondrial protein import and sorting machinery harbor aberrant mitochondria. Yeast strains were analyzed as in Figure 2. Bar, 5 μm.

defects in mitochondrial morphology. These include essential subunits of the TOM complex (translocase of the outer membrane), the SAM complex (sorting and assembly machinery of the outer membrane), and the TIM23 complex (translocase of the inner membrane).[1] The following proteins were found to be required for normal mitochondrial morphology: Tom22, a subunit of the TOM complex; Mim1, a protein required for TOM complex assembly; Sam35 and Sam50, two subunits of the SAM complex; and Pam18, Zim17, and Mge1, three components or cofactors of the TIM23 translocase and its associated import motor (Figure 3 and Table 1). Strains that showed normal mitochondria in the presence of doxycycline include *mas1*, lacking a subunit of the mitochondrial processing peptidase; *tim50*, lacking a subunit of the TIM23 translocase; and *tim22*, lacking a subunit of the TIM22 translocase. It is presently unclear whether residual amounts of these proteins are sufficient to maintain normal mitochondrial shape or whether these proteins are not required for mitochondrial morphogenesis.

Because 70% of the strains with down-regulated components of the mitochondrial protein sorting machinery showed aberrant mitochondria, we conclude that intact TOM, SAM, and TIM23 complexes are required for mitochondrial morphogenesis. This is in accordance with our earlier observation that a nonessential TOM component, Tom7, is required for wild-type-like mitochondrial distribution and morphology (Dimmer *et al.*, 2002). In case of Sam50, similar mitochondrial defects have been reported in a recent study using temperature-sensitive *sam50* mutants. It has been suggested that Sam50 together with Mas37 and the mitochondrial morphology protein Mdm10 is required for assembly of Tom40 and its association with Tom22 to generate a functional TOM complex (Meisinger *et al.*, 2004). Most of the known proteins required for maintenance of mitochondrial structure are located in the mitochondrial outer and inner membrane. Because these proteins have to be imported via the TOM complex and then sorted to the mitochondrial subcompartments, it is conceivable that defects of the mitochondrial protein sorting machinery lead to aberrant mitochondrial morphology as a secondary consequence.

[1] Many components of mitochondrial protein sorting machineries have alternative names. Here, we use the standard gene names according to the *Saccharomyces* Genome Database.

Dependence of Mitochondrial Morphology on the Actin Cytoskeleton

Mitochondrial transport in *S. cerevisiae* is dependent on actin filaments (Hermann and Shaw, 1998; Jensen *et al.*, 2000; Boldogh *et al.*, 2001b). Therefore, we expected that depletion of actin cytoskeleton-related factors would result in mitochondrial morphology defects. Mutants of 15 essential yeast genes required for integrity or function of the actin cytoskeleton are contained in the strain collection. We examined the organization of mitochondria and the actin cytoskeleton in all of these mutants by fluorescence microscopy. Based on their phenotypes, the mutants were grouped into four classes: class A mutants have aberrant mitochondria and disorganized actin filaments; class B mutants have aberrant mitochondria and normal actin filaments; class C mutants have normal mitochondria and disorganized actin filaments; and class D mutants have normal mitochondria and normal actin

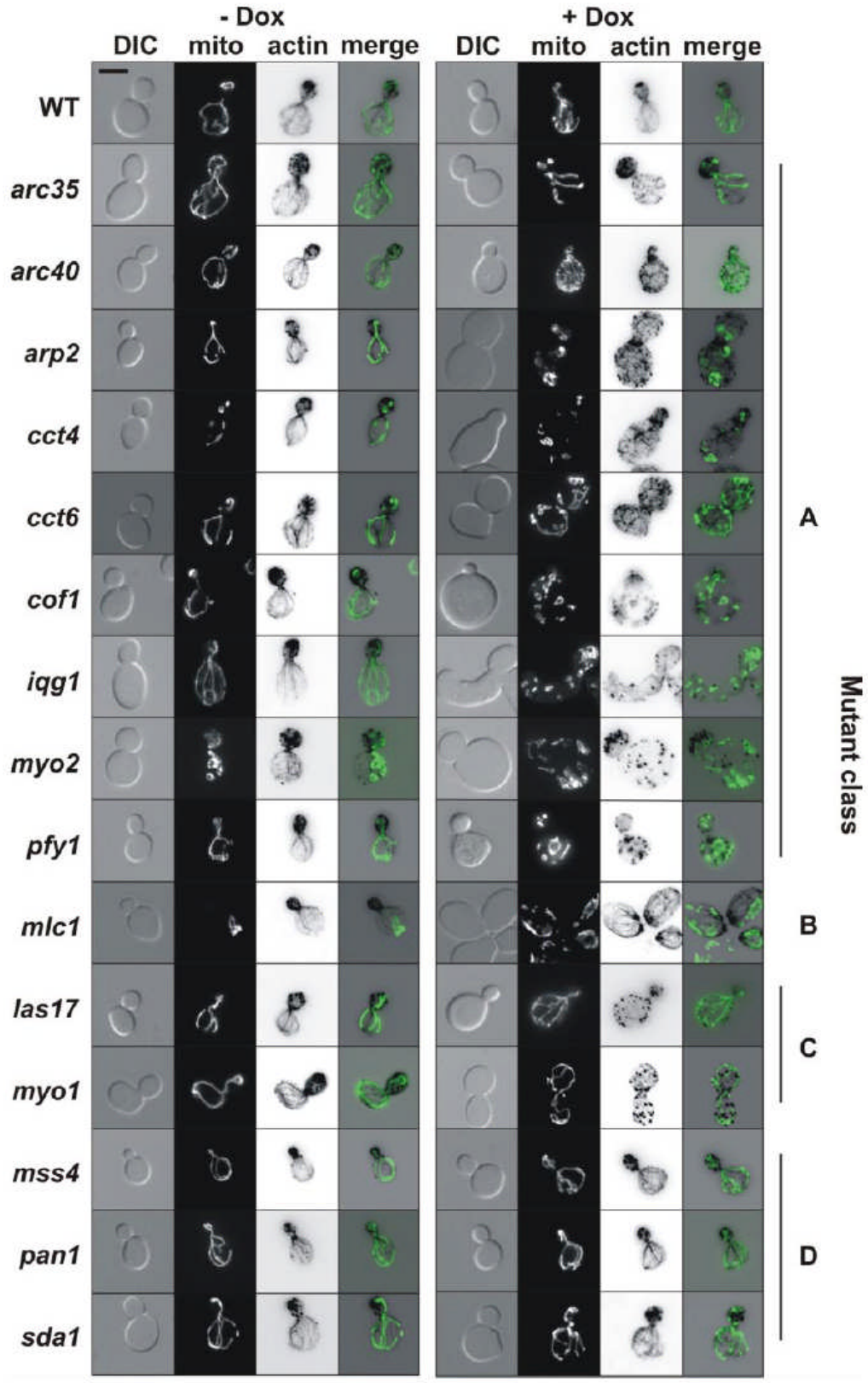
- Dox
+ Dox
DIC
mito
actin
merge
DIC
mito
actin
merge
WT
arc35
arc40
arp2
cct4
cct6
cof1
iqg1
myo2
pfy1
mlc1
las17
myo1
mss4
pan1
sda1
A
B
C
D
Mutant class

Figure 4.

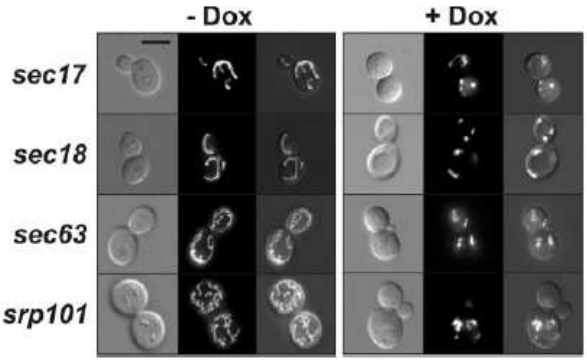

Figure 5. Yeast cells lacking components of the protein secretion and vesicular trafficking machinery harbor aberrant mitochondria. Yeast strains were analyzed as in Figure 2. Bar, 5 μm.

filaments (Figure 4). Class A comprises subunits of the ARP2/3 complex (Arc35, Arc40, Arp2), subunits of the CCT chaperone, which is required for assembly of actin and tubulin (Cct4, Cct6), a myosin-related motor protein (Myo2), and other factors required for organization of the actin cytoskeleton (Cof1/cofilin, Iqg1, Pfy1/profilin). Class B contains Mlc1, a myosin light chain associated with Myo2. Class C comprises a myosin-related motor protein (Myo1), and a component of cortical actin patches (Las17). Class D comprises three proteins involved in actin cytoskeleton assembly (Mss4, Pan1, Sda1). Class C and D components apparently do not play an important role in mitochondrial morphogenesis.

The large number of actin cytoskeleton mutants with aberrant mitochondria found in classes A and B underscores the fundamental importance of actin-dependent transport for mitochondrial distribution and morphology in yeast. Although the mitochondrial phenotype seen in cells lacking Cof1, Iqg1, Pfy1, and CCT subunits is most likely an indirect consequence of disturbed microfilament organization, a direct role in mitochondrial binding to actin has been suggested for the ARP2/3 complex (Boldogh *et al.*, 2001a; Fehrenbacher *et al.*, 2004) and Myo2 (Itoh *et al.*, 2002; Boldogh *et al.*, 2004; Itoh *et al.*, 2004). It is still a matter of debate whether ARP2/3 complex-driven motility or myosin-related motor proteins are of major importance for mitochondrial movement (Boldogh *et al.*, 2001a, 2004; Itoh *et al.*, 2004). Here we found that the *mlc1* mutant harbors intact actin cables, but highly aberrant mitochondria. Intriguingly, mitochondrial defects were also apparent in *mlc1* and *myo2* mutants in the absence of doxycycline (Figure 4), conditions that likely lead to nonphysiological expression levels of these proteins. The fact that mitochondrial defects are manifest in cells that display a relatively normal actin cytoskeleton is in favor of a direct role of Myo2 and its associated light chain, Mlc1, in linking the organelle to the cytoskeleton.

Figure 4 (facing page). Yeast cells lacking components linked to the actin cytoskeleton harbor aberrant mitochondria. Strains expressing mitochondria-targeted GFP were grown in the absence (− Dox) or presence (+ Dox) of doxycycline in YPD medium to logarithmic growth phase, fixed, stained with rhodamine phalloidin, and analyzed by fluorescence microscopy. First image, differential interference contrast (DIC) image; second image, mitochondrial morphology (mito); third image, organization of filamentous actin (a reversed fluorescence image is shown to better visualize faint actin cables); and fourth image, merged image of reversed actin fluorescence and mitochondrial fluorescence. On the right hand side, the mutant class is indicated: A, aberrant mitochondria and disorganized actin cytoskeleton; B, aberrant mitochondria and normal actin cytoskeleton; C, normal mitochondria and disorganized actin cytoskeleton; D, normal mitochondria and normal actin cytoskeleton. WT, wild type. Bar, 5 μm.

Connection of Vesicular Transport and Mitochondrial Morphology

Components of the vesicular trafficking system constitute the largest group of essential yeast proteins that were identified as mitochondrial biogenesis components. Thirty-five proteins mediating protein translocation into the ER and vesicle budding and fusion were found to be required for maintenance of mitochondrial morphology (Table 1 and Supplementary Table 1). On depletion of these proteins, mitochondria appeared fragmented and/or aggregated. This phenotype was very similar in all mutants. Examples shown in Figure 5 are Sec17 and Sec18, homologues of mammalian alpha-SNAP and NSF, which are required for priming of the vesicular fusion machinery, Sec63, a subunit of the SEC61 translocon, and Srp101, the alpha subunit of the signal recognition particle receptor.

Previous observations of mitochondria in mutants of the secretory pathway have yielded ambiguous results. For example, temperature-sensitive *sec18* mutants did not show defects in mitochondrial fusion (Nunnari *et al.*, 1997), whereas temperature-sensitive *srp101* mutants contained fragmented mitochondria (Prinz *et al.*, 2000), similar to the aberrant organelles observed here (Figure 5). It is thought that defects in mitochondrial structure do not affect the ER, because mutants such as *fzo1-1*, *Δmdm30*, *Δmdm31*, *Δmdm32*, *Δmdm33*, *mmm1-1*, *Δmmm2*, and *Δugo1* maintain a normal ER structure (Prinz *et al.*, 2000; Sesaki and Jensen, 2001; Fritz *et al.*, 2003; Messerschmitt *et al.*, 2003; Youngman *et al.*, 2004; Dimmer *et al.*, 2005). In contrast, the majority of vesicular transport mutants analyzed here showed strong defects in mitochondrial morphology. We conclude that the ER and secretory pathway play an important and general role in maintenance of a normal mitochondrial reticulum. We consider it likely that this role involves the supply of mitochondria with lipids.

Role of the Ubiquitin/26S Proteasome System for Mitochondrial Biogenesis

A large fraction, 32%, of the mutants lacking proteins of the ubiquitin/26S proteasome system showed strong defects in mitochondrial morphology. When only proteasome subunits are considered, this number increases to 64% (Figure 1), whereas strains lacking cell cycle-specific protein degradation factors, such as components of the anaphase-promoting complex, showed no mitochondrial defects (see Supplementary Table 1). In addition to seven proteasome subunits (Table 1) the following components were found to be required for normal mitochondrial structure: two subunits of the SCF ubiquitin ligase complex (Cdc34 and Cdc53/cullin), a ubiquitin-activating enzyme (Uba1), and a protein involved in recognition of ubiquitinated proteins (Ufd1). Mitochondria generally appeared highly fragmented or aggregated in these mutants, as shown for *cdc34*, *cdc53*, *pre1*, and *rpn8* (Figure 6). These results are in accordance with earlier observations that ubiquitin (Fisk and Yaffe, 1999), the 26S proteasome (Rinaldi *et al.*, 1998) and SCF-dependent protein degradation (Fritz *et al.*, 2003) are involved in maintenance of mitochondrial morphology.

Proteins of Unknown Function

Four mutants lacking proteins of unknown function showed severe mitochondrial defects, *ymr134*, *ydr339c*, *ynl149c* (and

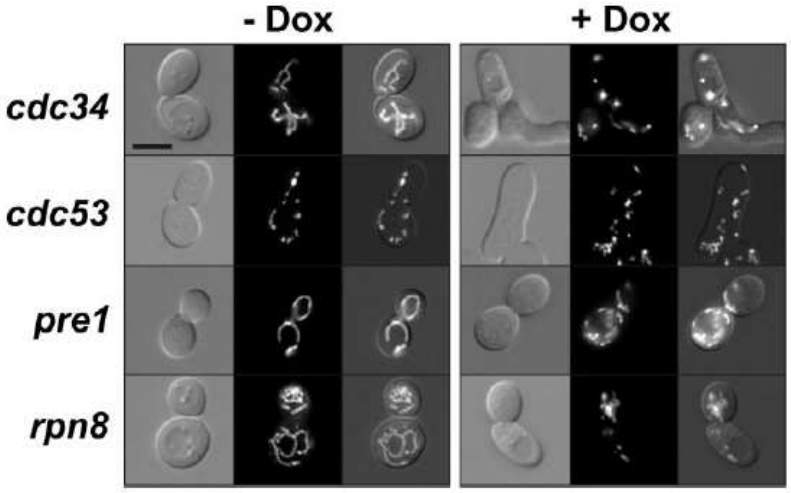

Figure 6. Yeast cells lacking components of the ubiquitin/26S proteasome system harbor aberrant mitochondria. Yeast strains were analyzed as in Figure 2. Bar, 5 μm.

its overlapping ORF *ynl150w*), and *yor060c* (Figure 7). These ORFs might encode novel essential proteins required for mitochondrial morphogenesis. However, the percentage of mutants showing aberrant mitochondria is very similar for ORFs of unknown function (8%) and for the group of all remaining mutants (7%; Supplementary Table 1). We consider it likely that the latter mutants show aberrant mitochondria due to pleiotropic effects of their mutations or general defects in cell physiology. It remains to be determined whether the novel components of unknown function might contribute to mitochondrial inheritance directly or indirectly.

CONCLUSIONS

With the screening of 4794 deletion mutants lacking nonessential genes (Dimmer *et al.*, 2002) and the screening of 768 promoter shutoff strains reported here we have now systematically analyzed 5562 of the ca. 6000 yeast genes for their role in mitochondrial distribution and morphology. Most of the known components mediating mitochondrial fusion, fission, and maintenance of mitochondrial structure are encoded by nonessential genes (Hermann and Shaw, 1998; Jensen *et al.*, 2000; Boldogh *et al.*, 2001b; Dimmer *et al.*, 2002; Shaw and Nunnari, 2002; Scott *et al.*, 2003). The present work allows us to define the essential cellular pathways that contribute to the complex process of mitochondrial inheritance. In particular, our results have revealed an important role of ergosterol biosynthesis, mitochondrial protein import, and vesicular trafficking. Apparently, these processes are much more important for mitochondrial morphogenesis than previously anticipated. Moreover, our results underscore the fundamental importance of the actin cytoskeleton and the proteasome for mitochondrial biogenesis.

Obviously, many of the identified components affect mitochondrial morphology in a rather indirect way, as, for example, subunits of the mitochondrial protein translocation machinery or the vesicular transport system. Moreover, depletion of essential gene products for several days may lead to pleiotropic defects, including aberrant mitochondrial morphology. It should be noted that such pleiotropic defects might play a role in all classes of proteins reported here. On the other hand, some of the components identified (e.g., Dpm1, Mot1, Ncp1, Prp31, Sec4, and Sec63) were recently found in the mitochondrial proteome (Sickmann *et al.*, 2003) although they were previously localized to other cellular compartments. This opens the exciting possibility that these

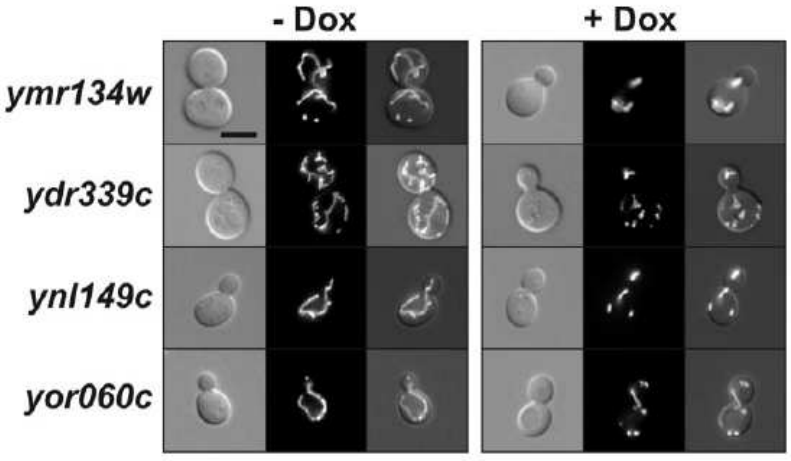

Figure 7. Yeast cells lacking components of unknown function harbor aberrant mitochondria. Yeast strains were analyzed as in Figure 2. Bar, 5 μm.

proteins have a dual localization and might play a more direct role in mitochondrial biogenesis. Clearly, much more work is required to reveal the molecular interactions that link mitochondrial morphogenesis with cellular processes such as synthesis and exchange of membrane lipids, mitochondrial protein import, cytoskeleton-dependent organelle transport, and protein degradation.

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Supplementary Material

Supplemental Table 1. Yeast mutants of essential genes screened for aberrant mitochondrial morphology. Genes were catalogued according to their cellular functions using criteria from the Saccharomyces Genome Database (SGD), Comprehensive Yeast Genome Database (CYGD) and manual annotation. The first column indicates the systematic gene name, the second column indicates the standard gene name, "+" indicates a mitochondrial morphology defect in the presence of doxycycline, "-" indicates wild type-like mitochondria in the presence of doxycycline, the right column gives a brief description of the protein's function according to SGD. An asterisk (*) indicates that less than 70% of cells were viable after incubation in doxycycline-containing medium. Numbers indicate for each group the number of mutants with aberrant mitochondrial morphology, the total number of mutants, and the percentage of mutants with aberrant mitochondrial morphology.

Total (119/768=15%)

Ergosterol biosynthesis (11/11=100%)

YGL001c	ERG26	+	C-3 sterol dehydrogenase
YGR060w	ERG25	+	C-4 sterol methyl oxidase
YGR175c	ERG1	+	Squalene monooxygenase
YHR042w	NCP1	+	NADP-cytochrome P450 reductase
YHR072w	ERG7	+	Lanosterol synthase
YLR100w	ERG27	+	3-keto sterol reductase
YML126c	ERG13	+	3-hydroxy-3-methylglutaryl coenzyme a synthase
YMR208w	ERG12	+	Mevalonate kinase
YMR220w	ERG8	+	Phosphomevalonate kinase
YNR043w	MVD1	+	Mevalonate pyrophosphate decarboxylase
YPL028w	ERG10	+	Acetyl-CoA-acetyltransferase, cytosolic

Mitochondrial protein import (7/10=70%)

YHR083w	SAM35	+	Sorting and assembly machinery (SAM) complex subunit
YLR008c	PAM18	+	Mitochondrial import motor
YNL026w	SAM50	+	Sorting and assembly machinery (SAM) complex subunit
YNL131w	TOM22	+	Outer membrane import receptor complex
YNL310c	ZIM17	+	Essential component of the mitochondrial import system
YOL026c	MIM1	+	Protein required for mitochondrial import
YOR232w	MGE1	+	Heat-shock protein-chaperone
YDL217c	TIM22	-	TIM22 subunit of the TIM22 complex
YLR163c	MAS1	-	Mitochondrial processing peptidase
YPL063w	TIM50	-	Mitochondrial inner membrane import translocase subunit

Actin cytoskeleton-related factors (10/15=67%)

YBR234c	ARC40	+	Essential subunit of the ARP2/3 complex
YDL029w	ARP2	+	Actin-like protein (ARP2/3 complex)
YDL143w	CCT4	+	Required for the assembly of actin and tubulins in vivo
YDR188w	CCT6	+	Required for the assembly of actin and tubulins in vivo
YGL106w	MLC1	+	Myo2p light chain
YLL050c	COF1	+	Cofilin, actin binding and severing protein
YNR035c	ARC35	+	Subunit of the ARP2/3 complex
YOR122c	PFY1	+	Profilin

YOR326w	MYO2	+	Myosin heavy chain
YPL242c	IQG1	+	Protein involved in cytokinesis
YDR208w	MSS4	-	Phosphatidylinositol-4-phosphate 5-kinase
YGR245c	SDA1	-	Required for normal organization of the actin cytoskeleton
YHR023w	MYO1	-	Myosin-1 isoform heavy chain
YIR006c	PAN1	-	Actin-cytoskeleton assembly protein
YOR181w	LAS17	-	Component of actin cortical patches

Vesicular transport and secretion (35/57=61%)

YBL050w	SEC17	+	Transport vesicle fusion protein
YBR080c	SEC18	+	Vesicular-fusion protein, functional homolog of NSF
YBR254c	TRS20	+	Targeting and fusion of ER to golgi transport vesicles
YDL092w	SRP14	+	Signal recognition particle subunit
YDL195w	SEC31	+	Component of the COPII coat of ER-golgi vesicles
YDR166c	SEC5	+	Required for exocytosis
YDR189w	SLY1	+	Suppressor of YPT1 and member of the Sec1p family
YDR238c	SEC26	+	Coatomer complex beta chain of secretory pathway vesicles
YDR292c	SRP101	+	Signal recognition particle receptor, alpha chain
YDR299w	BFR2	+	Involved in protein transport steps at the Brefeldin A blocks
YDR407c	TRS120	+	Targeting and fusion of ER to golgi transport vesicles
YDR498c	SEC20	+	Secretory pathway protein
YER008c	SEC3	+	Component of exocyst complex
YFL005w	SEC4	+	GTP-binding protein of the ras superfamily
YFL045c	SEC53	+	Folding and glycosylation of secretory proteins
YFR051c	RET2	+	Coatomer complex delta chain
YGL098w	USE1	+	Complex with the SNAREs Sec22p, Sec20p and Ufe1p
YGL137w	SEC27	+*	Coatomer complex beta chain of secretory pathway vesicles
YGL233w	SEC15	+	Vesicular traffic control protein
YGR172c	YIP1	+	Golgi membrane protein
YKL122c	SRP21	+	Signal recognition particle subunit
YKL154w	SRP102	+	Signal recognition particle receptor, beta chain
YLR026c	SED5	+	Syntaxin (T-SNARE)
YLR166c	SEC10	+	Required for exocytosis
YLR208w	SEC13	+	Protein transport protein
YLR378c	SEC61	+	ER protein-translocation complex subunit
YML105c	SEC65	+	Signal recognition particle subunit
YMR079w	SEC14	+	PI/PC transfer protein
YNL258c	DSL1	+	Required for Golgi-ER-transport
YNL272c	SEC2	+	Protein transport protein
YNL287w	SEC21	+*	Involved in ER to Golgi transport of selective cargo
YOR254c	SEC63	+	ER protein-translocation complex subunit
YPL210c	SRP72	+	Signal recognition particle protein
YPL243w	SRP68	+	Signal recognition particle protein
YPR055w	SEC8	+	Protein transport protein
YDL058w	USO1	-	Intracellular protein transport protein
YDL145c	COP1	-	Coatomer complex alpha chain of secretory pathway vesicles
YDR164c	SEC1	-	Protein transport protein
YDR170c	SEC7	-	Component of the non-clathrin vesicle coat
YDR246w	TRS23	-	Targeting and fusion of ER to golgi transport vesicles
YGL145w	TIP20	-	TIP20 required for ER to Golgi transport
YGR120c	SEC35	-	Conserved oligomeric golgi complex
YIL004c	BET1	-	Protein transport protein
YIR022w	SEC11	-	Signal sequence processing protein
YKL006c-a	SFT1	-	SNARE-like protein
YKL196c	YKT6	-	SNARE protein for Endoplasmic Reticulum-Golgi transport
YKR068c	BET3	-	Targeting and fusion of ER to golgi transport vesicles
YLR066w	SPC3	-	Signal peptidase subunit
YLR078c	BOS1	-	Secretory pathway protein
YML077w	BET5	-	Targeting and fusion of ER to golgi transport vesicles
YMR197c	VTI1	-	v-SNARE; involved in Golgi retrograde protein traffic

YMR218c	TRS130	-	Targeting and fusion of ER to golgi transport vesicles
YNL006w	LST8	-	Transport of permeases to the plasma membrane
YNL263c	YIF1	-	Slh1 Interacting Factor
YNR026c	SEC12	-	GDP/GTP exchange factor for Sar1p
YPL010w	RET3	-	Coatomer complex zeta chain
YPR105c	COG4	-	Conserved oligomeric golgi complex

Protein degradation (11/34=32%)

Proteasome subunits (7/11=64%)

YDL007w	RPT2	+	26S proteasome regulatory subunit
YER012w	PRE1	+	20S proteasome subunit C11 (beta4)
YJL001w	PRE3	+	20S proteasome subunit (beta1)
YMR314w	PRE5	+	20S proteasome subunit (alpha6)
YOL038w	PRE6	+	20S proteasome subunit (alpha4)
YOR259c	RPT4	+*	26S proteasome regulatory subunit
YOR261c	RPN8	+	26S proteasome regulatory subunit
YDL147w	RPN5	-	26S proteasome regulatory subunit
YFR004w	RPN11	-	26S proteasome regulatory subunit
YGL048c	RPT6	-	26S proteasome regulatory subunit
YML092c	PRE8	-	20S proteasome subunit Y7 (alpha2)

Cell cycle-specific protein degradation factors (0/11=0%)

YBL084c	CDC27	-	Subunit of anaphase-promoting complex/cyclosome
YDL008w	APC11	-	Subunit of anaphase-promoting complex/cyclosome
YDL064w	UBC9	-	Subunit of anaphase-promoting complex/cyclosome
YDR118w	APC4	-	Subunit of anaphase-promoting complex/cyclosome
YGL116w	CDC20	-	Cell division control protein
YGR098c	ESP1	-	Required for sister chromatide separation
YHR166c	CDC23	-	Subunit of anaphase-promoting complex/cyclosome
YKL022c	CDC16	-	Subunit of anaphase-promoting complex/cyclosome
YLR127c	APC2	-	Subunit of anaphase-promoting complex/cyclosome
YOR249c	APC5	-	Subunit of anaphase-promoting complex/cyclosome
YPL020c	ULP1	-	Ubl-specific protease

Other protein degradation factors (4/12=33%)

YDL132w	CDC53	+	Cullin; subunit of SCF ubiquitin ligase complex
YDR054c	CDC34	+	Skp1-Cullin-F-box ubiquitin protein ligase (SCF) subunit
YGR048w	UFD1	+	Ubiquitin fusion degradation protein
YKL210w	UBA1	+	E1-like (ubiquitin-activating) enzyme
YBR170c	NPL4	-	Recognizes ubiquitinated proteins in the ER
YDL126c	CDC48	-	Retrotranslocation of ubiquitinated proteins from the ER
YDR177w	UBC1	-	E2 ubiquitin-conjugating enzyme
YIL046w	MET30	-	Regulation of sulfur assimilation genes and cell cycle
YIR011c	STS1	-	Transport of Rna15p from the cytoplasm to the nucleus
YLR167w	RPS31	-	Ubiquitin/40S small subunit ribosomal protein
YOL133w	HRT1	-	Skp1-Cullin-F-box ubiquitin protein ligase (SCF) subunit
YOR057w	SGT1	-	Subunit of SCF ubiquitin ligase complex

Proteins of unknown function (5/65=8%)

YDR339c	+	Uncharacterized ORF
YMR134w	+	Uncharacterized ORF
YNL149c	+	Uncharacterized ORF
YNL150w	+	Dubious ORF, overlaps with YNL149c
YOR060c	+	Uncharacterized ORF
YBL073w	-	Dubious ORF

YBL077w	-	Dubious ORF
YBR190w	-	Dubious ORF
YCL053c	-	Dubious ORF
YCR013c	-	Dubious ORF
YCR072c	-	Uncharacterized ORF
YDL016c	-	Dubious ORF
YDL193w	-	Uncharacterized ORF
YDL196w	-	Dubious ORF
YDR196c	-	Uncharacterized ORF
YDR267c	-	Uncharacterized ORF
YDR327w	-	Dubious ORF
YDR367w	-	Uncharacterized ORF
YDR396w	-	Dubious ORF
YDR412w	-	Uncharacterized ORF
YDR413c	-	Dubious ORF
YDR437w	-	Uncharacterized ORF
YGL108c	-	Uncharacterized ORF
YGR046w	-	Uncharacterized ORF
YGR198w	-	Uncharacterized ORF
YGR251w	-	Uncharacterized ORF
YGR283c	-	Uncharacterized ORF
YHR036w	-	Uncharacterized ORF
YHR122w	-	Uncharacterized ORF
YJL009w	-	Dubious ORF
YJL097w	-	Uncharacterized ORF
YJL202c	-	Dubious ORF
YJR067c	-	Uncharacterized ORF
YJR141w	-	Uncharacterized ORF
YKL033w	-	Uncharacterized ORF
YKL083w	-	Dubious ORF
YKL088w	-	Uncharacterized ORF
YKL111c	-	Dubious ORF
YKR022c	-	Uncharacterized ORF
YKR071c	-	Uncharacterized ORF
YLL037w	-	Dubious ORF
YLR076c	-	Dubious ORF
YLR101c	-	Dubious ORF
YLR132c	-	Uncharacterized ORF
YLR140w	-	Dubious ORF
YLR198c	-	Dubious ORF
YLR243w	-	Uncharacterized ORF
YLR419w	-	Uncharacterized ORF
YLR440c	-	Uncharacterized ORF
YML125c	-	Uncharacterized ORF
YNL171c	-	Dubious ORF
YNL181w	-	Uncharacterized ORF
YNL260c	-	Uncharacterized ORF
YNL313c	-	Uncharacterized ORF
YOL022c	-	Uncharacterized ORF
YOR004w	-	Uncharacterized ORF
YOR102w	-	Dubious ORF
YOR146w	-	Dubious ORF
YOR169c	-	Dubious ORF
YOR218c	-	Dubious ORF
YOR262w	-	Uncharacterized ORF
YOR287c	-	Uncharacterized ORF
YPL142c	-	Dubious ORF
YPR085c	-	Uncharacterized ORF
YPR169w	-	Uncharacterized ORF

Other proteins (40/576=7%)

YBL030c	PET9	+	Mitochondrial ADP/ATP carrier
YBR253w	SRB6	+	DNA-directed RNA polymerase II suppressor protein
YDL047w	SIT4	+	Ser/thr protein phosphatase
YDR168w	CDC37	+	Cell division control protein
YDR232w	HEM1	+	5-aminolevulinate synthase
YDR236c	FMN1	+	Riboflavin kinase
YDR302w	GPI11	+	Glycosylphosphatidylinositol (GPI) biosynthesis
YER165w	PAB1	+	mRNA polyadenylate-binding protein
YFL002c	SPB4	+	ATP-dependent RNA helicase of DEAH box family
YGL103w	RPL28	+	Large (60S) ribosomal subunit
YGL155w	CDC43	+	Geranylgeranyltransferase beta subunit
YGL238w	CSE1	+	Importin-beta-like protein
YGR091w	PRP31	+	Pre-mRNA splicing factor
YKL052c	ASK1	+	Outer kinetochore protein - part of Dam1 complex
YKL165c	MCD4	+	Involved in GPI anchor synthesis
YKR002w	PAP1	+	Poly(A) polymerase
YKR008w	RSC4	+	Member of RSC complex
YKR037c	SPC34	+*	Outer kinetochore protein - part of Dam1 complex
YLR033w	RSC58	+	Chromatin Remodeling Complex subunit
YLR212c	TUB4	+	Gamma tubulin
YLR223c	IFH1	+	Pre rRNA processing machinery control protein
YML127w	RSC9	+	Remodels the Structure of Chromatin
YMR168c	CEP3	+	Kinetochore protein complex, 71KD subunit
YNL061w	NOP2	+	Nucleolar protein
YNL256w	FOL1	+	Dihydroneopterin aldolase
YNR046w	TRM112	+	Subunit of adoMet tRNA methyltransferase complex
YOL120c	RPL18a	+	Protein component of the large (60S) ribosomal subunit
YOL142w	RRP40	+	Protein involved in ribosomal RNA processing
YOR168w	GLN4	+	Glutamine tRNA synthetase
YOR236w	DFR1	+	Dihydrofolate reductase
YOR370c	MRS6	+	Geranylgeranyltransferase regulatory subunit
YPL082c	MOT1	+	Transcriptional accessory protein
YPL204w	HRR25	+	Involved in regulating vesicular trafficking
YPL211w	NIP7	+	Required for efficient 60S ribosome subunit biogenesis
YPL231w	FAS2	+*	Fatty-acyl-CoA synthase, alpha chain
YPL252c	YAH1	+	Iron-sulfur protein of the mitochondrial matrix
YPR034w	ARP7	+	Component of SWI-SNF global transcription activator complex
YPR082c	DIB1	+	Required for mitosis
YPR107c	YTH1	+	Protein of the 3^ processing complex
YPR183w	DPM1	+	Dolichyl-phosphate beta-D-mannosyltransferase
YAL003w	EFB1	-	Translation elongation factor eEF1 beta
YAL025c	MAK16	-	Nuclear viral propagation protein
YAL033w	POP5	-	Required for processing of tRNAs and rRNAs
YAL034w-a	MTW1	-	Determines metaphase spindle length
YAL038w	CDC19	-	Pyruvate kinase
YAL043c	PTA1	-	Pre-tRNA processing protein / PF I subunit
YAR008w	SEN34	-	tRNA splicing endonuclease gamma subunit
YAR019c	CDC15	-	Protein kinase of the MAP kinase kinase family
YBL004w	UTP20	-	U3 snoRNP protein
YBL014c	RRN6	-	RNA polymerase I specific transcription initiation factor
YBL018c	POP8	-	Required for processing of tRNAs and rRNAs
YBL020w	RFT1	-	Nuclear division protein
YBL034c	STU1	-	Mitotic spindle protein
YBL035c	POL12	-	DNA-directed DNA polymerase alpha, 70KD subunit
YBL040w	ERD2	-	ER lumen protein-retaining receptor
YBL074c	AAR2	-	A1 cistron splicing factor
YBL076c	ILS1	-	Cytoplasmic isoleucyl-tRNA synthetase
YBR002c	RER2	-	Cis-prenyltransferase, a key enzyme in dolichol synthesis
YBR011c	IPP1	-	Cytoplasmic inorganic pyrophosphatase (PPase)
YBR029c	CDS1	-	CDP-diacylglycerol synthase

YBR049c	REB1	-	Transcription factor
YBR055c	PRP6	-	snRNP (U4/U6)-associated splicing factor
YBR060c	ORC2	-	Origin recognition complex, 72KD subunit
YBR070c	ALG14	-	Second step of dolichyl-linked oligosaccharide synthesis
YBR079c	RPG1	-	Translation initiation factor eIF3
YBR086c	IST2	-	Plasma membrane protein, involved in osmotolerance
YBR087w	RFC5	-	DNA replication factor C, 40KD subunit
YBR088c	POL30	-	Proliferating Cell Nuclear Antigen (PCNA)
YBR102c	EXO84	-	Exocyst protein essential for secretion
YBR110w	ALG1	-	Beta-mannosyltransferase
YBR135w	CKS1	-	Cyclin-dependent kinases regulatory subunit
YBR136w	MEC1	-	Cell cycle checkpoint protein
YBR140c	IRA1	-	Inhibitory regulator protein of the ras-cyclic AMP pathway
YBR142w	MAK5	-	ATP-dependent RNA helicase
YBR143c	SUP45	-	Translational release factor
YBR153w	RIB7	-	HTP reductase
YBR155w	CNS1	-	Component of Hsp90p chaperone machinery
YBR167c	POP7	-	Nuclear Rnase P subunit
YBR168w	PEX32	-	Peroxisomal integral membrane protein
YBR192w	RIM2	-	Protein of the mitochondrial carrier family (MCF)
YBR193c	MED8	-	Transcriptional regulation mediator
YBR196c	PGI1	-	Glucose-6-phosphate isomerase
YBR198c	TAF5	-	TFIID and SAGA unit
YBR202w	CDC47	-	Cell division control protein
YBR211c	AME1	-	Regulator of microtubule stability
YBR236c	ABD1	-	mRNA cap methyltransferase
YBR237w	PRP5	-	Pre-mRNA processing RNA-helicase
YBR243c	ALG7	-	UDP-N-acetylglucosamine-1-phosphate transferase
YBR252w	DUT1	-	dUTP pyrophosphatase precursor
YBR256c	RIB5	-	Riboflavin synthase, alpha chain
YBR257w	POP4	-	Involved in processing of tRNAs and rRNAs
YCL004w	PGS1	-	Phosphatidylglycerophosphate synthase
YCL017c	NFS1	-	Biogenesis of Iron-Sulfur cluster protein
YCL059c	KRR1	-	Required for 40S ribosome biogenesis
YCR035c	RRP43	-	Protein involved in rRNA processing
YCR042c	TAF2	-	Component of TFIID complex
YDL015c	TSC13	-	ER protein involved in very long chain fatty acid synthesis
YDL028c	MPS1	-	Serine/threonine/tyrosine protein kinase
YDL030w	PRP9	-	Pre-mRNA splicing factor
YDL031w	DBP10	-	Putative RNA helicase involved in ribosome biogenesis
YDl043c	PRP11	-	Pre-mRNA splicing factor
YDL045c	FAD1	-	Flavin adenine dinucleotide (FAD) synthetase
YDL055c	PSA1	-	Mannose-1-phosphate guanyltransferase
YDL060w	TSR1	-	Processing of 20S pre-rRNA in the cytoplasm
YDL087c	LUC7	-	U1 snRNP protein with a role in 5´ splice site recognition
YDL098c	SNU23	-	Putative RNA binding zinc finger protein
YDL102w	CDC2	-	DNA-directed DNA polymerase delta, catalytic 125 KD subunit
YDL103c	QRI1	-	UDP-N-acetylglucosamine pyrophosphorylase
YDL105w	QRI2	-	Role in function of the Smc5p-Rhc18p DNA repair complex
YDL108w	KIN28	-	Cyclin dependent ser/thr protein kinase
YDL111c	RRP42	-	Protein involved in rRNA processing
YDL139c	SCM3	-	Suppressor of chromosome misseggregation
YDL140c	RPO21	-	DNA-directed RNA polymerase II, 215 KD subunit
YDL141w	BPL1	-	Biotin holocarboxylase synthetase
YDL148c	NOP14	-	Possible role in ribosome biogenesis
YDL150w	RPC53	-	DNA-directed RNA polymerase III, 47 KD subunit
YDL153c	SAS10	-	Involved in silencing
YDL164c	CDC9	-	DNA ligase
YDL166c	FAP7	-	Oxidative stress response
YDL207w	GLE1	-	RNA export mediator
YDL208w	NHP2	-	Nucleolar rRNA processing protein
YDL209c	CWC2	-	Involved in mRNA splicing

YDL220c	CDC13	-	Cell division control protein
YDR013w	PSF1	-	Part of GINS, replication multiprotein complex
YDR021w	FAL1	-	Required for maturation of 18S rRNA
YDR023w	SES1	-	Seryl-tRNA synthetase
YDR037w	KRS1	-	Lysyl-tRNA synthetase
YDR044w	HEM13	-	Coproporphyrinogen III oxidase
YDR045c	RPC11	-	RNA polymerase III subunit C11
YDR047w	HEM12	-	Uroporphyrinogen decarboxylase
YDR050c	TPI1	-	Triose-phosphate isomerase
YDR052c	DBF4	-	Regulatory subunit for Cdc7p protein kinase
YDR060w	MAK21	-	Protein required for large (60S) ribosomal subunit biogenesis
YDR062w	LCB2	-	Serine C-palmitoyltransferase subunit
YDR087c	RRP1	-	Processing rRNA precursors species to mature rRNAs
YDR088c	SLU7	-	Pre-mRNA splicing factor involved in 3' splice site choices
YDR091c	RLI1	-	Protein promoting preinitiation complex assembly
YDR113c	PDS1	-	Cell cycle regulator
YDR141c	DOP1	-	Involved in establishing cellular polarity and morphogenesis
YDR145w	TAF12	-	TFIID and SAGA subunit
YDR167w	TAF10	-	TFIID and SAGA subunit
YDR182w	CDC1	-	Cell division control protein
YDR190c	RVB1	-	RUVB-like protein
YDR201w	SPC19	-	Outer kinetochore protein - part of Dam1 complex
YDR211w	STN1	-	Involved in telomere length regulation
YDR228c	PCF11	-	Component of pre-mRNA 3^-end processing factor CF I
YDR235w	PRP42	-	U1 snRNP associated protein, required for pre-mRNA splicing
YDR240c	SNU56	-	U1 snRNP protein
YDR243c	PRP28	-	Pre-mRNA splicing factor RNA helicase of DEAD box family
YDR280w	RRP45	-	Protein involved in rRNA processing
YDR288w	NSE3	-	Protein required for cell viability
YDR301w	CFT1	-	Pre-mRNA 3´-end processing factor
YDR308c	SRB7	-	DNA-directed RNA polymerase II holoenzyme
YDR311w	TFB1	-	TFIIH subunit 75KD
YDR324c	UTP4	-	U3 snoRNP protein
YDR325w	YCG1	-	Yeast Condensin G
YDR341c	YDR341c	-	Cytosolic arginyl-tRNA synthetase
YDR353w	TRR1	-	Thioredoxin reductase
YDR356w	NUF1	-	Spindle pole body component
YDR361c	BCP1	-	Nuclear export of cytoskeleton organization protein Mss4p
YDR365c	ESF1	-	18 S rRNA Factor
YDR373w	FRQ1	-	Regulator of phosphatidylinositol-4-OH kinase protein
YDR376w	ARH1	-	Mitochondrial protein putative ferredoxin-NADP+ reductase
YDR397c	NCB2	-	Negative regulator of RNA polymerase II holoenzyme
YDR398w	UTP5	-	U3 snoRNP protein
YDR416w	SYF1	-	Synthetic lethal with CDC40
YDR429c	TIF35	-	Translation initiation factor eIF3
YDR434w	GPI17	-	Glycosylphosphatidylinositol transamidase complex
YDR449c	UTP6	-	U3 snoRNP protein
YDR454c	GUK1	-	Guanylate kinase
YDR460w	TFB3	-	TFIIH subunit (transcription/repair factor)
YDR464w	SPP41	-	Negative regulator of PRP3 and PRP4 gene expression
YDR473c	PRP3	-	Essential splicing factor
YDR478w	SNM1	-	RNA binding protein of Rnase MRP
YDR489w	SLD5	-	Part of GINS, replication multiprotein complex
YDR527w	RBA50	-	RNA polymerase II associated protein 50
YDR531w	YDR531w	-	Pantothenate kinase
YEL002c	WBP1	-	Oligosaccharyl transferase beta subunit precursor
YEL019c	MMS21	-	DNA repair protein
YEL032w	MCM3	-	Replication initiation protein
YEL034w	HYP2	-	Translation initiation factor eIF-5A
YEL055c	POL5	-	Required for the synthesis of rRNA
YEL058w	PCM1	-	Phosphoacetylglucosamine mutase
YER003c	PMI40	-	Mannose-6-phosphate isomerase

YER006w	NUG1	-	Nuclear GTPase (involved in ribosome biogenesis)
YER009w	NTF2	-	Nuclear transport factor
YER022w	SRB4	-	DNA-directed RNA polymerase II holoenzyme
YER023w	PRO3	-	Delta 1-pyrroline-5-carboxylate reductase
YER026c	CHO1	-	CDP-diacylglycerol serine O-phosphatidyltransferase
YER043c	SAH1	-	S-adenosyl-L-homocysteine hydrolase,
YER048c	CAJ1	-	DnaJ homolog
YER082c	UTP7	-	U3 snoRNP protein
YER093c	TSC11	-	Lipid biosynthesis, binding of TOR
YER112w	LSM4	-	Sm-like protein
YER127w	LCP5	-	Ngg1p interacting protein
YER146w	LSM5	-	Sm-like protein
YER168c	CCA1	-	tRNA nucleotidyltransferase
YER171w	RAD3	-	DNA helicase/ATPase
YER172c	BRR2	-	RNA helicase related protein
YFL008w	SMC1	-	Chromosome segregation protein
YFL018w-a	LPD1	-	Dihydrolipoamided dehydrogenase precursor
YFL024c	EPL1	-	Histone H4/H2A acetyltransferase complex
YFL029c	CAK1	-	Cdc-activating protein kinase
YFL035c	MOB2	-	Required for maintenance in ploidy
YFL035c-a	MOB2	-	Required for maintenance in ploidy
YFR003c	YPI1	-	Inhibitor of the type I protein phosphatase Glc7p
YFR005c	SAD1	-	SnRNP assembly defective
YFR031c	SMC2	-	Chromosome segregation protein
YFR037c	RSC8	-	Subunit of the RSC complex
YGL018c	JAC1	-	Molecular chaperone
YGL044c	RNA15	-	Component of pre-mRNA 3´-end processing factor
YGL047w	ALG13	-	Second step of dolichyl-linked oligosaccharide synthesis
YGL061c	DUO1	-	Outer kinetochore protein - part of Dam1 complex
YGL065c	ALG2	-	Mannosyltransferase
YGL068w	MNP1	-	Putative mitochondrial-nucleoid specific ribosomal protein
YGL073w	HSF1	-	Heat shock transcription factor
YGL075c	MPS2	-	Required for spindle pole body assembly
YGL091c	NBP35	-	Nucleotide binding protein
YGL092w	NUP145	-	Nuclear pore protein
YGL093w	SPC105	-	Spindle pole body protein
YGL112c	TAF6	-	TFIID and SAGA subunit
YGL122c	NAB2	-	Nuclear polyadenylated RNA-binding protein
YGL130w	CEG1	-	mRNA guanylyltransferase
YGL142c	GPI10	-	Required for Glycosyl Phosphatdyl Inositol synthesis
YGL171w	ROK1	-	ATP-dependent RNA helicase
YGL172w	NUP49	-	Nuclear pore protein
YGL207w	SPT16	-	General chromatin factor
YGL245w	YGL245w	-	Glutamyl-tRNA synthetase
YGL247w	BRR6	-	Nuclear envelope integral membrane protein
YGR002c	GOD1	-	SWR Complex member
YGR005c	TFG2	-	Transcription initiation factor TFIIF subunit
YGR030c	POP6	-	Required for processing of tRNAs and rRNAs
YGR065c	VHT1	-	Biotin (vitamin H) permease
YGR074w	SMD1	-	U6 snRNP protein
YGR075c	PRP38	-	Component of the U4/U6.U5 tri-snRNP particle
YGR090w	UTP22	-	Possibly involved in maturation of pre-18S rRNA
YGR094w	VAS1	-	Valyl-tRNA synthetase
YGR095c	RRP46	-	Involved in rRNA processing
YGR099w	TEL2	-	Involved in controlling telomere length and position effect
YGR103w	NOP7	-	Synthesis of 60S ribosomal subunits
YGR116w	SPT6	-	Transcription elongation protein
YGR119c	NUP57	-	Nuclear pore protein
YGR128c	UTP8	-	U3 snoRNP protein
YGR147c	NAT2	-	N-acetyltransferase for N-terminal methionine
YGR158c	MTR3	-	Involved in mRNA transport
YGR173w	GIR1	-	Similarity to mammalian GTP-binding protein

YGR179c	OKP1	-	Outer kinetochore protein
YGR186w	TFG1	-	TFIIF subunit, 105KD
YGR195w	SKI6	-	Required for 3´ end formation of 5.8S rRNA
YGR211w	ZPR1	-	Protein binds to translation elongation factor eEF-1
YGR216c	GPI1	-	N-acetylglucosaminyl phosphatidylinositol synthesis
YGR255c	COQ6	-	Monooxygenase
YGR264c	MES1	-	Methionyl-tRNA synthetase
YGR267c	FOL2	-	GTP cyclohydrolase I
YGR274c	TAF1	-	TFIIH subunit 145KD
YGR278w	CWC22	-	Involved in pre-mRNA splicing,
YGR280c	PXR1	-	Component of the ribosomal RNA processing machinery
YHR005c	GPA1	-	GTP-binding protein alpha subunit of the pheromone pathway
YHR019c	DED81	-	Asparaginyl-tRNA synthetase
YHR040w	BCD1	-	Required for box C/D snoRNA accumulation
YHR058c	MED6	-	RNA polymerase II transcriptional regulation mediator
YHR062c	RPP1	-	Required for processing of tRNAs and rRNAs
YHR068w	DYS1	-	Deoxyhypusine synthase
YHR069c	RRP4	-	Required for 3´ end formation of 5.8S rRNA
YHR070w	TRM5	-	tRNA (mG37) methyltransferase
YHR072w-a	NOP10	-	Nucleolar rRNA processing protein
YHR074w	QNS1	-	Glutamine-dependent NAD(+) synthetase
YHR085w	IPI1	-	May be involved in rRNA processing
YHR088w	RPF1	-	Assembly of the large ribosomal subunit
YHR089c	GAR1	-	Nucleolar rRNA processing protein
YHR090c	YNG2	-	Component of NuA4 histone acetyltransferase complex
YHR099w	TRA1	-	Component of the Ada-Spt transcriptional regulatory complex
YHR101c	BIG1	-	Big cells phenotype
YHR107c	CDC12	-	Septin
YHR118c	ORC6	-	Origin recognition complex, 50KD subunit
YHR143w-a	RPC10	-	DNA-directed RNA polymerases I, II, III 7.7 KD subunit
YHR164c	DNA2	-	DNA helicase
YHR165c	PRP8	-	U5 snRNP protein, pre-mRNA splicing factor
YHR169w	DBP8	-	ATP-dependent helicase involved in rRNA processing
YHR172w	SPC97	-	Spindle pole body component
YHR174w	ENO2	-	Enolase II (2-phosphoglycerate dehydratase)
YHR186c	KOG1	-	Subunit of TORC1
YHR188c	GPI16	-	Glycosylphosphatidylinositol transamidase complex
YHR196w	UTP9	-	U3 snoRNP protein
YHR197w	IPI2	-	Ribosome export protein
YHR205w	SCH9	-	Serine/threonine protein kinase involved in stress response
YIL003w	CFD1	-	Cytoplasmic Fe-S cluster assembly factor
YIL021w	RPB3	-	DNA-directed RNA-polymerase II, 45 kDa
YIL026c	IRR1	-	Nuclear cohesin protein
YIL048w	NEO1	-	P-type ATPase, a proposed aminophospholipid translocase
YIL061c	SNP1	-	U1snRNP 70K protein homolog
YIL078w	THS1	-	Threonyl-tRNA synthetase
YIL106w	MOB1	-	Required for completion of mitosis and maintenance of ploidy
YIL115c	NUP159-	-	Nuclear pore protein
YIL126w	STH1	-	Subunit of the RSC complex
YIL144w	TID3	-	Outer kinetochore protein - part of Ndc80p complex
YIL147c	SLN1	-	Two-component signal transducer
YIL150c	MCM10	-	Required for S-phase initiation or completion
YIR008c	PRI1	-	DNA polymerase alpha 48KD subunit (DNA primase)
YIR010w	DSN1	-	Important for chromosome segregation
YIR012w	SQT1	-	Suppresses mutants of the ribosomal protein QSR1
YIR015w	RPR2	-	Rnase P subunit
YJL011c	RPC17	-	RNA polymerase III subunit C17
YJL033w	HCA4	-	Can suppress the U14 snoRNA rRNA processing function
YJL039c	NUP192-	-	Nucleoporin localize at the inner site of the nuclear membrane
YJL042w	MHP1	-	Microtubule-associating protein
YJL050w	MTR4	-	Involved in necleocytoplasmic transport of mRNA
YJL061w	NUP82	-	Nuclear pore protein

YJL069c	UTP18	-	Possibly involved in maturation of pre-18S rRNA
YJL072c	PSF2	-	Part of GINS, replication multiprotein complex
YJL074c	SMC3	-	Required for structural maintenance of chromosomes
YJL076w	NET1	-	Required for rDNA silencing and nucleolar integrity
YJL081c	ARP4	-	Nuclear actin-related protein
YJL087c	TRL1	-	tRNA ligase
YJL090c	DPB11	-	Involved in DNA replication and S-phase checkpoint
YJL091c	GWT1	-	Formation of glucosaminyl(acyl)phosphatidylinositol
YJL194w	CDC6	-	Cell division control protein
YJL203w	PRP21	-	Pre-mRNA splicing factor
YJR002w	MPP10	-	Component of the U3 small nucleolar ribonucleoprotein
YJR007w	SUI2	-	Translation initiation factor eIF2
YJR017c	ESS1	-	Processing/termination factor1
YJR022w	LSM8	-	Splicing factor
YJR041c	YJR041c	-	Metabolism of the rRNA primary transcript
YJR042w	NUP85	-	Nuclear pore protein
YJR058c	APS2	-	AP-2 complex subunit, sigma2 subunit, 17 KD
YJR068w	RFC2	-	DNA replication factor C, 41KD subunit
YJR072c	YJR072c	-	Association with pre-ribosomal particles
YJR076c	CDC11	-	Septin
YKL009w	MRT4	-	mRNA turnover 4
YKL014c	URB1	-	Required for the normal accumulation of 25S and 5.8S rRNAs
YKL018w	SWD2	-	Subunit of the COMPASS complex
YKL021c	MAK11	-	Involved in cell growth and replication of M1 dsRNA virus
YKL035w	UGP1	-	UTP--glucose-1-phosphate uridylyltransferase
YKL045w	PRI2	-	DNA directed DNA polymerase alpha 58KD subunit
YKL059c	MPE1	-	3' end formation of mRNA
YKL078w	DHR2	-	RNA helicase, involved in ribosomal RNA maturation
YKL082c	YKL082c	-	Protein putative involved in cell differentiation
YKL095w	YJU2	-	Putative spliceosomal component involved in mRNA splicing
YKL099c	UTP11	-	U3 snoRNP protein
YKL108w	SLD2	-	Chromosomal DNA replication protein
YKL112w	ABF1	-	ARS - binding factor
YKL125w	RRN3	-	RNA polymerase I specific transcription factor
YKL139w	CTK1	-	Carboxy-terminal domain (CTD) kinase, alpha subunit
YKL141w	SDH3	-	Cytochrome b subunit of succinate dehydrogenase
YKL144c	RPC25	-	DNA-directed RNA polymerase III, 25 KD subunit
YKL172w	EBP2	-	Pre rRNA processing and ribosomal subunit assembly
YKL180w	RPL17a	-	Ribosomal protein L17.e
YKL186c	MTR2	-	mRNA transport protein
YKL189w	HYM1	-	Component of the RAM signaling network
YKL193c	SDS22	-	Mitotic function of type I protein phosphatase
YKL203c	TOR2	-	Phosphatidylinositol 3-kinase
YKR025w	RPC37	-	Pol III transcription
YKR062w	TFA2	-	TFIIE subunit (transcription initiation factor), 43 KD
YKR079c	TRZ1	-	Protein required for cell viability
YKR083c	DAD2	-	Outer kinetochore protein - part of Dam1 complex
YKR086w	PRP16	-	RNA-dependent ATPase
YLL003w	SFI1	-	Function in budding yeast spindle pole body duplication
YLL004w	ORC3	-	Origin recognition complex, 62KD subunit
YLL008w	DRS1	-	RNA-helicase of the DEAD box family
YLL018c	DPS1	-	Aspartyl-tRNA synthetase
YLL034c	RIX7	-	Biogenesis and nuclear export of 60S ribosomal subunits
YLL035w	GRC3	-	Possibly involved in rRNA processing
YLR002c	NOC3	-	Maturation and intranuclear transport of pre-ribosomes
YLR005w	SSL1	-	TFIIH subunit (transcription initiation factor), factor B
YLR007w	NSE1	-	Essential nuclear protein required for DNA repair
YLR009w	RPL24	-	60S large subunit ribosomal protein L24.e.A
YLR010c	TEN1	-	Protein involved in telomeric pathways
YLR022c	SDO1	-	Required for cell viability localised to cytoplasm and nucleus
YLR029c	RPL15A	-	60s large subunit ribosomal protein L15.e.c12
YLR045c	STU2	-	Supressor of a cs tubulin mutation

YLR060w	FRS1	-	Phenylalanyl-tRNA synthetase
YLR071c	RGR1	-	DNA-directed RNA polymerase II holoenzyme subunit
YLR086w	SMC4	-	Stable maintenance of chromosomes
YLR088w	GAA1	-	Required for attachment of GPI anchor onto proteins
YLR103c	CDC45	-	Required for initiation of chromosomal DNA replication
YLR105c	SEN2	-	Subunit of the tRNA splicing endonuclease
YLR106c	MDN1	-	Associated with 60S pre-ribosomes (large subunit precursor)
YLR115w	CFT2	-	Cleavage and polyadenylation specificity factor, part of CF II
YLR117c	CLF1	-	Pre-mRNA splicing factor
YLR145w	RMP1	-	Involved in RNA processing in mitochondria
YLR147c	SMD3	-	Required for pre-mRNA splicing
YLR153c	ACS2	-	Acetyl-coenzyme A synthetase
YLR175w	CBF5	-	Putative rRNA pseuduridine synthase
YLR186w	EMG1	-	Essential for mitotic growth
YLR195c	NMT1	-	N-myristoyl transferase
YLR196w	PWP1	-	Resembles members of beta-transducine superfamily
YLR229c	CDC42	-	GTP-binding protein of RAS superfamily
YLR249w	YEF3	-	Translation elongation factor eEF3
YLR259c	HSP60	-	Heat shock protein - chaperone, mitochondrial
YLR272c	LOC7	-	Subunit of condensine protein complex
YLR274w	CDC46	-	Cell division control protein
YLR275w	SMD2	-	U1 snRNP protein of the Sm class
YLR276c	DBP9	-	Involved in biogenesis of the 60S ribosomal subunit
YLR277c	YSH1	-	Component of pre-mRNA polyadenylation factor PF I
YLR291c	GCD7	-	Translation initiation factor eIF2B
YLR298c	YHC1	-	Associated with the U1 snRNP complex
YLR305c	STT4	-	Phosphatidylinositol-4-kinase
YLR310c	CDC25	-	GDP/GTP exchange factor for RAS1p ans RAS2p
YLR314c	CDC3	-	Cell division control protein
YLR316c	TAD3	-	Subunit of tRNA-specific adenosine-34-deaminase
YLR323c	CWC24	-	Essential protein, component of a complex containing Cef1p
YLR336c	SGD1	-	Involved in HOG pathway
YLR347c	KAP95	-	Karyopherin-beta
YLR355c	ILV 5	-	Ketol-acid reducto-isomerase
YLR359w	ADE13	-	Adenylosuccinate lyase
YLR383w	RHC18	-	Recombination repair protein
YLR424w	SPP382	-	Essential protein present in native splicing complexes
YLR430w	SEN1	-	Positive effector of tRNA-splicing endonuclease
YLR438w	CAR2	-	Ornithine aminotransferase
YLR457c	NBP1	-	Nap1p binding protein
YLR459w	CDC91	-	Involved in attachment of GPI anchors to proteins
YML015c	TAF11	-	TFIID subunit 40KD
YML023c	NSE5	-	Protein putative involved in DNA repair
YML025c	YML6	-	Mitochondrial ribosomal protein of the large subunit
YML031w	NDC1	-	Nuclear envelope protein
YML043c	RRN11	-	RNA polymerase I specific transcription initiation factor
YML046w	PRP39	-	Pre-mRNA splicing factor
YML049c	RSE1	-	Involved in RNA splicing and ER to Golgi transport
YML064c	TEM1	-	GTP binding protein of ras superfamily
YML065w	ORC1	-	Origin recognition complex, 104KD subunit
YML069w	POB3	-	Protein that binds to DNA polymeraseI (PolI)
YML085c	TUB1	-	Alpha-1-tubulin
YML091c	RPM2	-	Ribonuclease P precursor, mitochondrial
YML098w	TAF13	-	TFIID subunit 19KD
YML114c	TAF65	-	TBP Associated Factor 65 KDa
YML130c	ERO1	-	Required for protein disulfide bond formation in the ER
YMR001c	CDC5	-	Protein kinase, involved in regulation of DNA replication
YMR005w	TAF4	-	Required for protein synthesis
YMR013c	SEC59	-	Dolichol kinase
YMR028w	TAP42	-	Component of the TOR signaling pathway
YMR033w	ARP9	-	Actin-related protein
YMR043w	MCM1	-	Transcription factor of the MADS box family

YMR047c	NUP116	-	Nuclear pore protein
YMR059w	SEN15	-	tRNA splicing endonuclease
YMR061w	RNA14	-	component of pre-mRNA 3^-end processing factor CF I
YMR076c	PDS5	-	precocious dissociation of sister chromatides
YMR093w	UTP15	-	Nucleolar protein
YMR094w	CTF13	-	Kinetochore protein complex, 58KD subunit
YMR112c	MED11	-	Mediator complex subunit
YMR113w	FOL3	-	Dihydrofolate synthetase
YMR117c	SPC24	-	Outer kinetochore protein - part of Ndc80p complex
YMR128w	ECM16	-	Putative DEAH-box RNA helicase
YMR146c	TIF34	-	Translation initiation factor eIF3
YMR149w	SWP1	-	Oligosaccharyl transferase glycoprotein complex
YMR186w	HSC82	-	Heat shock protein
YMR200w	ROT1	-	Mutant suppresses tor2 mutation
YMR211w	DML1	-	May function in the segregation of chromosomes
YMR213w	CEF1	-	Required during G2/M transition
YMR227c	TAF7	-	TFIID subunit 67KD
YMR235c	RNA1	-	GTPase activating protein
YMR236w	TAF9	-	TFIID and SAGA subunit
YMR239c	RNT1	-	Double stranded ribonuclease
YMR240c	CUS1	-	U2 snRNP protein
YMR268c	PRP24	-	Pre-mRNA splicing factor
YMR270c	RRN9	-	RNA polymerase I specific transcription initiation factor
YMR288w	HSH155	-	Component of a multiprotein splicing factor
YMR290c	HAS1	-	Required for 40S ribosomal subunit biogenesis
YMR308c	PSE1	-	Beta karyopherin
YMR309c	NIP1	-	Associated with 40s ribosomal subunit
YNL002c	RLP7	-	Nucleolar protein related to ribosomal protein L7
YNL038w	GPI15	-	GlycosylPhosphatidylInositol anchor biosynthesis protein
YNL088w	TOP2	-	Essential type II topoisomerase
YNL102w	POL1	-	DNA directed polymerase alpha, 180KD subunit
YNL110c	NOP15	-	Constituent of 66S pre-ribosomal particles
YNL118c	DCP2	-	Suppressor protein of yeast pet mutant
YNL124w	NAF1	-	Nuclear Assembly Factor
YNL126w	SPC98	-	Spindle pole body component
YNL151c	RPC31	-	DNA-directed RNA polymerase III, 31 KD subunit
YNL158w	YNL158w	-	Required for maturation of Gas1p and Pho8p
YNL161w	CBK1	-	Protein kinase involved in cell wall biosynthesis
YNL163c	RIA1	-	Translation elongation factor eEF4
YNL182c	IPI3	-	Part of noncoding RNA-processing machinery
YNL188w	KAR1	-	Cell division control protein
YNL207w	RIO2	-	Protein required for cell viability
YNL216w	RAP1	-	DNA-binding protein with repressor and activator activity
YNL221c	POP1	-	Protein component of ribonuclease P and ribonuclease MRP
YNL222w	SSU72	-	Suppressor of cs mutant of sua7
YNL232w	CSL4	-	Core component of the 3´-5´exosome
YNL244c	SUI1	-	Translation initiation factor eIF3
YNL245c	CWC25	-	Involved in pre-mRNA splicing
YNL247w	YNL247w	-	Cysteine-tRNA ligase synthetase
YNL251c	NRD1	-	Involved in regulation of nuclear pre-mRNA abundance
YNL261w	ORC5	-	Origin recognition complex, 50KD subunit
YNL282w	POP3	-	Required for processing of tRNAs and rRNAs
YNL290w	RFC3	-	DNA replication factor C, 40KD subunit
YNL308c	KRI1	-	KRRI-Interacting protein1
YNL312w	RFA2	-	DNA replication factor A, 36KD subunit
YNL317w	PFS2	-	Polyadenylation factor I subunit 2
YNR003c	RPC34	-	DNA-directed RNA polymerase III, 34 KD subunit
YNR038w	DBP6	-	RNA helicase required for 60S ribosomal subunit assembly
YNR053c	NOG2	-	Associates with pre-60S ribosomal subunits in the nucleolus
YNR054c	ESF2	-	Eighteen S rRNA processing factor
YOL005c	RPB11	-	DNA-directed RNA polymerase II subunit, 13.6 kD
YOL010w	RCL1	-	RNA terminal phosphate cyclase-like protein

YOL021c	DIS3	-	Required for 3´ end formation of 5.8S rRNA
YOL034w	SMC5	-	Structural maintenance of chromosomes (SMC) protein
YOL069w	NUF2	-	Outer kinetochore protein-part of Ndc80p complex
YOL077c	BRX1	-	Nucleolar protein
YOL078w	AVO1	-	May have a role in regulation of cell growth
YOL094c	RFC4	-	DNA replication factor C, 37KD subunit
YOL097c	WRS1	-	Tryptophanyl-tRNA synthetase
YOL102c	TPT1	-	tRNA 2'-phosphotransferase
YOL123w	HRP1	-	CF Ib (RNA3´ Cleavage factor Ib)
YOL130w	ALR1	-	Transporter of magnesium and other divalent cations
YOL135c	MED7	-	RNA Polymerase II transcriptional regulation mediator
YOL139c	CDC33	-	Translation initiation factor eIF4E
YOL144w	NOP8	-	Nucleolar protein
YOR048c	RAT1	-	5´-3´exoribonuclease
YOR063w	RPL3	-	Protein component of the large (60S) ribosomal subunit
YOR074c	CDC21	-	Thymidylate synthase
YOR077w	RTS2	-	Involved in UV response and DNA replication
YOR095c	RKI1	-	D-ribose-5-phosphate ketol-isomerase
YOR098c	NUP1	-	Nuclear pore protein
YOR103c	OST2	-	Oligosaccharyltransferase epsilon subunit
YOR110w	TFC7	-	RNA polymerase III transcription initiation factor
YOR116c	RPO31	-	DNA-directed RNA polymerase III, 160 KD subunit
YOR119c	RIO1	-	Essential protein that plays a role in cell cycle progression
YOR143c	THI80	-	Thiamine pyrophosphokinase
YOR145c	PNO1	-	Protein required for cell viability
YOR149c	SMP3	-	Protein kinase C pathway protein
YOR151c	RPB2	-	DNA-directed RNA polymerase II, 140 kDa chain
YOR159c	SME1	-	Required for mRNA splicing
YOR174w	MED4	-	Transcription regulation mediator
YOR176w	HEM15	-	Ferrochelatase precursor
YOR204w	DED1	-	ATP-dependent RNA helicase
YOR206w	NOC2	-	Intranuclear movement of ribosomal precursor particles
YOR210w	RPB10	-	DNA-directed polymerase I, II, III 8.3 subunit
YOR224c	RPB8	-	RNA polymerase subunit ABC14.5
YOR244w	ESA1	-	Histone acetyltransferase
YOR272w	YTM1	-	Microtubule-interacting protein
YOR278w	HEM4	-	Uroporphyrinogen III synthase,
YOR281c	PLP2	-	Likely to be involved in regulation of pheromone response
YOR294w	RRS1	-	Regulator of ribosome synthesis
YOR335c	ALA1	-	Cytoplasmic alanyl-tRNA synthetase,
YOR336w	KRE5	-	Killer toxin-resistance protein
YOR340c	RPA43	-	DNA-directed RNA polymerase I, 36 KD subunit
YOR361c	PRT1	-	Translation initiation factor eIF3
YOR372c	NDD1	-	Protein required for nuclear division
YPL007c	TFC8	-	RNA Polymerase III transcription initiation factor TFIIIC
YPL011c	TAF3	-	Component of the TBP-associated protein complex
YPL012w	RRP12	-	Protein required for normal pre-rRNA processing
YPL043w	NOP4	-	Nucleolar protein
YPL075w	GCR1	-	Transcriptional activator
YPL076w	GPI2	-	N-acetylglucosamyl-phosphatidylinositol biosynthetic protein
YPL083c	SEN54	-	tRNA splicing endonuclease alpha subunit
YPL093w	NOG1	-	Associates with free 60S ribosomal subunits in the nucleolus
YPL124w	SPC29	-	Spindle pole body component
YPL126w	NAN1	-	Part of small (ribosomal) subunit (SSU) processosome
YPL128c	TBF1	-	Telomere TTAGGG repeat-binding factor 1
YPL151c	PRP46	-	Splicing factor
YPL169c	MEX67	-	Factor for nuclear mRNA export
YPL190c	NAB3	-	Polyadenylated RNA-binding protein
YPL217c	BMS1	-	Required for distinct steps of 40S ribosome biogenesis
YPL218w	SAR1	-	GTP-binding protein of the ARF family
YPL228w	CET1	-	RNA 5´-triphosphatase (mRNA capping enzyme, beta subunit)
YPL235w	RVB2	-	RUVB-like protein

YPR016c	TIF6	-	Translation initiation factor elF5
YPR019w	CDC54	-	Member of the Cdc46p/Mcm2p/Mcm3p family
YPR033c	HTS1	-	Mitochondrial histidine tRNA synthetase
YPR048w	TAH18	-	Potential role in DNA replication
YPR086w	SUA7	-	TFIIB subunit (transcription initiation factor), factor E
YPR094w	RDS3	-	Protein required for cell viability
YPR110c	RPC40	-	DNA-directed RNA polymerase I, III 40 KD subunit
YPR112c	MRD1	-	Protein that associates with 35S precursor rRNA
YPR133c	IWS1	-	Protein is Spt6-interacting putative elongation factor
YPR137w	RRP9	-	Protein associated with the U3 small nucleolar RNA
YPR144c	NOC4	-	Nucleolar protein
YPR161c	SGV1	-	Ser/thr protein kinase
YPR162c	ORC4	-	Origin recognition complex, 56KD subunit
YPR168w	NUT2	-	Negative transcription regulator from artificial reporters
YPR175w	DPB2	-	DNA-directed DNA polymerase epsilon, subunit B
YPR178w	PRP4	-	U4/U6 snRNP 52 KD protein
YPR180w	AOS1	-	Forms heterodimeric activating enzyme for Smt3p
YPR186c	PZF1	-	TFIIIA (transcription initiation factor)
YPR187w	RPO26	-	DNA-directed RNA polymerase I, II, III 18 KD subunit
YPR190c	RPC82	-	DNA-directed RNA polymerase III, 82 KD subunit

Teilarbeit B

Katrin Altmann, Martina Frank, Daniel Neumann, Stefan Jakobs, and Benedikt Westermann (2008)

The class V myosin motor protein, Myo2, plays a major role in mitochondrial movement in *Saccharomyces cerevisiae*

Journal of Cell Biology 181, 119-130

Darstellung des Eigenanteils

Alle Experimente und sämtliche Abbildungen, bis auf nachfolgend erwähnte, sind Resultate meiner Arbeit. Unter meiner Anleitung entstanden in Zusammenarbeit mit Martina Frank die visuellen Aktinbindungsassays (Abbildung 2E und 2F; Abbildung 4B). Die Bewegungsaufnahmen von Mitochondrien der Punkmutante L1301P entstanden am Max-Planck-Institut für Biophysikalische Chemie in Göttingen. Dr. Stefan Jakobs stellte mir seine Instrumente zur Verfügung, während in Zusammenarbeit mit Daniel Neumann die Bildaufnahmen entstanden (siehe Abbildung 5A).

Verfasst wurde diese Teilarbeit von Prof. Dr. Benedikt Westermann.

The class V myosin motor protein, Myo2, plays a major role in mitochondrial motility in *Saccharomyces cerevisiae*

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The actin cytoskeleton is essential for polarized, bud-directed movement of cellular membranes in *Saccharomyces cerevisiae* and thus ensures accurate inheritance of organelles during cell division. Also, mitochondrial distribution and inheritance depend on the actin cytoskeleton, though the precise molecular mechanisms are unknown. Here, we establish the class V myosin motor protein, Myo2, as an important mediator of mitochondrial motility in budding yeast. We found that mutants with abnormal expression levels of Myo2 or its associated light chain, Mlc1, exhibit aberrant mitochondrial morphology and loss of mitochondrial DNA. Specific mutations in the globular tail of Myo2 lead to aggregation of mitochondria in the mother cell. Isolated mitochondria lacking functional Myo2 are severely impaired in their capacity to bind to actin filaments in vitro. Time-resolved fluorescence microscopy revealed a block of bud-directed anterograde mitochondrial movement in cargo binding–defective *myo2* mutant cells. We conclude that Myo2 plays an important and direct role for mitochondrial motility and inheritance in budding yeast.

Introduction

Mitochondria play a key role in cellular energy metabolism; they are the site of many anabolic and catabolic pathways, they are essential for the assembly of iron/sulfur clusters, and they participate in calcium signaling and regulation of cell death programs. Because of this multitude of cellular functions, the presence of mitochondria is essential for viability in virtually every eukaryotic cell. Mitochondria cannot be made de novo. Rather, they have to grow and divide from preexisting membranes, and their inheritance during cytokinesis involves ordered, cytoskeleton-dependent partitioning mechanisms (Nunnari and Walter, 1996; Warren and Wickner, 1996; Catlett and Weisman, 2000). Furthermore, cytoskeleton-based motility concomitant with frequent membrane fusion and fission is required to adapt mitochondrial copy number, morphology, and intracellular position to the cellular demands (Bereiter-Hahn, 1990; Yaffe, 1999; Chan, 2006; Dimmer and Scorrano, 2006). However, the molecular mechanisms governing mitochondrial motility, inheritance, and distribution within the cell are only poorly understood.

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Abbreviations used in this paper: DIC, differential interference contrast; Dox, doxycycline; mtDNA, mitochondrial DNA; mtGFP, mitochondria-targeted GFP.

The online version of this paper contains supplemental material.

The budding yeast *Saccharomyces cerevisiae* has proven to be an excellent model organism to study the molecular basis of mitochondrial dynamics, and most of the evolutionarily conserved key components were first discovered in yeast (Hermann and Shaw, 1998; Jensen et al., 2000; Okamoto and Shaw, 2005; Merz et al., 2007). However, although many mechanistic details of the machineries mediating mitochondrial fusion and fission have been revealed in recent years (Shaw and Nunnari, 2002; Westermann, 2003; Hoppins et al., 2007), the identity of the proteins driving cytoskeleton-dependent mitochondrial movement in yeast is still under debate.

The evidence for a central role of the actin cytoskeleton in mitochondrial motility in budding yeast is clear cut. Numerous mutations in genes encoding actin or factors involved in actin filament dynamics lead to aberrant mitochondrial distribution and morphology (Drubin et al., 1993; Lazzarino et al., 1994; Simon et al., 1995; Smith et al., 1995; Hermann et al., 1997; Simon et al., 1997; Yang et al., 1999; Singer et al., 2000; Boldogh et al., 2001a; Fehrenbacher et al., 2004; Altmann and Westermann, 2005). Treatment of wild-type cells with actin filament–depolymerizing drugs produces defects in mitochondrial shape and movement (Boldogh et al., 1998), and fluorescence microscopy of fixed and live yeast cells revealed that mitochondria colocalize with

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and move along actin cables (Drubin et al., 1993; Lazzarino et al., 1994; Simon et al., 1997; Fehrenbacher et al., 2004). Isolated mitochondria bind to actin filaments in vitro in an ATP-sensitive manner (Lazzarino et al., 1994; Simon et al., 1995; Boldogh et al., 1998).

The myosin family of actin-based motors consists of at least 15 structurally and functionally distinct classes. In particular, class V family members have been shown to participate in numerous membrane trafficking events (Reck-Peterson et al., 2000). The yeast genome encodes five myosin-related motor proteins (Winsor and Schiebel, 1997). The *MYO1* gene encodes a class II myosin that, depending on the strain background, is either essential or nonessential for viability; the essential *MYO2* and the nonessential *MYO4* genes encode class V myosins and the nonessential *MYO3* and *MYO5* genes encode class I myosins. A myosin light chain that associates with Myo1 and Myo2 heavy chains is encoded by the essential *MLC1* gene (Stevens and Davis, 1998; Luo et al., 2004). In yeast, class V myosins are of major importance for polarized growth and actin-based organelle segregation. Cargos transported by Myo2 or Myo4 include post-Golgi secretory vesicles, the trans-Golgi network, the cortical endoplasmic reticulum, vacuolar membranes, peroxisomes, mRNA–protein complexes, and microtubule plus ends (Pruyne et al., 2004). Thus, it is tempting to speculate that mitochondria might also be transported by class V myosins. However, several genetic attempts to identify a myosin-like protein responsible for mitochondrial movement have failed so far. Δ*myo1*, Δ*myo3*, Δ*myo4*, and Δ*myo5* single deletion mutants (Simon et al., 1995; Dimmer et al., 2002; Boldogh et al., 2004) and a Δ*myo3* Δ*myo5* double mutant (Goodson et al., 1996) do not display major defects in mitochondrial distribution and morphology. Moreover, mitochondria have been reported to appear normal in certain conditional *myo2* mutants, namely *myo2-66* (Simon et al., 1995), *myo2-338* (Itoh et al., 2002), and *myo2-Δ6IQ* (Boldogh et al., 2004), and in double mutant *myo2-66* Δ*myo4* (Simon et al., 1995). Based on these findings, it is assumed that myosins do not play an important role in mitochondrial motility and inheritance in budding yeast (Boldogh et al., 2001b; Pruyne et al., 2004; Boldogh and Pon, 2006).

However, it has been reported that the *myo2-573* allele induces defects in mitochondrial distribution toward the bud (Itoh et al., 2002, 2004), and close inspection of mutants containing the *myo2-66* allele revealed an accumulation of mitochondria in the mother cell (Boldogh et al., 2004). Based on these results, a model has been proposed that assigns to Myo2 a rather indirect role in mitochondrial inheritance. According to this model, Myo2 drives movement of yet unknown retention factors from the mother cell to the bud tip, where these factors serve to anchor mitochondria to prevent retrograde movement back into the mother cell (Boldogh et al., 2004; Boldogh and Pon, 2006). In this scenario, entry of mitochondria into the bud would be mediated by myosin-independent mechanisms, and Myo2 would not be required for interaction of mitochondria with microfilaments.

We recently screened a collection of yeast strains containing essential genes under control of a titratable promoter to identify novel components involved in mitochondrial morphogenesis. The observation that depletion of Myo2 or Mlc1 results in the formation of highly aberrant mitochondria (Altmann and Westermann, 2005) prompted us to investigate whether Myo2 plays a direct role in mediating interactions of mitochondria with the cytoskeleton and bud-directed mitochondrial movement. Our results described here suggest that Myo2 is much more important for mitochondrial motility and inheritance than previously anticipated.

Results

Depletion of Myo2 induces defects in mitochondrial morphology

To investigate the role of essential myosin-related motor proteins in mitochondrial morphogenesis, we took advantage of promoter shutoff strains that carry the *MLC1*, *MYO1*, or *MYO2* gene under control of the $TetO_7$ promoter (Mnaimneh et al., 2004). Addition of doxycycline (Dox) to the medium efficiently represses the $TetO_7$ promoter, whereas promoter activity is high in the absence of Dox (Gari et al., 1997). Mitochondrial defects were never observed in $TetO_7$-*myo1* cells (Altmann and Westermann, 2005). In contrast, mitochondria have aberrant morphology in $TetO_7$-*mlc1* and $TetO_7$-*myo2* strains under repressive conditions but also in the absence of Dox (Altmann and Westermann, 2005). The latter conditions presumably result in nonphysiological overexpression of Myo2, which is expected to cause toxic effects because Mlc1 amounts become limiting (Stevens and Davis, 1998). Consistently, nonphysiological expression levels of Myo2 and Mlc1 lead to severe growth defects both in the absence and presence of Dox (Fig. 1 A).

To test the dependence of mitochondrial morphology on *MYO2* expression, we incubated $TetO_7$-*myo2* cells expressing mitochondria-targeted GFP (mtGFP) for different time periods in Dox-containing medium. Then, cells were fixed, microfilaments were stained with rhodamine phalloidin, and mitochondria and the actin cytoskeleton were visualized by fluorescence microscopy. Wild-type control cells displayed their characteristic tubular branched mitochondrial network, with actin patches concentrated in the bud and actin cables extending throughout the mother cell (Fig. 1 B). It should be mentioned that even extended growth on Dox-containing medium does not affect the actin cytoskeleton in wild-type cells (Altmann and Westermann, 2005). Already, in the absence of Dox, 35% of $TetO_7$-*myo2* cells contained misshapen, clumped, and often ring-shaped mitochondria in the presence of an apparently normal actin cytoskeleton (Fig. 1 B). This fraction of cells reached a maximum of 47% after 15 h of promoter repression. At later time points, an increasing fraction of cells contained a disorganized actin cytoskeleton in addition to altered mitochondria, presumably because of pleiotropic defects of Myo2 depletion. After 22 h of promoter repression, no cells with wild type–like mitochondria could be found (Fig. 1 B). Very similar results were obtained with the $TetO_7$-*mlc1* strain (Fig. 1 B). Interestingly, aberrant mitochondria could also be found in 36% of cells overexpressing *MYO2* from the *GAL* promoter compared with 4% in the control strain. We conclude that maintenance of mitochondrial morphology is sensitive to both overexpression and depletion of Myo2. The fact that a large fraction of cells contain aberrant

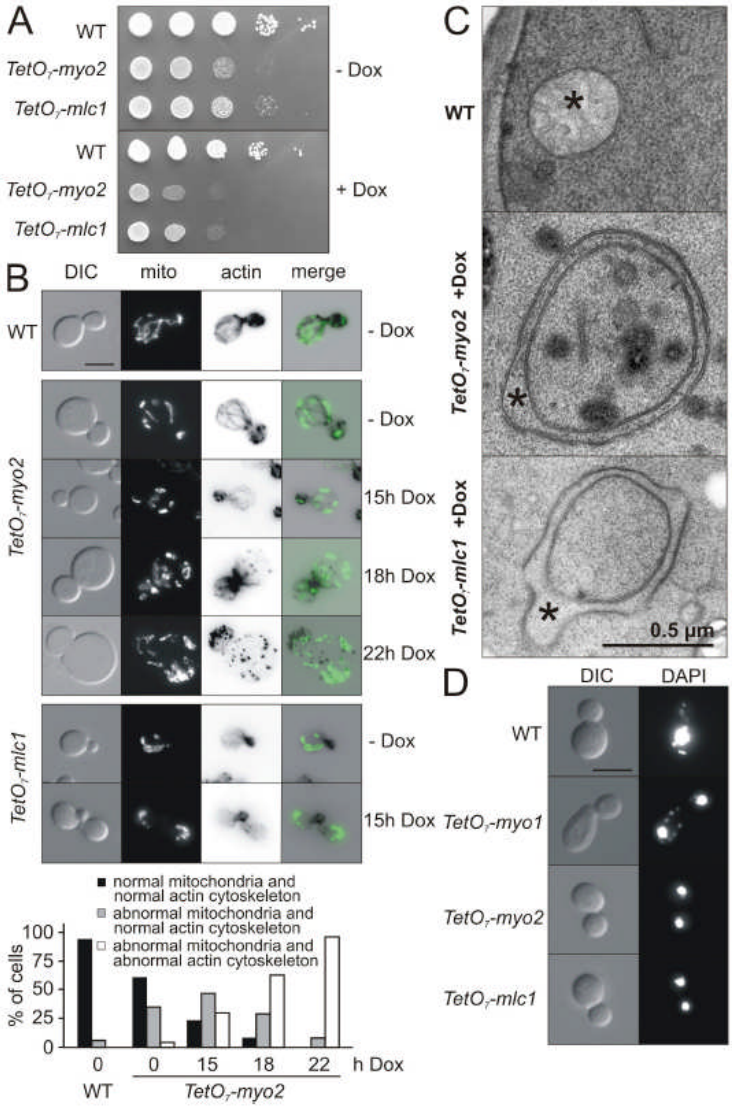

Figure 1. **Myo2 and Mlc1 are involved in mitochondrial morphology and inheritance.** (A) Serial dilutions of wild-type (WT), *TetO7-myo2*, and *TetO7-mlc1* cell suspensions were spotted onto plates with glucose-containing medium with or without 30 µg/ml Dox and incubated for three days at 30°C. (B) WT, *TetO7-myo2*, and *TetO7-mlc1* cells expressing mtGFP were grown for the indicated time periods in liquid glucose-containing medium in the absence or presence of 30 µg/ml Dox. Actin was stained with rhodamine phalloidin in fixed cells and cells were observed by fluorescence microscopy. (left) DIC image. (second from the left) Mitochondrial morphology (mito). (second from the right) Organization of filamentous actin (a reversed fluorescence image is shown to better visualize faint actin cables). (right) Merged image of reversed actin fluorescence and mitochondrial fluorescence. The graph at the bottom is a quantification of phenotypes of 100 cells per time point. Bar, 5 µm. (C) WT, *TetO7-myo2*, and *TetO7-mlc1* cells were grown to logarithmic growth phase in glucose-containing medium. *TetO7-myo2* and *TetO7-mlc1* cells were incubated in the presence of 30 µg/ml Dox for 15 h to deplete Myo2 or Mlc1. Ultrathin sections were analyzed by electron microscopy. All images are shown at the same magnification. Asterisks indicate the matrix space. (D) WT and *TetO7*-myosin strains were grown in glucose-containing medium, cellular DNA was stained with DAPI, and cells were analyzed by DIC and fluorescence microscopy. Nuclei are seen as large fluorescent spots; mtDNA nucleoids are seen as small fluorescent foci. Bar, 5 µm.

mitochondria in the presence of a normally organized actin cytoskeleton suggests that mitochondrial phenotypes seen in *TetO7-myo2* and *TetO7-mlc1* cells are not secondary consequences of cytoskeletal defects.

Next, we analyzed the ultrastructure of mitochondria in *TetO7-myo2* and *TetO7-mlc1* cells by electron microscopy. Promoter activity was repressed by growth in Dox-containing medium, cells were fixed and stained, and ultrathin sections were prepared and analyzed by transmission electron microscopy. Wild-type control cells displayed normal mitochondrial tubules with diameters of ~300–500 nm (Fig. 1 C). In contrast, cells depleted of Myo2 or Mlc1 frequently showed ring-shaped mitochondria with a very narrow matrix space of <100 nm in width (Fig. 1 C). This demonstrates that loss of Myo2 has severe effects on the internal structure of mitochondria. It is conceivable that the aberrant structures seen in electron micrographs correspond to the ring-shaped mitochondria observed by fluorescence microscopy (Fig. 1 B). Cross sections of complete yeast cells revealed an accumulation of coated vesicles in addition to aberrant mitochondrial morphology (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200709099/DC1), which suggests that secretion and bud growth are also impaired under these conditions.

We observed some residual growth of *TetO7-myo2* and *TetO7-mlc1* cells on Dox-containing medium (Fig. 1 A). This suggests

that a basal expression of Myo2 and Mlc1 occurs even under repressive conditions. However, *TetO7-myo2* and *TetO7-mlc1* cells were unable to grow on nonfermentable carbon sources (unpublished data). As many mutants defective in mitochondrial morphology lose their mitochondrial genome (Berger and Yaffe, 2000), we asked whether respiratory deficiency of *TetO7*-myosin strains might be caused by the loss of mitochondrial DNA (mtDNA). To test this, cellular DNA was visualized by DAPI staining and fluorescence microscopy in wild-type, *TetO7-myo1*, *TetO7-myo2*, and *TetO7-mlc1* cells. Numerous spots of fluorescent mtDNA nucleoids were observed in wild-type and *TetO7-myo1* cells but never in *TetO7-myo2* and *TetO7-mlc1* cells (Fig. 1 D). Thus, nonphysiologically high expression levels in the absence of Dox lead to the loss of the mitochondrial genome, which indicates that normal Myo2 and Mlc1 levels are required for maintenance of mtDNA. We conclude that Myo2 and its associated light chain Mlc1 are important for normal mitochondrial distribution and morphology, maintenance of the internal structure of mitochondria, and maintenance of mtDNA.

Myo2 and Mlc1 are required for binding of mitochondria to actin filaments in vitro

The interaction of isolated mitochondria with actin filaments in vitro has been shown to be ATP-sensitive, reversible, and dependent on mitochondria-associated proteins (Lazzarino et al., 1994). As these properties are compatible with the view that myosin-related motor proteins mediate organelle–cytoskeleton interactions, we asked whether Myo2 and Mlc1 are involved in this process. First, we incubated purified wild-type mitochondria with filamentous actin in the absence or presence of ATP, sedimented the organelles through a sucrose cushion, and detected bound actin by immunoblotting. Mitochondria were found to interact with actin filaments in an ATP-sensitive manner (Fig. 2 A, lanes 1 and 2; and Fig. 2 E). This binding activity could be removed by the extraction of mitochondria with high-salt buffer (Fig. 2 A, lanes 3 and 4; and Fig. 2 E) and was restored by the readdition of salt extract (Fig. 2 A, lanes 5 and 6; and Fig. 2 E). These results are very similar to observations made previously by Boldogh et al. (1998) and confirm that mitochondria–cytoskeleton interactions are mediated by proteins peripherally bound to the mitochondrial surface. Next, we tested mitochondria purified from *TetO7-myo2* and *TetO7-mlc1* cells grown under repressing conditions. Depletion of Myo2 reduced the mitochondrial actin binding capacity to 51% compared with the wild type (Fig. 2, B and E). Similarly, depletion of Mlc1 reduced actin binding activity to 55% (Fig. 2 B and E), demonstrating that Myo2 and its associated light chain are required for an efficient interaction of mitochondria with actin filaments. Remarkably, addition of salt extract prepared from wild-type mitochondria improved the actin binding activity of Myo2-depleted mitochondria from 51 to 95% (Fig. 2, C and E). This suggests that Myo2 is the peripheral mitochondria-associated factor that becomes limiting in *TetO7-myo2* mitochondria. Moreover, incubation of wild-type mitochondria with affinity-purified antibodies directed against Myo2 abolished ATP-sensitive mitochondrial actin binding activity completely, whereas the same amount of antibodies directed against the mitochondrial inner membrane protein Mdm31 (Dimmer et al., 2005) had no effect (Fig. 2, D and E). This result demonstrates that Myo2 on the mitochondrial surface is required to establish interactions of the organelle with the cytoskeleton.

To corroborate these findings and observe the mitochondria–cytoskeleton interactions more directly, we used a visual in vitro assay. Mitochondria were isolated from mtGFP-expressing wild-type, *TetO7-myo2*, and *TetO7-mlc1* cells that were grown under repressive conditions. Purified mitochondria were incubated with Alexa Fluor 568–labeled actin filaments in the absence or presence of ATP and observed by fluorescence microscopy. In the absence of ATP, 91% of wild-type mitochondria were bound to actin. In contrast, only 45% of *TetO7-myo2* and 51% of *TetO7-mlc1* mitochondria were found in the vicinity of actin filaments (Fig. 2, F and G). In the presence of ATP, only 19–29% of mitochondria were found adjacent to actin filaments (Fig. 2 G), again indicating the ATP sensitivity of binding. Remarkably, only 16% of mitochondria pretreated with Myo2 antibodies were found next to actin filaments, whereas preincubation of mitochondria with Mdm31 antibodies as a control had no effect (Fig. 2, F and G). The fact that Myo2 antibodies blocked ATP-sensitive mitochondrial actin binding activity completely suggests that minor activity seen with Myo2-depleted mitochondria (Fig. 2, B, C, E, F, and G) is caused by residual expression of Myo2 in *TetO7-myo2* cells under repressive conditions. We conclude from this series of experiments that Myo2 and its associated light chain are directly required for binding of mitochondria to the actin cytoskeleton.

Specific mutations in the Myo2 globular tail affect mitochondrial distribution and morphology

The carboxy terminal globular tail of Myo2 mediates specific binding of the myosin motor to cargo membranes such as vacuoles (Catlett and Weisman, 1998) and secretory vesicles (Schott et al., 1999). It consists of two structurally and functionally distinct subdomains; the proximal half binds to vacuolar membranes, whereas the distal half interacts with secretory vesicles (Catlett et al., 2000; Pashkova et al., 2005, 2006). We asked whether the Myo2 globular tail has a role in mitochondrial distribution and morphology. To test this, we used a series of point mutants carrying substitutions of single amino acid residues in regions critical for cargo binding. Alleles *myo2(Q1233R)*, *myo2(G1248D)*, *myo2(D1297N)*, *myo2(D1297G)*, *myo2(L1301P)*, *myo2(N1304S)*, *myo2(N1304D)*, and *myo2(N1307D)* cause specific defects in vacuolar inheritance (Catlett and Weisman, 1998; Catlett et al., 2000; Pashkova et al., 2006). These mutants do not show any growth defects, which indicates that essential cellular functions, such as secretion or organization of the cytoskeleton, are not severely impaired (Catlett and Weisman, 1998; Catlett et al., 2000; Pashkova et al., 2006). Alleles *myo2(L1331S)*, *myo2(L1411S)*, *myo2(Y1415E)*, *myo2(K1444A)*, and *myo2(Q1447R)* carry

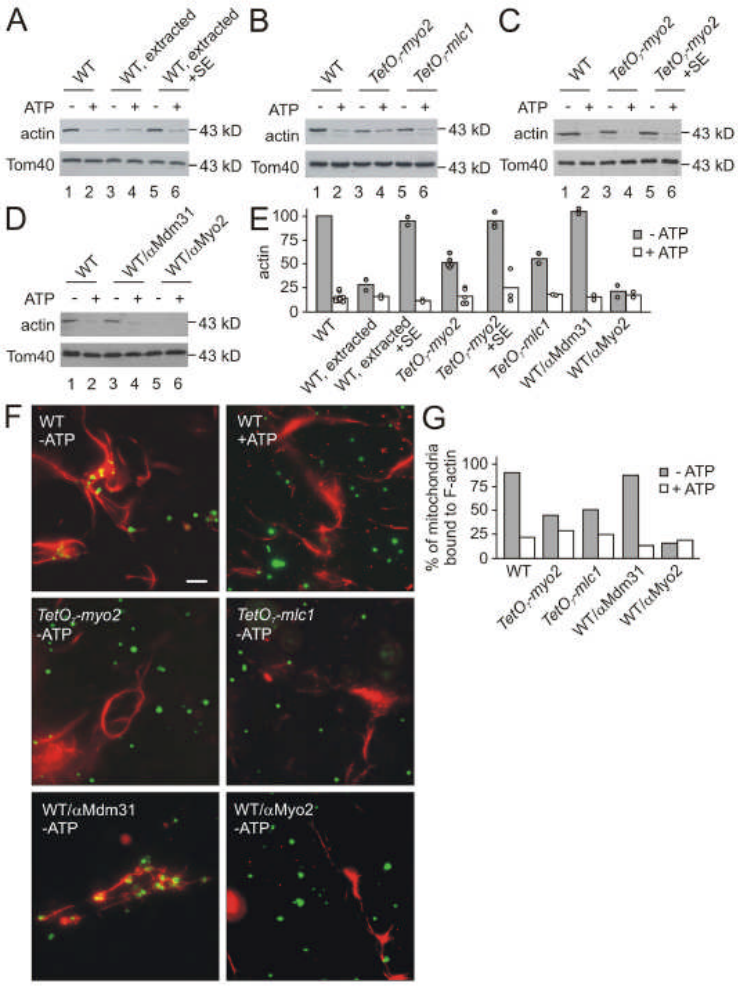

Figure 2. **Myo2 is required for interaction of mitochondria with actin filaments in vitro.** (A) Wild-type (WT) mitochondria were incubated with isolated actin filaments in the absence or presence of ATP. After centrifugation of mitochondria through a sucrose cushion, bound actin was detected by immunoblotting. The mitochondrial protein Tom40 served as a loading control. Lanes 1 and 2 show standard conditions; lanes 3 and 4 show mitochondria that were extracted with 1 M KCl before incubation with actin filaments; lanes 5 and 6 show salt-extracted mitochondria that were incubated with salt extract (SE) before incubation with actin filaments. (B) WT, *TetO7-myo2*, and *TetO7-mlc1* cells were precultured in glucose-containing medium and incubated for 15 h in the presence of 30 µg/ml Dox before isolation of mitochondria. Binding of actin to mitochondria was analyzed under standard conditions in A. (C) Binding of actin to wild-type and *TetO7-myo2* mitochondria was analyzed as in A. Lanes 1 and 2 show wild-type mitochondria analyzed under standard conditions; lanes 3 and 4 show *TetO7-myo2* mitochondria analyzed under standard conditions; lanes 5 and 6 show *TetO7-myo2* mitochondria that were incubated with wild-type SE before incubation with actin filaments. (D) Binding of actin to wild-type mitochondria was analyzed as in A. Lanes 1 and 2 show standard conditions; lanes 3 and 4 show mitochondria preincubated with affinity-purified Mdm31 antibodies at a concentration of 120 ng per mg of mitochondrial protein; lanes 5 and 6 show mitochondria preincubated with the same amount of affinity-purified Myo2 antibodies. (E) Replicate actin sedimentation experiments were quantified by densitometry of actin immunoblots. In each experiment, the signal obtained with wild-type mitochondria in the absence of ATP was set to 100. Bars represent mean values; circles represent individual data points of all measurements that have been performed. Arbitrary units are indicated. (F) Mitochondria isolated from mtGFP-expressing cells were incubated with Alexa Fluor 568–labeled actin filaments and observed by fluorescence microscopy. Depletion of Myo2 and Mlc1 and antibody pretreatments were performed as in A–E. Displayed are merged images of GFP and Alexa Fluor 568 fluorescence. An image of a sample incubated in the presence of ATP is only shown for wild-type mitochondria. Bar, 5 µm. (G) The graph shows a quantification of the experiment in Fig. 2 E. 200 mitochondria were scored per sample.

amino acid substitutions in the secretory vesicle binding site (Pashkova et al., 2006). Vacuole movement is fully functional in these mutants; however, they show severe growth defects because of defective vesicular transport (Pashkova et al., 2006).

Subdomain-specific *myo2* mutants expressing mtGFP were incubated at 30 and 37°C, and mitochondrial morphology was observed by fluorescence microscopy. Wild-type cells and secretion-specific mutants contained branched tubular mitochondria in 84–99% of the cells at both temperatures (Fig. 3, A and B; and Table I). Virtually all budded cells contained mitochondria partitioned to the daughter cell (Table I). In contrast, a large percentage of cells contained aggregated and/or unpartitioned mitochondria in the following six vacuole-specific mutants: *myo2(Q1233R)*, *myo2(D1297G)*, *myo2(L1301P)*, *myo2(N1304S)*, *myo2(N1304D)*, and *myo2(N1307D)* (Fig. 3, A and B; and Table I). Mitochondrial defects were most pronounced in *myo2(L1301P)* and *myo2(Q1233R)*; at a nonpermissive temperature, 83 or 73%, respectively, of mutant cells contained mitochondria that were aggregated or clumped in the mother cell, whereas buds were largely devoid of mitochondria (Fig. 3, A and B; and Table I). Only two vacuole-specific alleles, *myo2(G1248D)* and *myo2(D1297N)*, did not produce any significant mitochondrial defects. We conclude that the subdomain of the Myo2 tail responsible for vacuolar movement is also important for mitochondrial distribution and morphology.

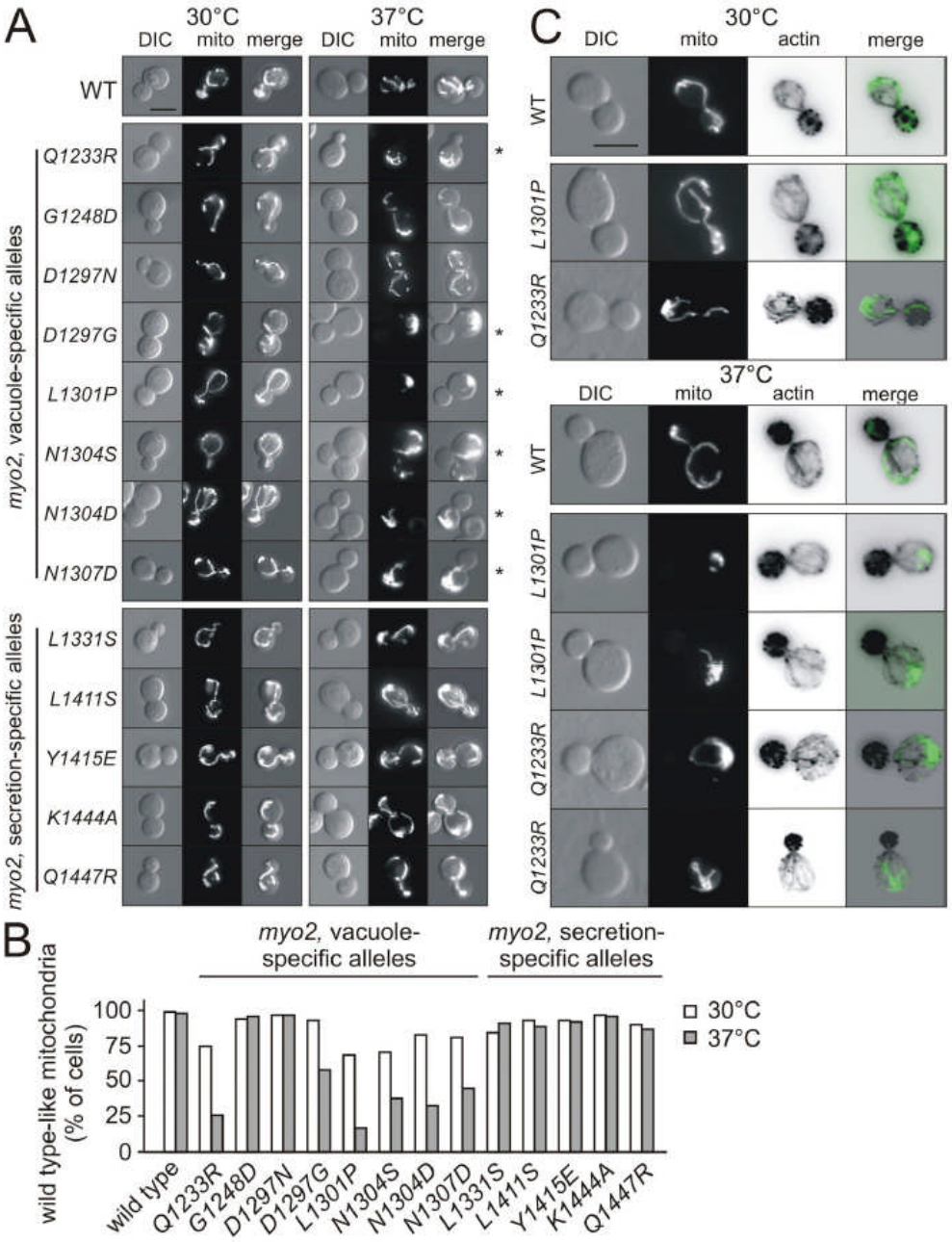

Figure 3. **Specific mutations in the Myo2 cargo-binding domain produce mitochondrial morphology and inheritance defects.** (A) Wild-type (WT) and *myo2* mutant cells expressing mtGFP were cultured in glucose-containing medium overnight at 30°C. Cultures were then diluted with fresh medium and either kept at 30°C (left) or shifted to 37°C for 3 h (right). Cells were analyzed by DIC and fluorescence microscopy. Asterisks indicate characteristic mitochondrial partitioning defects. Bar, 5 µm. (B) The graph shows a quantification of mitochondrial phenotypes scored in the experiment shown in A. Displayed is an excerpt of data presented in Table I. (C) Wild-type, *myo2-L1301P*, and *myo2-Q1233R* cells were grown as in A and analyzed as in Fig. 1 B. Bar, 5 µm.

Table I. **Mitochondrial distribution and morphology in *myo2* mutants**

Strain	Mitochondrial distribution and morphology					
	Temperature	Wild type–like	Aggregated or fragmented	Aggregated in mother, bud empty	Tubular in mother, bud empty	No. of cells scored
	°C	*% of cells*	*% of cells*	*% of cells*	*% of cells*	
Wild type	30	99	0	0	1	300
	37	98	0	0	2	300
Alleles producing vacuolar transport defects						
myo2(Q1233R)	30	75	0	12	13	300
	37	26	1	45	28	300
myo2(G1248D)	30	94	0	0	6	300
	37	96	0	0	4	300
myo2(D1297N)	30	97	0	0	3	300
	37	97	1	1	1	300
myo2(D1297G)	30	93	0	2	5	300
	37	58	2	32	8	300
myo2(L1301P)	30	69	3	15	13	300
	37	17	0	52	31	300
myo2(N1304S)	30	70	2	8	20	300
	37	38	1	42	19	300
myo2(N1304D)	30	83	1	11	15	300
	37	33	0	44	23	300
myo2(N1307D)	30	81	1	4	14	300
	37	45	0	30	25	300
Alleles producing vesicular transport defects						
myo2(L1331S)	30	84	16	0	0	100
	37	91	9	0	0	100
myo2(L1411S)	30	93	7	0	0	100
	37	89	11	0	0	100
myo2(Y1415E)	30	93	7	0	0	100
	37	92	8	0	0	100
myo2(K1444A)	30	97	3	0	0	100
	37	96	4	0	0	100
myo2(Q1447R)	30	90	10	0	0	100
	37	87	13	0	0	100

The fact that two vacuole-specific alleles did not produce mitochondrial defects suggests that the mitochondrial binding site on the Myo2 tail largely overlaps with, but is not identical to, the vacuolar binding site.

In further experiments, we concentrated on mutants *myo2(L1301P)* and *myo2(Q1233R)*, as these alleles produced the clearest mitochondrial phenotypes. To exclude the possibility that mitochondrial aggregation is caused by defects in the organization of the actin cytoskeleton, mutant and wild-type cells expressing mtGFP were incubated at 30 or 37°C, cells were fixed, microfilaments were stained with rhodamine phalloidin, and mitochondria and the actin cytoskeleton were observed by fluorescence microscopy. Again, mitochondria formed aggregates in *myo2(L1301P)* and *myo2(Q1233R)* mutants at 37°C, and buds were devoid of mitochondria. However, the actin cytoskeleton appeared normal under all conditions (Fig. 3 C). We conclude that specific mutations in the proximal half of the Myo2 globular tail affect mitochondrial distribution and morphology.

The globular tail of Myo2 is required for binding of mitochondria to actin filaments in vitro

We asked whether the globular tail of Myo2 is critical for interaction of mitochondria with actin filaments. Mitochondria were isolated from the wild type and *myo2(L1301P)* and *myo2(Q1233R)* mutants, incubated with actin filaments in the absence or presence of ATP, and centrifuged through a sucrose cushion, and bound actin was quantified by immunoblotting. We observed that actin-binding activity of mutant mitochondria was reduced to 61 or 37%, respectively, in comparison to the wild type (Fig. 4, A and B). Similar effects were found when mitochondrial actin-binding activity was observed directly in the visual assay. While in the absence of ATP, 90% of wild-type mitochondria were found associated with actin filaments, and this number was reduced to 34% for *myo2(L1301P)* mitochondria and 36% for *myo2(Q1233R)* mitochondria (Fig. 4, C and D). These results point to an important role of the proximal half of the Myo2 globular tail in mediating mitochondria–cytoskeleton interactions.

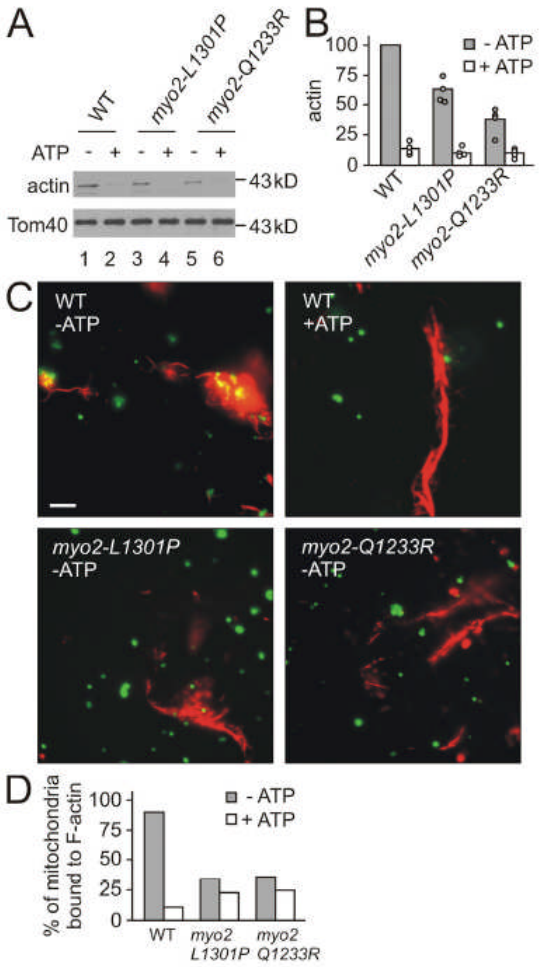

Figure 4. **Mutations in the cargo-binding domain of Myo2 impair binding of mitochondria to actin filaments in vitro.** (A) Wild-type (WT), *myo2-L1301P*, and *myo2-Q1233R* cultures were grown in glucose-containing minimal medium at 30°C and shifted to 37°C for 3 h before isolation of mitochondria. Binding of actin filaments to mitochondria under standard conditions was analyzed as in Fig. 2 A. (B) Replicate actin sedimentation experiments were quantified as in Fig. 2 E. Bars represent mean values; circles represent individual data points of all measurements that have been performed. (C) Cultures of mtGFP-expressing cells were grown as in A and the binding of mitochondria to actin filaments was analyzed as in Fig. 2 F. An image of a sample analyzed in the presence of ATP is shown only for wild-type mitochondria. Bar, 5 µm. (D) The graph shows a quantification of the experiment in Fig. 4 C. 200 mitochondria were scored per sample.

Myo2 is required for directed movement of mitochondria into the bud

Next, we investigated whether Myo2 is required for anterograde movement of mitochondria from the mother cell into the bud. Logarithmically growing wild-type and *myo2(L1301P)* cells expressing mtGFP were shifted to 37°C for 3 h and then observed by time-lapse live cell microscopy for 30 min at ambient temperature. 3D data stacks of budded cells were obtained every 3 min by confocal microscopy. In wild-type cells, mitochondria were observed to move around and undergo frequent shape changes by fusion and fission. Importantly, the organelles were well partitioned in mother and daughter cells, both in cells carrying large and small buds (Fig. 5 A). In contrast, movement of mitochondria was restricted to a much smaller area in *myo2(L1301P)* cells, and even large buds were often found to be devoid of mitochondria (Fig. 5 A). Close inspection of data stacks obtained from wild-type cells ($n = 5$) revealed that an average of 6.4 mitochondria per hour passed the bud neck. However, in *myo2(L1301P)* cells ($n = 11$), only 0.36 mitochondria per hour entered the bud. These data demonstrate that anterograde, bud-directed mitochondrial movement is severely impaired by mutation of the cargo binding site of Myo2.

To corroborate these findings, we quantified the number of buds lacking mitochondria in *myo2* mutants. Wild-type cells and cells with secretion-specific *myo2* alleles carried buds devoid of mitochondria at a frequency of 2–6% (Fig. 5 B). In accordance with the mitochondrial morphology defects described above (compare Fig. 3 A and Table I), six *myo2* strains defective in vacuolar inheritance produced significant mitochondrial partitioning defects (Fig. 5 B). Again, these phenotypes were most pronounced in *myo2(L1301P)* and *myo2(Q1233R)* mutants, which displayed buds devoid of mitochondria in 78 or 72% of cells at 37°C, respectively. We conclude that Myo2-mediated anterograde movement of mitochondria is important for mitochondrial inheritance during budding of yeast cells.

Discussion

Several lines of evidence support a direct role of the class V myosin, Myo2, as a mediator of mitochondrial motility in *S. cerevisiae*. Depletion of Myo2 or mutation of its cargo-binding domain produces severe mitochondrial morphology and inheritance defects in cells containing an apparently normal actin cytoskeleton. In particular, cells carrying specific *myo2* mutant alleles show pronounced mitochondrial defects that are similar to vacuolar inheritance defects reported in the literature (Pashkova et al., 2006). The fact that entry of mitochondria into the bud is largely blocked in these mutants strongly argues against an indirect role of Myo2 as a transporter of mitochondrial retention factors because these would become effective only after mitochondria have reached the bud tip. Notably, mitochondria lacking functional Myo2 are defective in ATP-sensitive binding to actin filaments in vitro. A critical role of Myo2 on the mitochondrial surface is demonstrated by the following two observations: actin-binding activity of Myo2-depleted mitochondria can be restored by the addition of salt extracts prepared from wild-type mitochondria; and interactions of wild-type mitochondria with actin filaments can be blocked completely with antibodies directed against Myo2. In summary, these results demonstrate that Myo2 is of major importance for mitochondrial movement in yeast.

A key role of Myo2 in mitochondrial transport is compatible with several observations that have been reported early after the discovery of the actin dependence of mitochondrial inheritance. Yeast actin mutants that exhibit specific mitochondrial defects contain amino acid exchanges under or near the myosin

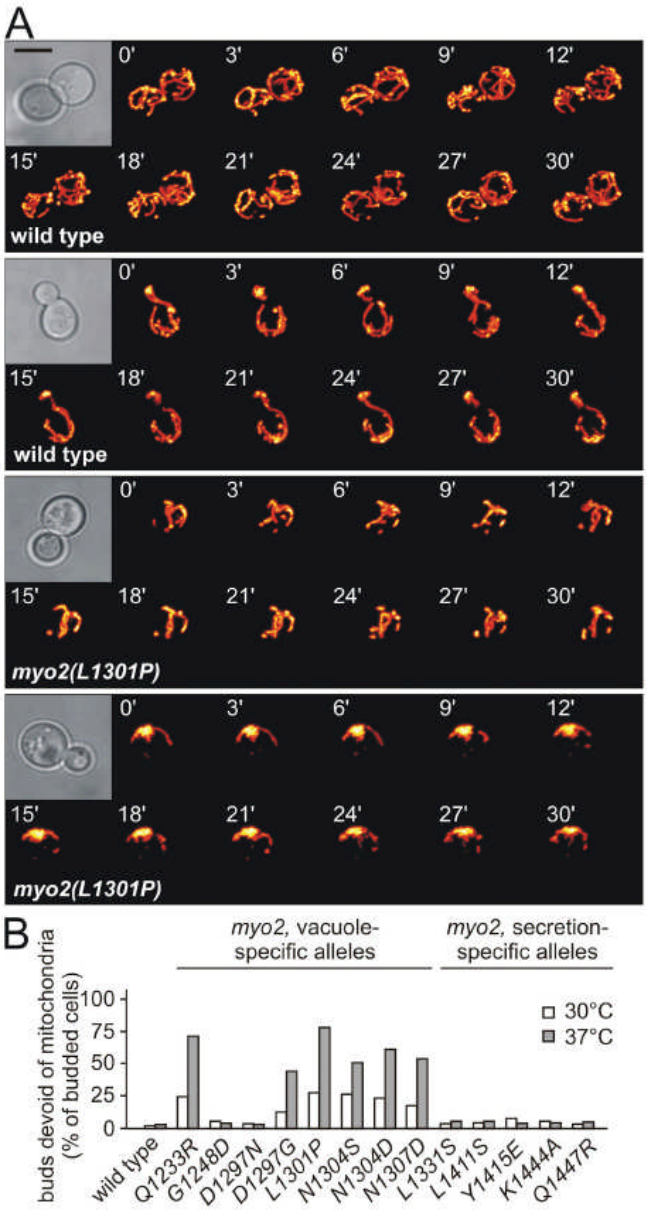

Figure 5. **Mutations in the cargo-binding domain of Myo2 impair anterograde mitochondrial movement and partitioning of mitochondria to the bud.** (A) Wild-type and *myo2-L1301P* cells expressing mtGFP were precultured in glucose-containing minimal medium at 30°C, shifted to 37°C for 3 h, and observed by confocal time-lapse microscopy at ambient temperature. The first image of each series is a bright-field image; mitochondria are shown as maximum intensity projections of several optical planes. One representative cell carrying a large bud and one representative cell carrying a small bud are shown for the wild type and *myo2(L1301P)*. Time is indicated in minutes. Bar, 5 µm. (B) Wild-type and *myo2* mutant cells expressing mtGFP were grown as in Fig. 3 A and buds were scored for the presence of mitochondria. 200 budded cells were analyzed for each strain at each temperature.

footprint (Drubin et al., 1993). This observation suggested immediately that actin–myosin interactions might underlie the cytoplasmic organization of mitochondria (Drubin et al., 1993). Furthermore, saturation of myosin-binding sites on actin filaments with the globular head domain of skeletal muscle myosin was observed to block binding of mitochondria in vitro (Lazzarino et al., 1994), and a mitochondria-associated motor activity was found to display an ATP concentration dependence similar to that of myosin family members in a microfilament sliding assay (Simon et al., 1995). However, it has been argued that Myo2 is unlikely to be the major mitochondrial motor because reduction of the length of the Myo2 lever arm in the *myo2-Δ6IQ* mutant is expected to decrease the velocity of the motor but was found to have no effect on the mean velocity of mitochondrial movement (Boldogh et al., 2004). However, it has been pointed out that Myo2-dependent movement of secretory vesicles in the *myo2-Δ6IQ* mutant is even faster than that of mitochondria in wild-type cells, which suggests that, in the case of mitochondria, Myo2 velocity may not be the limiting factor (Frederick and Shaw, 2007). We consider it likely that several motor molecules must cooperate

to move a large organelle, such as a mitochondrion. In this case, velocity would not be expected to be limited by the speed of a single, processively moving motor molecule.

The fact that mutations in the proximal half of the Myo2 globular tail impair binding of mitochondria to actin filaments suggests that the sites responsible for establishing interactions of the motor with mitochondria and vacuoles overlap. Although the identity of the mitochondrial Myo2 receptor remains to be identified, two previously characterized proteins potentially cooperate with Myo2 in mitochondrial distribution and morphology. Ypt11 is a small Rab-type GTPase, and Mmr1 is a protein concentrated on mitochondria in the bud. Both components act as high-dose suppressors of *myo2* mutants, and a Δ*ypt11* Δ*mmr1* double mutant shows severe mitochondrial partitioning defects (Itoh et al., 2002, 2004). However, Δ*ypt11* and Δ*mmr1* single mutants do not display strong mitochondrial phenotypes (Dimmer et al., 2002; Itoh et al., 2002, 2004), indicating that neither protein on its own is sufficient to act as a mitochondrial Myo2 receptor. Moreover, it has not been demonstrated whether Ypt11 and Mmr1 are directly required for interaction of mitochondria with the cytoskeleton. As Ypt11 does not seem to associate with mitochondria (Itoh et al., 2002, 2004), it appears that additional, yet unknown proteins are involved in anchoring Myo2 to the mitochondrial surface.

Boldogh et al. (2001a) have proposed an alternative mechanism to explain mitochondrial motility in budding yeast. They found that subunits of the Arp2/3 complex, the cell's most important initiator of actin polymerization, are located on the mitochondrial surface and that mitochondrial motility is impaired when actin dynamics are perturbed. These observations raised the possibility that mitochondria might move by an actin polymerization–dependent mechanism similar to the intracellular movement of certain bacterial pathogens, such as *Listeria monocytogenes* (Boldogh et al., 2001a, 2006). However, actin-dependent movement of mitochondria differs from other actin polymerization–dependent motility processes in an important aspect: mitochondrial movement occurs along preexisting actin cables (Fehrenbacher et al., 2004), whereas intracellular bacterial pathogens and endocytic membranes are propelled by growth of a newly generated comet-like actin tail (Kaksonen et al., 2006; Stevens et al., 2006). Moreover, it is difficult to reconcile this model with our observations that Myo2 is required for interaction of mitochondria with actin filaments in vitro, and that anterograde mitochondrial movement and entry into the bud is impaired in cargo binding–defective *myo2* mutants in vivo. Thus, we consider it unlikely that Arp2/3 complex–dependent actin polymerization is the main mechanism mediating mitochondrial motility in yeast. Recent evidence suggests that somewhat redundant mechanisms for mitochondrial movement have evolved in fungi. For example, microtubule-dependent transport of mitochondria in the filamentous fungus *Neurospora crassa* is mediated by an evolutionarily conserved kinesin-related motor protein. When this motor is lacking, expression of an unconventional fungi-specific kinesin that replaces its function is induced (Fuchs and Westermann, 2005). In analogy, we consider it possible that Myo2 is the major transporter of mitochondria in yeast, and actin polymerization–driven motility might contribute to a minor extent.

Our work establishes for the first time a direct role of Myo2 as a motor protein moving mitochondria in yeast. Some observations reported in the literature provide evidence for an involvement of myosin-related proteins in mitochondrial motility in other organisms also. A putative unconventional myosin has been detected on motile mitochondria in locust photoreceptors (Stürmer and Baumann, 1998), partitioning of mitochondria and other organelles during spermatogenesis is defective in *Caenorhabditis elegans* class VI myosin mutants (Kelleher et al., 2000), and a plant-specific class XI myosin was found to colocalize with mitochondria and chloroplasts in maize cells (Wang and Pesacreta, 2004). Interestingly, cellular fractionation experiments and immunoelectron microscopy revealed that a class V myosin encoded by the *dilute* gene is associated with mitochondria in mammalian melanoma cells (Nascimento et al., 1997). Thus, it will be interesting to see in the future whether yeast and mammalian cells use similar mechanisms to mediate actin-dependent mitochondrial motility.

Materials and methods

Yeast strains

Growth and manipulation of yeast strains was performed according to standard procedures (Burke et al., 2000). All strains used in this study are derivatives of BY4741, BY4742, or BY4743 (Brachmann et al., 1998). $TetO_7$ promoter strains (Mnaimneh et al., 2004) and isogenic wild-type R1158 were obtained from BioCat. Plasmid BG1805 containing the *MYO2* gene under control of the *GAL1* promoter was obtained from BioCat and transformed into strain BY4742. To construct yeast strains expressing *myo2* mutant alleles, a heterozygous *myo2* deletion strain (Giaever et al., 2002) was obtained from EUROSCARF and transformed with plasmid pRS416-MYO2 (Catlett et al., 2000). The resulting strain was sporulated, tetrads were dissected, and a haploid strain was isolated that contained a genomic *myo2::kanMX4* deletion allele and the *MYO2* wild-type allele on the plasmid. This strain served as a recipient for plasmid pRS413-MYO2 (Catlett and Weisman, 1998) to construct the wild-type control and for pRS413-MYO2–based plasmids containing *myo2* mutant alleles (Catlett and Weisman, 1998; Catlett et al., 2000; Pashkova et al., 2006). After counterselection against pRS416-MYO2 by growth on 5-fluoroorotic acid–containing medium, strains were obtained that expressed *myo2* alleles from single copy plasmids under control of the endogenous *MYO2* promoter.

Staining of cellular structures

To visualize mitochondria, yeast strains were transformed with plasmid pYX142-mtGFP (Westermann and Neupert, 2000). mtDNA nucleoids were stained in methanol-fixed cells according to published procedures (Jones and Fangman, 1992). The actin cytoskeleton was stained with rhodamine phalloidin (Invitrogen) as described previously (Amberg, 1998).

Microscopy

Electron microscopy of yeast cells (Dürr et al., 2006) and isolated mitochondria (Meeusen et al., 2004) was performed as described previously. Differential interference contrast (DIC) and epifluorescence microscopy was performed using a microscope (Axioplan 2; Carl Zeiss, Inc.) equipped with a Plan-Neofluar 100× 1.30 NA Ph3 oil objective (Carl Zeiss, Inc.). Samples were embedded in 1% low-melting-point agarose and observed at room temperature. Images were recorded with a monochrome camera (Evolution VF Mono Cooled; Intas Science Imaging Instruments GmbH) and processed with Image Pro Plus 5.0 and Scope Pro 4.5 software (Media Cybernetics, Inc.). CorelDRAW graphics suite version 12.0 (Corel Corporation) was used for mounting of the figures; image manipulations other than minor adjustments of brightness and contrast were not performed.

For time-lapse live cell microscopy, cells were grown in glucose-containing minimal medium to early logarithmic growth phase and transferred to a percolation chamber that was constantly flushed with fresh medium and kept at ambient temperature. Cells were mounted in 1% low-melting-point agarose to inhibit spatial movements of the cells. For image acquisition, a beam-scanning confocal microscope (TCS SP2; Leica) equipped

with a Plan-apo 63× 1.2 NA water immersion lens (Leica) was used. Images were averaged fourfold. Displayed are maximal intensity projections of 3D data stacks. Except smoothing and contrast stretching using the image acquisition software Imspector (Max-Planck-Innovation GmbH), no further image processing was applied.

Analysis of mitochondria–actin interactions in vitro

Phalloidin-stabilized actin filaments were prepared by polymerizing nonmuscular human actin (Tebu-Bio GmbH) according to the manufacturer's instructions. To fluorescently label actin filaments, 15 µg of Alexa Fluor 568–labeled actin (Invitrogen) was mixed with 100 µg of nonlabeled actin before polymerization.

For actin/mitochondria cosedimentation, isolated mitochondria were further purified by sucrose density gradient centrifugation as described previously (Altmann et al., 2007). The purity of representative mitochondria preparations was checked by electron microscopy (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709099/DC1). Preparation of salt-washed mitochondria and mitochondrial salt extracts (Boldogh et al., 1998), binding of filamentous actin (at a concentration of 100 µg/ml) to purified mitochondria, and cosedimentation of actin with mitochondria (Lazzarino et al., 1994) were performed according to published procedures. Affinity-purified Myo2 antibodies used for pretreatment of isolated mitochondria were a gift from L.S. Weisman (University of Michigan, Ann Arbor, MI). Actin was detected by immunoblotting with monoclonal PanActin Ab-5 antibodies (Thermo Fisher Scientific), and mitochondria were detected with polyclonal Tom40 antibodies (a gift from D. Rapaport, Universität Tübingen, Tübingen, Germany). ECL-generated bands were quantified by densitometry using Scion Image software (Scion Corporation).

For visualization of actin–mitochondria interactions, 200 µg of mitochondria isolated from strains expressing mtGFP were incubated with 2.25 µg of fluorescently labeled actin filaments in 50 µl RM buffer (0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, 2 mM $MgCl_2$, 0.1 M KCl, 1 mg/ml of fatty acid–free bovine serum albumin, protease inhibitor cocktail, and 1 mM PMSF). To assay ATP sensitivity, either 50 U/ml apyrase (−ATP) or 2 mM ATP, 0.1 mg/ml creatine kinase, and 10 mM creatine phosphate (+ATP) were added. Samples were incubated for 10 min at 30°C, embedded in low-melting-point agarose, and observed by fluorescence microscopy.

Online supplemental material

Fig. S1 shows cross sections of complete wild-type and *$TetO_7$-myo2* cells analyzed by electron microscopy. Fig. S2 shows preparations of purified wild-type and *$TetO_7$-myo2* mitochondria analyzed by electron microscopy. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709099/DC1.

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Supplementary Material

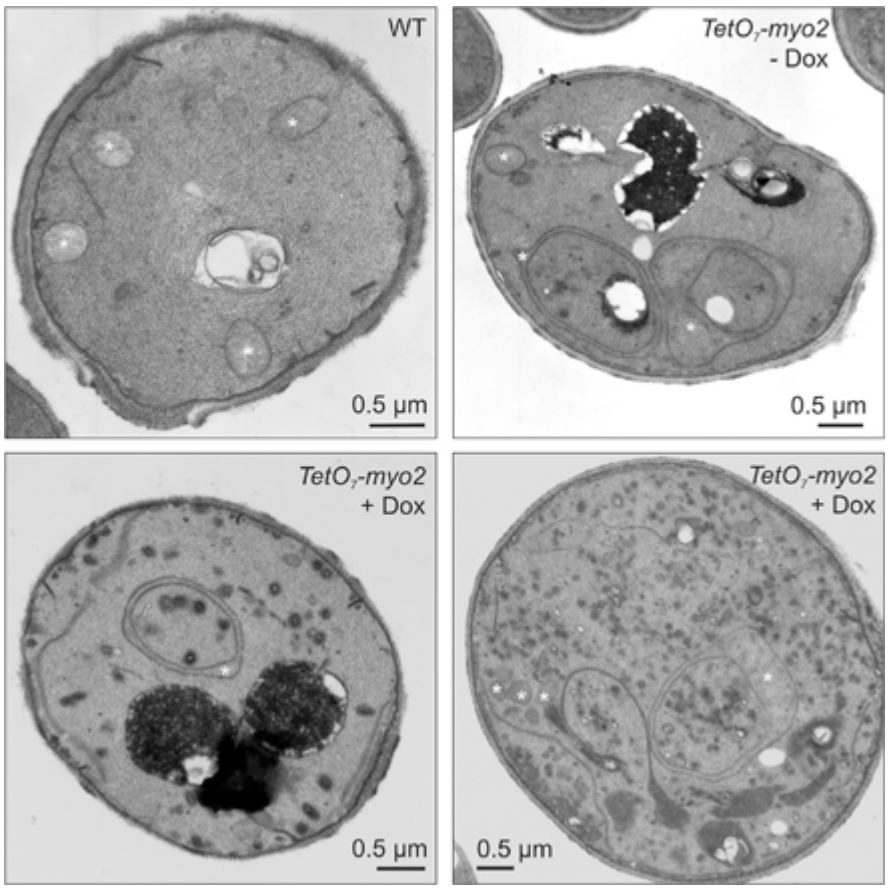

Figure S1.**Wild-type and *TetO$_7$-myo2* cells were grown to logarithmic growth phase in glucose-containing medium.** *TetO$_7$-myo2* cells were incubated in the presence of 30 µg/ml Dox for 15 h to deplete Myo2. Ultrathin sections were analyzed by electron microscopy. Asterisks indicate the matrix space.

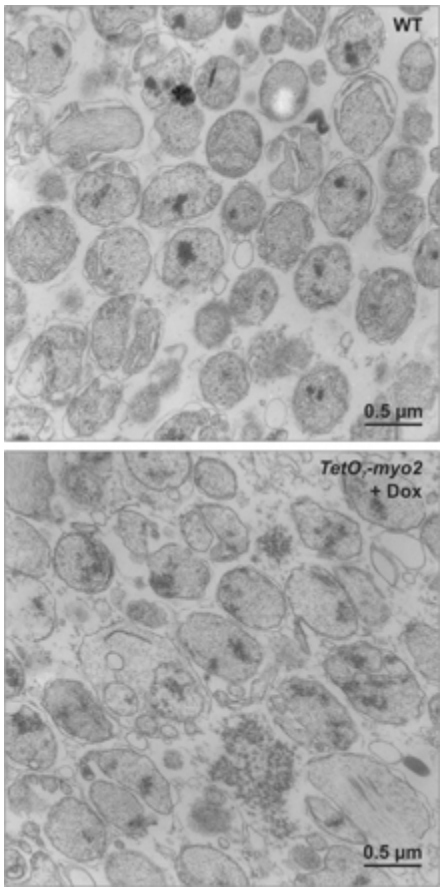

Figure S2.**Mitochondria were isolated by differential centrifugation from wild-type and *TetO$_7$-myo2* cells grown in Dox-containing medium, further purified by sucrose gradient centrifugation, and analyzed by electron microscopy.**

Teilarbeit C

Kai Stefan Dimmer, Stefan Jakobs, Frank Vogel, Katrin Altmann und Benedikt Westermann (2005)

Mdm31 and Mdm32 are inner membrane proteins required for maintenance of mitochondrial shape and stability of mitochondrial DNA nucleoids in yeast

The Journal of Cell Biology 168, 103-115

Darstellung des Eigenanteils

Mein Anteil an Teilarbeit C (Abbildung 6b) beschäftigte sich mit der Frage, ob in Mdm31-, bzw. Mdm32-Deletionsmutanten die Bindung von mtDNA-Nukleoiden und den mitochondrialen Außenmembranproteinen des membrandurchspannenden Komplexes aufgehoben wird.

Verfasst wurde diese Teilarbeit von Benedikt Westermann.

Mdm31 and Mdm32 are inner membrane proteins required for maintenance of mitochondrial shape and stability of mitochondrial DNA nucleoids in yeast

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The *MDM31* and *MDM32* genes are required for normal distribution and morphology of mitochondria in the yeast *Saccharomyces cerevisiae*. They encode two related proteins located in distinct protein complexes in the mitochondrial inner membrane. Cells lacking Mdm31 and Mdm32 harbor giant spherical mitochondria with highly aberrant internal structure. Mitochondrial DNA (mtDNA) is instable in the mutants, mtDNA nucleoids are disorganized, and their association with Mmm1-containing complexes in the outer membrane is abolished. Mutant mitochondria are largely immotile, resulting in a mitochondrial inheritance defect. Deletion of either one of the *MDM31* and *MDM32* genes is synthetically lethal with deletion of either one of the *MMM1*, *MMM2*, *MDM10*, and *MDM12* genes, which encode outer membrane proteins involved in mitochondrial morphogenesis and mtDNA inheritance. We propose that Mdm31 and Mdm32 cooperate with Mmm1, Mmm2, Mdm10, and Mdm12 in maintenance of mitochondrial morphology and mtDNA.

Introduction

Mitochondria are ubiquitous and essential organelles of eukaryotic cells. Because they cannot be generated de novo, they have to be inherited during cell division (Warren and Wickner, 1996). Inheritance of mitochondria involves active transport of the organelles along cytoskeletal tracks, concomitant with frequent membrane division and fusion events (Yaffe, 1999). The mitochondrial genome, which encodes a small subset of mitochondrial proteins, has to be partitioned to the daughter cell in an active and ordered manner (Azpiroz and Butow, 1993; Okamoto et al., 1998; Berger and Yaffe, 2000; Garrido et al., 2003; Meeusen and Nunnari, 2003). As mitochondria are double membrane-bounded organelles, transport processes occurring at the mitochondrial surface and partitioning events of matrix components must be coordinated across two membranes. For example, mitochondrial DNA (mtDNA) is located in protein-containing complexes, termed nucleoids, in the matrix. It has been suggested that inheritance of these nucleoids requires a segregation machinery in the cytosol (Berger and Yaffe, 2000; Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003). Moreover, it is conceivable that maintenance of the structure of the inner membrane depends on an intimate coordination with the behavior of the outer membrane, involving interactions of proteins in both membranes. However, the molecular processes coordinating the behavior of the double membranes during mitochondrial inheritance are not well understood.

Mitochondria form highly dynamic interconnected networks in many cell types from yeast to man (Bereiter-Hahn, 1990; Nunnari et al., 1997; Jakobs et al., 2003). In recent years a growing number of proteins controlling mitochondrial motility and behavior have been identified, mainly in the baker's yeast *Saccharomyces cerevisiae* (Hermann and Shaw, 1998; Jensen et al., 2000; Scott et al., 2003). In yeast, establishment, maintenance, and motility of the branched mitochondrial network depend on the actin cytoskeleton (Boldogh et al., 2001). Some mitochondrial outer membrane proteins have been suggested to play a role in microfilament-dependent inheritance of mitochondria and mtDNA. Yeast mutants lacking Mdm10, Mdm12, or Mmm1 have giant spherical mitochondria (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997), which show severely compromised intracellular motility (Boldogh et al.,

The online version of this article includes supplemental material.

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Abbreviations used in this paper: AAC, ADP/ATP carrier; mtDNA, mitochondrial DNA; mtGFP, mitochondria-targeted GFP; PK, proteinase K.

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1998, 2003). As these proteins are often localized next to mtDNA nucleoids, and as mtDNA nucleoids are disorganized in mutants, it has been proposed that Mdm10, Mdm12, and Mmm1 are parts of a cytoskeleton-dependent double membrane-spanning transport machinery required for inheritance of mitochondria and mtDNA (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003). Mmm2 (alternative name Mdm34) has been identified as another protein that participates in this process (Dimmer et al., 2002; Youngman et al., 2004). Mmm2 is located in a separate complex in the outer membrane, and mutants lacking Mmm2 harbor aberrant mitochondria and disorganized mtDNA nucleoids (Youngman et al., 2004).

It can be predicted that there must be partners in the inner membrane that physically and/or functionally interact with the outer membrane proteins Mmm1, Mmm2, Mdm10, and Mdm12 in mediating the inheritance of mitochondrial membranes and mtDNA nucleoids. It has been suggested that Mmm1 in yeast spans both mitochondrial membranes and exposes a small NH_2-terminal segment to the matrix (Kondo-Okamoto et al., 2003). However, the NH_2-terminal extension is absent in other homologous proteins, such as MMM1 in *Neurospora crassa* (Prokisch et al., 2000), and it is not required for maintenance of normal tubular networks and mtDNA nucleoids in yeast (Kondo-Okamoto et al., 2003). Thus, there must be other, yet unknown, inner membrane proteins participating in these processes. By screening a comprehensive yeast gene deletion library, we recently isolated several novel genes important for mitochondrial distribution and morphology, MDM (Dimmer et al., 2002). Here, we show that *MDM31* and *MDM32* encode novel components of the mitochondrial inner membrane. We propose that Mdm31 and Mdm32 functionally cooperate with the outer membrane machinery mediating maintenance of mitochondrial morphology and inheritance of mtDNA.

Results

MDM31 and *MDM32* encode two members of a novel protein family

The *MDM31* (systematic name *YHR194W*) and *MDM32* (systematic name *YOR147W*) genes encode two related proteins of 66.7 and 75.6 kD, respectively. Both proteins share 16.4% amino acid identity with each other. Related genes encoding homologous proteins can be found in the genomes of *Candida albicans*, *Schizosaccharomyces pombe*, *N. crassa*, and other ascomycetes fungi (Fig. 1 A; for an alignment see online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200410030/DC1). Remarkably, these more distantly related fungi have only one homologous gene, which is more closely related to *MDM31* (between 27.8% amino acid identity for *S. pombe* and 52.3% for *C. albicans*). Other species of the family *Saccharomycetaceae* have two related isoforms (Cliften et al., 2003; Kellis et al., 2003). Thus, the second isoform apparently has arisen by a relatively recent gene duplication event.

All members of the Mdm31 protein family have a similar domain structure (Fig. 1 B). The NH_2 termini have the characteristics of typical mitochondrial presequences. They are rich in positively charged residues, lack acidic charges, and have a high content of hydroxylated residues. Computational prediction of mitochondrial presequences by the MitoProt II program (Claros and Vincens, 1996) gives very high probabilities for mitochondrial targeting (between 0.9518 for *S. pombe* Mdm31 and 0.9989 for Mdm32). Hydropathy analysis (Hofmann and Stoffel, 1993) predicts two transmembrane segments, one close to the NH_2 terminus of the matured protein and another one at the very COOH terminus. The predicted domain structure is very similar for all five family members with the exception of the *S. pombe* protein that lacks a hydrophobic segment at its COOH terminus.

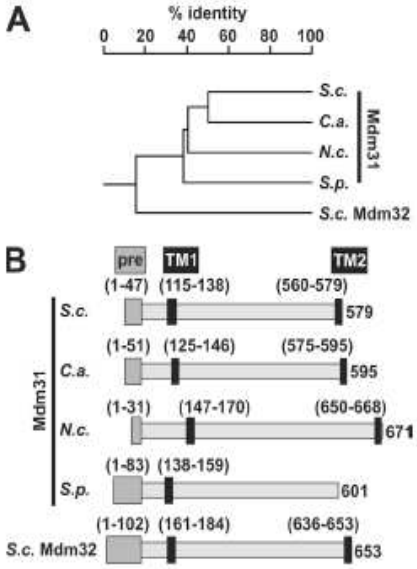


Figure 1. **Mdm31 and Mdm32 are members of a novel protein family.** (A) Homology tree of the Mdm31 protein family. Homologous proteins were identified by BLAST search (Altschul et al., 1997), and the tree was constructed using DNAMAN software (Lynnon BioSoft). Genome annotation numbers are Ca49C10.08 for *Candida albicans*, NCU07955.1 for *N. crassa*, and SPAC3H1.04c for *S. pombe*. (B) Domain structure of Mdm31 protein family members. Mitochondrial presequences (pre; indicated by gray boxes) were predicted using the MitoProt II program (Claros and Vincens, 1996). Transmembrane helices (TM; indicated by black boxes) were predicted using the TMpred program (Hofmann and Stoffel, 1993). Numbers of amino acid residues defining the borders of predicted domains are indicated; domains are drawn to scale.

Mdm31 and Mdm32 are located in the mitochondrial inner membrane

To determine the intracellular location of Mdm31 and Mdm32, wild-type yeast cells were fractionated into mitochondria, microsomes, and cytosol. Cell fractions were analyzed by Western blotting using specific antisera against Mdm31 and Mdm32. Both proteins cofractionated with the mitochondrial ADP/ATP carrier (AAC; Fig. 2 A), demonstrating a mitochondrial location. To determine the intramitochondrial location, isolated mitochondria were subfractionated. When intact mitochondria were treated with proteinase K (PK), both Mdm31 and Mdm32 were protected against proteolytic degradation (Fig. 2 B, lane 2), indicating that they are located in the interior

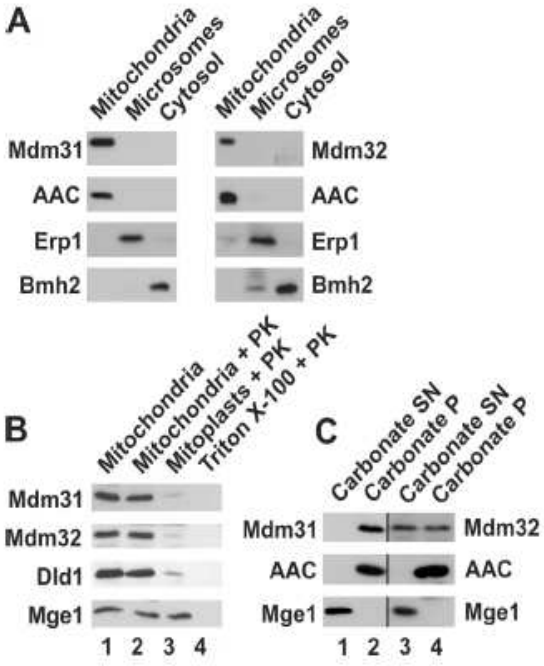


Figure 2. **Mdm31 and Mdm32 are located in the mitochondrial inner membrane.** (A) Subfractionation of yeast cells. Wild-type cells were subfractionated by differential centrifugation into mitochondria, microsomes, and cytosol. Mitochondria were further purified on a sucrose gradient, microsomes were purified on a percoll gradient. 50 μg of protein of each fraction was analyzed by Western blotting. The ADP/ATP carrier (AAC) served as a marker for mitochondria, Erp1 for ER, and Bmh2 for soluble cytosolic proteins. White lines indicate that intervening lanes have been spliced out. (B) Subfractionation of mitochondria. Isolated wild-type mitochondria were subfractionated, proteins were precipitated with TCA, and 50 μg of each fraction was analyzed by Western blotting. Lane 1, intact mitochondria; lane 2, intact mitochondria treated with proteinase K (PK); lane 3, mitoplasts generated by hypotonic swelling and treated with PK; lane 4, mitochondria solubilized with Triton X-100 and treated with PK. Markers used were Dld1 as an inner membrane protein exposed to the intermembrane space and Mge1 as a soluble matrix protein. (C) Carbonate fractionation of mitochondria. Isolated wild-type mitochondria were extracted with carbonate, proteins were precipitated with TCA, and 50 μg of each fraction was analyzed by Western blotting. Lanes 1 and 3, soluble protein–containing fraction after carbonate extraction (supernatant, SN); lanes 2 and 4, membrane protein–containing fraction after carbonate extraction (P, pellet). Markers used were AAC as an integral inner membrane protein and Mge1 as a soluble matrix protein.

of the organelle. When the outer membrane was selectively opened by hypotonic swelling, Mdm31 and Mdm32 were accessible to PK (Fig. 2 B, lane 3), indicating that a major domain is exposed to the intermembrane space. When the mitochondrial membranes were lysed with detergent, the proteins were completely degraded by PK (Fig. 2 B, lane 4). Upon carbonate extraction, all of Mdm31 and about half of Mdm32 cofractionated with mitochondrial membranes (Fig. 2 C), demonstrating that they are integral membrane proteins. It should be noted that partial extraction by carbonate has been observed also for other mitochondrial membrane proteins (Mokranjac et al., 2003). We conclude that Mdm31 and Mdm32 are located in the mitochondrial inner membrane. Protected fragments in protease-treated mitoplasts could never be observed in immunoblots of endogenous protein or after in vitro import of radiolabeled protein (unpublished data). We suggest that major parts of Mdm31 and Mdm32 are located in the intermembrane space, and the short NH_2 termini are exposed to the matrix.

Cells lacking Mdm31 and Mdm32 show severe defects in mitochondrial distribution and morphology

To examine the role of Mdm31 and Mdm32 in mitochondrial distribution and morphology, Δ*mdm31* and Δ*mdm32* deletion mutants and a Δ*mdm31*/Δ*mdm32* double mutant were constructed. All mutants were viable, both on fermentable and nonfermentable carbon sources (see section Mdm31 and Mdm32 are required for organization of mtDNA nucleoids) and showed identical phenotypes. Examination of mutant strains expressing mitochondria-targeted GFP (mtGFP) by confocal microscopy revealed highly aberrant mitochondrial structures (Fig. 3 A). Most of the cells harbored one or few giant spherical mitochondria (Fig. 3 A, b, c, e, and f). Often, the organelles contained one or few small hollow inclusions (Fig. 3 A, e and f). Some cells contained several, relatively small mitochondria (Fig. 3 A, d). Branched tubular mitochondrial networks resembling the wild type (Fig. 3 A, a) were not observed in the mutants. A quantification of mitochondrial phenotypes is given in Table I.

Many mutant cells generated buds that were devoid of mitochondria. Occasionally, a giant mitochondrion was positioned at the bud neck, which it could not pass (Fig. 3 A, b). To quantify these effects, logarithmically growing cultures of mtGFP-expressing cells were analyzed by fluorescence microscopy. Cells were counted that showed mitochondria both in the mother and the daughter cell, mitochondria stuck at the bud neck, or mitochondria-free buds. For comparison, we included in this analysis a Δ*mmm1* strain, which has giant spherical mitochondria (Burgess et al., 1994) similar to the Δ*mdm31* and Δ*mdm32* mutants. Only 50–60% of the buds contained mitochondria in Δ*mdm31*, Δ*mdm32*, Δ*mdm31*/Δ*mdm32*, and Δ*mmm1* cells. In most of the remaining cells, mitochondria were somewhere deposited in the mother cell. A few percent of the cells showed mitochondria positioned directly at the bud neck (Table II).

To exclude the possibility that the observed phenotypes were caused by defects in the organization of the actin cytoskeleton, or that deletion of the *MDM31* and *MDM32* genes has pleiotropic effects on the structure of several cell organelles, we stained filamentous actin, the ER, and vacuoles. All these structures appeared normal in the mutants (Fig. 3 B). We conclude that Mdm31 and Mdm32 play an important and specific role in controlling mitochondrial distribution and morphology.

It has been speculated that Mdm31 and Mdm32 might be novel components of the mitochondrial membrane fusion machinery (Mozdy and Shaw, 2003). To test this possibility, we monitored fusion of mitochondria in vivo by mating of mutant cells preloaded with different fluorescent mitochondrial markers (Nunnari et al., 1997). Mixing of the markers could be observed in zygotes lacking Mdm31 or Mdm32, as well as in zygotes lacking both proteins (Fig. 3 C). This demonstrates that

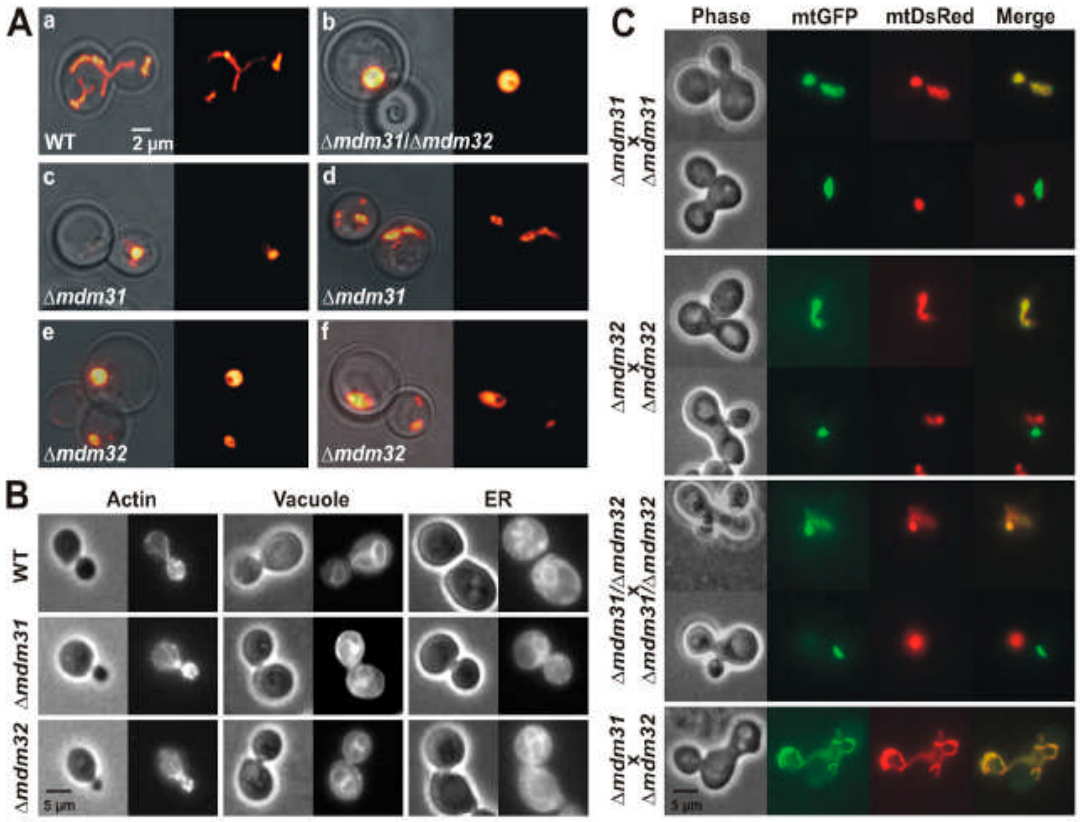


Figure 3. **Cells lacking Mdm31 and Mdm32 harbor aberrant mitochondria.** (A) Mitochondrial morphology. Wild-type (a), Δ*mdm31* (c and d), Δ*mdm32* (e and f), and Δ*mdm31*/Δ*mdm32* (b) cells expressing mtGFP were grown to log phase in YPD (yeast extract, peptone, and glucose) medium and analyzed by confocal fluorescence microscopy. (left) Maximum intensity projections of several optical planes covering the entire cell, merged with a bright field transmission image. (right) A representative single optical plane. (B) Morphology of microfilaments, vacuole, and the ER. Cells were grown to log phase in glucose-containing medium. Then, they were either fixed and stained with rhodamine-phalloidin (for actin), or living cells were stained with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (for vacuole), or cells expressing ER-targeted GFP were examined directly. Left, phase-contrast image; right, fluorescence microscopy. (C) Mitochondrial fusion. Cells of opposite mating type preloaded with mtGFP or mitochondria-targeted DsRed (mtDsRed) were mated, and zygotes were analyzed by phase-contrast and fluorescence microscopy.

Mdm31 and Mdm32 do not play an essential role in mitochondrial fusion. Interestingly, several zygotes were found in which the fluorescently labeled mitochondria of both parental cells remained separate (Fig. 3 C). However, these nonfused mitochondria were never seen close together. This observation suggests that in the latter cases fusion did not occur because the mitochondria did not approach each other. Heterologous crosses of Δ*mdm31* and Δ*mdm32* single deletion mutants showed complementation in zygotes, i.e., mitochondria looked like wild type and fused in an efficient manner (Fig. 3 C). We suggest that the function of Mdm31 and Mdm32 is required for efficient fusion in cells, even though these proteins are not integral components of the mitochondrial fusion machinery.

Mitochondria lacking Mdm31 and Mdm32 show dramatically altered internal structure

As Mdm31 and Mdm32 are inner membrane proteins, we considered it likely that also the internal structure of mutant mitochondria is altered. To examine this possibility, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells were examined by electron microscopy and compared with the wild type. Electron micrographs of wild-type cells grown on glucose-containing medium showed characteristic cross sections of tubular mitochondria containing cristae as invaginations of the inner membrane (Fig. 4 A). In contrast, the ultrastructure of Δ*mdm31* (Fig. 4 C), Δ*mdm32* (Fig. 4, B and D), and Δ*mdm31*/Δ*mdm32* (Fig. 4, E–H) mutant cells was dramatically altered. The organelles were generally very large. These giant organelles were largely devoid of cristae. Only in some organelles a few small cristae were found (Fig. 4 E, arrows). Frequently, circular-shaped double membrane structures were seen inside the organelles (Fig. 4, B–G). These structures were of varying sizes, but the spacing between the membranes was remarkably constant and was identical to the size of the intermembrane space. This finding suggests that the double membranes were derived from the mitochondrial outer and inner membranes, and that the compartment surrounded by the circular membranes topologically corresponds to the exterior of the organelle. Consistently, these structures appeared as holes in sections obtained by confo-

Table I. **Quantification of mitochondrial morphology in Δ*mdm31* and Δ*mdm32* mutant cells**

Strain	Mitochondrial morphology (percentage of cells)					
	Wild type–like	Spherical	Ring-like/with holes	Aggregated/fragmented	Elongated/tubular	Net-like
WT	100	-	-	-	-	-
Δ*mdm31*	-	54	32	6	8	-
Δ*mdm32*	-	54	39	-	7	-
Δ*mdm31*/Δ*mdm32*	-	73	21	-	6	-
Δ*mdm33*	-	7	77	7	9	-
Δ*mdm31*/Δ*mdm33*	-	68	27	1	4	-
Δ*mdm32*/Δ*mdm33*	-	78	21	1	-	-
Δ*dnm1*	-	-	-	-	3	97
Δ*mdm31*/Δ*dnm1*	-	75	12	4	9	-
Δ*mdm32*/Δ*dnm1*	-	58	35	-	6	1
Δ*fzo1*	-	1	-	99	-	-
Δ*mdm31*/Δ*fzo1*	-	67	17	14	2	-
Δ*mdm32*/Δ*fzo1*	-	66	26	7	1	-

$n > 100$.

cal microscopy (Fig. 3 A). Occasionally, an internal membrane was connected with the inner membrane surrounding the organelle (Fig. 4, D, G, and H). In these cases, the intermembrane space was continuous with the space between the membranes of the circular inclusion (Fig. 4 G, arrows; enlarged image in Fig. 4 H). We conclude that deletion of the *MDM31* and *MDM32* genes has dramatic consequences on the organization of the mitochondrial membranes and the global structure of the organelle.

Mdm31 and Mdm32 are required for normal mitochondrial motility

Two lines of evidence suggested that motility of mitochondria is compromised in cells lacking Mdm31 and Mdm32. First, mutant cells often carried buds devoid of mitochondria (Fig. 3 A and Table II), and second, in many mutant zygotes, mitochondria did not fuse because they did not approach each other (Fig. 3 C). To examine mitochondrial movement directly, the behavior of mitochondria was followed over time by confocal time-lapse microscopy of mtGFP-expressing cells. Cells grown logarithmically in glucose-containing medium were transferred to a microscope chamber that was continuously flushed with fresh medium. Wild-type mitochondria are highly dynamic under these experimental conditions (Jakobs et al., 2003). Their shapes and positions were observed to change completely within a few minutes (Fig. 5 A). In contrast, mutant mitochondria of Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* strains were almost immotile. They hardly changed their positions within time periods of 15 to 30 min (Fig. 5, B–D). However, sometimes subtle shape changes were observed in the mutant mitochondria. Generally, these shape changes started with the occurrence of small protrusions (Fig. 5, B–D, arrows), probably by a force pulling on the organelle. In most cases, these protrusions were retracted soon afterwards (Fig. 5, C and D). Together, these results demonstrate that mitochondrial motility is severely compromised in Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutants.

Sometimes, the initial deformation resulted in a successful translocation event of the entire organelle (Fig. 5 B). To test whether or not the residual translocation activity might be mediated by the actin-dependent transport machinery of mitochondria, we examined the binding of mitochondria to actin filaments in vitro. Isolated mitochondria of wild type, Δ*mdm31*, and Δ*mdm32* strains were incubated with filamentous actin in the presence or absence of ATP. Then, mitochondria were sedimented by centrifugation through a sucrose cushion, and bound actin was detected by immunoblotting. Mitochondria lacking Mdm31 or Mdm32 were able to interact with actin filaments in an ATP-dependent manner, similar to wild-type mitochondria (Fig. 5 E). This suggests that mitochondrial motility defects in the mutants are due to structural aberrations of the organelle rather than defects of the machinery mediating interactions with the cytoskeleton.

Table II. **Quantification of mitochondria-free buds in Δ*mdm31* and Δ*mdm32* mutant cells**

Strain	Bud with mitochondria	Mitochondria stuck at bud neck	Mitochondria-free buds
	% of cells	% of cells	% of cells
WT	99	-	1
Δ*mdm31*	55	4	41
Δ*mdm32*	67	2	31
Δ*mdm31*/Δ*mdm32*	60	8	32
Δ*mmm1*	54	10	36

$n > 100$.

Δ*mdm31* and Δ*mdm32* mutations are epistatic to Δ*fzo1*, Δ*dnm1*, and Δ*mdm33* mutations

To investigate functional relationships of *MDM31* and *MDM32* with other genes encoding components important for mitochondrial structure and behavior, we constructed a series of double mutants. Δ*mdm31* and Δ*mdm32* strains were crossed with the following deletion strains: Δ*fzo1*, a mutant defective in mitochondrial fusion (Hermann et al., 1998; Rapaport et al., 1998); Δ*dnm1*, a mutant defective in outer membrane division (Otsuga et al., 1998); and Δ*mdm33*, a mutant defective in inner mem-

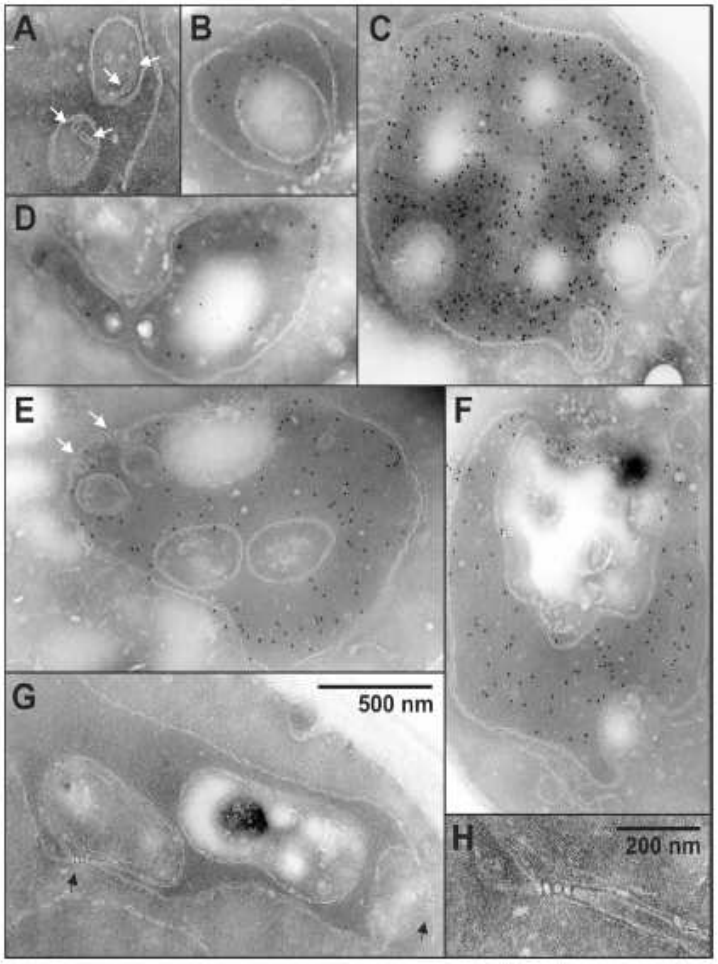


Figure 4. **Ultrastructure of mitochondria in cells lacking Mdm31 and Mdm32.** (A) Cross section of mitochondria in wild-type cells. (C) Cross section of a giant mitochondrion in a Δ*mdm31* cell. (B and D) Cross sections of mitochondria in Δ*mdm32* cells. (E–H) Cross sections of mitochondria in Δ*mdm31*/Δ*mdm32* cells. (A–F) mtGFP was labeled with immunogold to identify the matrix compartment. White arrows in A and E point to inner membrane cristae; black arrows in G point to membrane bridges between the inner membrane und circular inclusions. All images are displayed at the same magnification with the exception of H, which is an enlargement of G.

brane division (Messerschmitt et al., 2003). Resulting diploids were subjected to tetrad dissection, and mitochondrial morphology of haploid progeny was analyzed by fluorescence microscopy. In all cases, the parental mutants had clearly distinguishable phenotypes. Double mutants obtained from all crosses displayed mitochondria indistinguishable from their Δ*mdm31* and Δ*mdm32* parents (Table I). This finding indicates that the Δ*mdm31* and Δ*mdm32* mutations are epistatic to Δ*fzo1*, Δ*dnm1*, and Δ*mdm33* mutations; i.e., in the absence of Mdm31 or Mdm32, mitochondrial morphology does not depend on Fzo1, Dnm1, or Mdm33. We propose that the function of Mdm31 and Mdm32 is superior to mitochondrial fusion and division.

Δ*mdm31* and Δ*mdm32* mutations are synthetically lethal with Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* mutations

We asked if *MDM31* and *MDM32* have overlapping functions with *MMM1*, *MMM2*, *MDM10*, and *MDM12*, because mutants lacking these genes have very similar phenotypes (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998, 2003; Aiken Hobbs et al., 2001; Youngman et al., 2004). Δ*mdm31* and Δ*mdm32* mutants were crossed with Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* strains. Upon tetrad dissection, we observed in all crosses a 1:1:4 segregation into parental ditype tetrads, nonparental ditype tetrads, and tetratype tetrads (Table III). Spores containing both deleted alleles were not viable; i.e., Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* mutations are synthetically lethal with Δ*mdm31* and Δ*mdm32* mutations. Synthetic lethality of two mutations in different genes often indicates that the gene products are required for the same cellular processes (Guarente, 1993; Hartman et al., 2001). The synthetic lethal phenotype was confirmed in a plasmid shuffling experiment using the Δ*mdm32*/Δ*mmm1* double mutant (unpublished data). These results show that the function of Mdm31 and Mdm32 is essential for cell viability in the absence of Mmm1, Mmm2, Mdm10, and Mdm12.

Mdm31 and Mdm32 are required for organization of mtDNA nucleoids and localization of mtDNA adjacent to Mmm1 foci

Using strains obtained from the yeast gene deletion collection, we reported previously that Δ*mdm31* and Δ*mdm32* mutants are

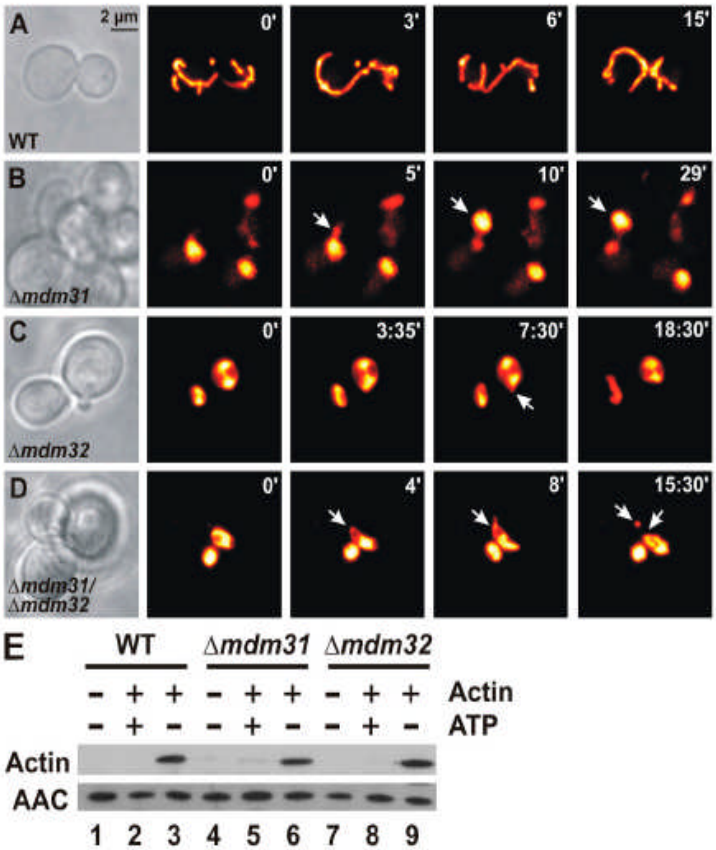


Figure 5. **Movement of mitochondria is compromised in cells lacking Mdm31 and Mdm32.** (A) Wild-type cells expressing mtGFP were grown to log phase in glucose-containing medium, transferred to a microscope chamber that was continuously flushed with fresh medium, and analyzed by confocal time-lapse microscopy. Left, bright field image; right, representative time points of the remodelling process shown as maximum intensity projections of several optical planes. Δ*mdm31* (B), Δ*mdm32* (C), and Δ*mdm31*/Δ*mdm32* (D) cells were analyzed as in A. Arrows point to shape changes of aberrant organelles. (E) Interaction of mitochondria with actin filaments in vitro. Isolated mitochondria of wild-type (WT), Δ*mdm31*, and Δ*mdm32* cells were incubated without or with isolated actin filaments in the presence or absence of ATP. After centrifugation of mitochondria through a sucrose cushion, bound actin was detected by immunoblotting. The mitochondrial protein AAC served as a loading control.

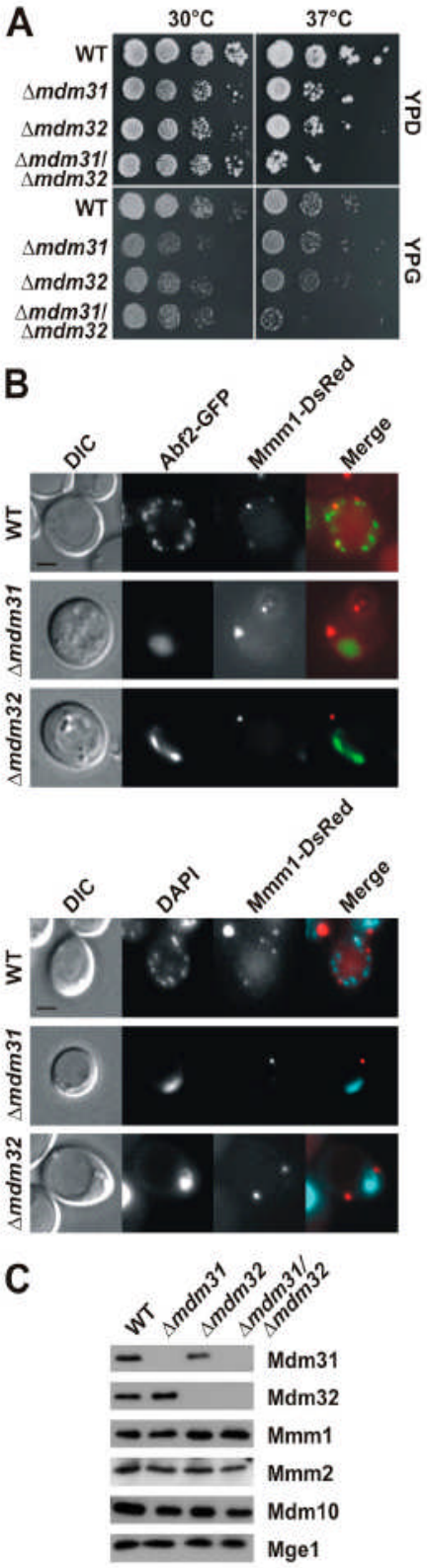
respiratory-deficient (Dimmer et al., 2002). Here, we observed that it is possible to grow newly made Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutants on nonfermentable carbon sources. Serial dilutions of wild-type and mutant cultures were spotted onto plates containing either glucose or glycerol as carbon source and incubated at 30 or 37°C. Mutant strains showed a moderate growth defect under most conditions, and the Δ*mdm31*/Δ*mdm32* double mutant showed a more severe growth defect on nonfermentable carbon sources at elevated temperature (Fig. 6 A). As inheritance of mtDNA depends on the integrity of the mitochondrial compartment (Berger and Yaffe, 2000), we reasoned that our initial observation of a petite phenotype in the mutants may be due to the gradual loss of the mitochondrial genome over several generations. To test this idea, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* strains were grown in liquid medium containing glucose to allow for loss of mtDNA. Cultures were maintained in the logarithmic growth phase at 30°C. At different time points, aliquots were taken and plated at an appropriate dilution onto glucose-containing medium. Subsequently, colonies were replica-plated onto glycerol-containing medium, and the percentage of colonies able to grow was determined as a measure of the fraction of respiratory-competent cells in the initial culture. After 3 d in glucose-containing medium, only ~50% of the mutant cells were respiratory-competent (very similar numbers were obtained for all three mutant strains). We conclude that Mdm31 and Mdm32 are required for normal inheritance of mtDNA.

We asked whether or not mtDNA is normally organized and distributed in cells lacking Mdm31 and Mdm32. mtDNA was stained in living cells by expressing an Abf2-GFP fusion protein, which binds to mtDNA nucleoids (Okamoto et al., 1998), or with a DNA-specific dye, DAPI. Wild-type cells contained normal mtDNA nucleoids, seen as 10–20 small fluores-

Table III. **Tetrad analyses**

	Parental ditype	Nonparental ditype	Tetratype
Expected	1	1	4
Δ*mdm31* × Δ*mmm1*	0.92 (*n* = 4)	0.92 (*n* = 4)	4.16 (*n* = 18)
Δ*mdm32* × Δ*mmm1*	1.37 (*n* = 8)	0.69 (*n* = 4)	3.94 (*n* = 23)
Δ*mdm31* × Δ*mmm2*	0.9 (*n* = 9)	1.5 (*n* = 15)	3.6 (*n* = 36)
Δ*mdm32* × Δ*mmm2*	0.86 (*n* = 5)	0.34 (*n* = 2)	4.78 (*n* = 28)
Δ*mdm31* × Δ*mdm10*	1.24 (*n* = 6)	1.03 (*n* = 5)	3.72 (*n* = 18)
Δ*mdm32* × Δ*mdm10*	0.5 (*n* = 3)	1.33 (*n* = 8)	4.17 (*n* = 25)
Δ*mdm31* × Δ*mdm12*	0.77 (*n* = 4)	0.58 (*n* = 3)	4.65 (*n* = 24)
Δ*mdm32* × Δ*mdm12*	0.6 (*n* = 3)	1.2 (*n* = 6)	4.2 (*n* = 21)

Parental ditype, two wild-type spores and two non-viable double mutant spores; nonparental ditype, four single mutant spores (two of each type), all four spores viable; tetratype, one wild-type spore, two single mutant spores (one of each type), and one non-viable double mutant spore. The ratios of the observed classes and the total number of tetrads for each class are indicated.



cent dots (Fig. 6 B). In contrast, Δ*mdm31* and Δ*mdm32* mutants contained only one or two misshapen mtDNA-containing structures per cell. The staining pattern of these structures was diffuse, and they were generally rather large (Fig. 6 B). Very similar results were obtained after DAPI staining of methanol-fixed cells (unpublished data). We conclude that Mdm31 and Mdm32 are required for establishment and/or maintenance of mtDNA nucleoid structure.

It has been reported that Mmm1 is located in distinct foci on the mitochondrial outer membrane. These foci are often found next to mtDNA nucleoids (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003), and their formation depends on the presence of the outer membrane protein Mmm2 (Youngman et al., 2004). It is thought that Mmm1-containing foci (in cooperation with yet unknown inner membrane proteins) contribute to the structural organization and inheritance of mtDNA nucleoids (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003; Youngman et al., 2004). The aberrant mtDNA nucleoids seen in Δ*mdm31* and Δ*mdm32* mutants and the genetic interactions with Δ*mmm1* prompted us to investigate whether the formation of Mmm1 foci and/or their localization next to mtDNA depends on the presence of Mdm31 and Mdm32. First, we tested whether or not the steady-state level of Mmm1 is altered in mitochondria of cells lacking Mdm31 and Mdm32. Immunoblot analysis showed that Mmm1 was present in similar amounts in mitochondria isolated from wild-type, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells (Fig. 6 C). The same result was obtained for Mmm2 and Mdm10 (Fig. 6 C). Mdm12 is required for localization of Mmm1 to mitochondria (Boldogh et al., 2003). As the level of Mmm1 was not changed in Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutant mitochondria, we conclude that also Mdm12 must be present in sufficient amounts. Thus, synthesis, mitochondrial targeting, and stability of Mmm1, Mmm2, Mdm10, and Mdm12 are not compromised in Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutants.

Next, we analyzed the intracellular distribution of Mmm1 and mtDNA by fluorescence microscopy. Consistent with previous reports (Aiken Hobbs et al., 2001; Meeusen and Nunnari,

Figure 6. **Mdm31 and Mdm32 are required for maintenance of normal mtDNA nucleoids.** (A) Growth phenotypes. Wild-type, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells were grown overnight in glucose-containing medium. Then, 10-fold serial dilutions were spotted onto plates containing glucose (YPD) or glycerol (YPG) as carbon source. YPD plates were incubated for 2 d and YPG plates for 3 d at the indicated temperatures. (B) Nucleoid structure and localization of Mmm1-containing complexes. Wild-type, Δ*mdm31*, and Δ*mdm32* cells expressing an Mmm1-DsRed fusion protein under control of the *MMM1* promoter were grown to log phase in glucose-containing medium. For staining of mtDNA nucleoids, expression of an Abf2-GFP fusion protein under control of a *GAL*-promoter was induced by shifting the cells to galactose-containing medium for 1 h (top), or cells were incubated in the presence of 1 μg/ml DAPI for 15 min. It should be noted that nuclear DNA is not stained under these conditions (Aiken Hobbs et al., 2001). Cells were washed in glucose-containing medium and analyzed by differential interference contrast (DIC) and fluorescence microscopy. Bars, 2 μm. (C) Steady-state levels of mitochondrial proteins. Mitochondria were isolated from wild-type (WT), Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells, and equal amounts of mitochondrial protein were analyzed by immunoblotting using the indicated antisera. The mitochondrial matrix protein Mge1 served as a loading control.

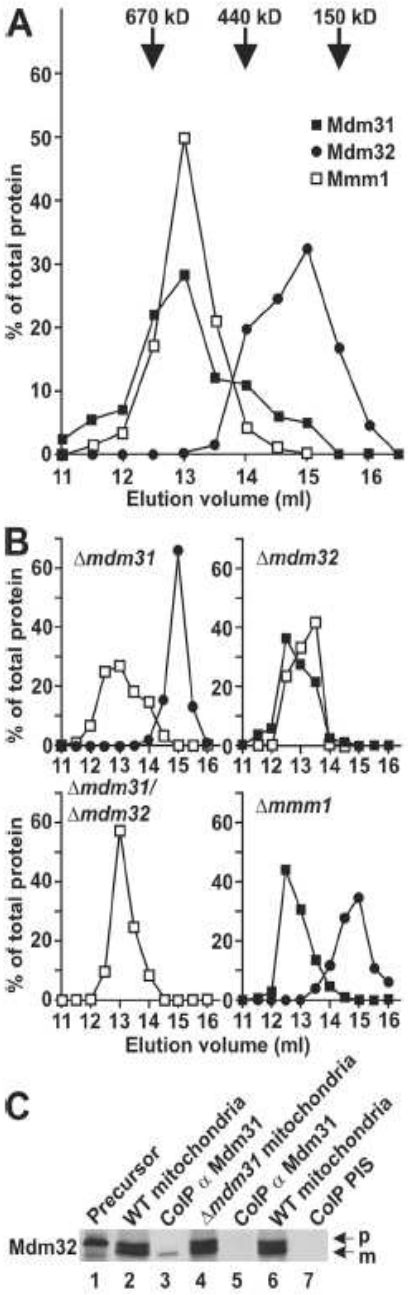


Figure 7. **Mdm31 and Mdm32 are parts of two separate complexes in the inner membrane.** (A) Gel filtration analysis of wild-type mitochondria. Isolated mitochondria were solubilized in digitonin-containing buffer and loaded onto a gel filtration column. After chromatography, proteins were precipitated with TCA, and fractions from the same column run were analyzed by Western blotting using affinity-purified antisera against Mdm31 (closed squares), Mdm32 (closed circles), and Mmm1 (open squares). Signals were quantified by densitometry and plotted as a percentage of total Mdm31, Mdm32, and Mmm1 protein in the extract. Arrows indicate molecular masses of calibration standards. (B) *Δmdm31*, *Δmdm32*, *Δmdm31/Δmdm32*, and *Δmmm1* mitochondria were analyzed as in A. (C) Coimmunoprecipitation of Mdm32 with Mdm31. Radiolabeled Mdm32 (lane 1) was imported into wild-type mitochondria (lane 2). Mitochondria were lysed with Triton X-100 and subjected to coimmunoprecipitation with affinity-purified antibodies against Mdm31 (lane 3). In control reactions, coimmunoprecipitation was analyzed in *Δmdm31* mitochondria (lanes 4 and 5), and preimmuneserum was used after import of Mdm32 into wild-type mitochondria (lanes 6 and 7). Signals were analyzed by SDS-PAGE and autoradiography. The amount of precursor protein in lane 1 corresponds to 10% of the material that was used for the import reactions; the amount of import reactions loaded in lanes 2, 4, and 6 corresponds to 10% of the material that was used for coimmunoprecipitation. p, precursor form of Mdm32; m, mature form of Mdm32. White lines indicate that intervening lanes have been spliced out.

2003; Youngman et al., 2004), wild-type cells expressing an Mmm1-DsRed fusion protein showed a punctate staining pattern. The majority of Mmm1 foci was located next to mtDNA nucleoids stained by Abf2-GFP or DAPI (Fig. 6 B). Mmm1 punctae were seen also in *Δmdm31* and *Δmdm32* cells, demonstrating that Mdm31 and Mdm32 are not required for Mmm1 foci formation (Fig. 6 B). However, mtDNA nucleoids were disorganized and Mmm1 foci were only rarely seen in the vicinity of mtDNA. Most Mmm1-DsRed–expressing mutant cells showed Mmm1 foci distantly located from diffusely organized mtDNA (Fig. 6 B). We conclude that Mdm31 and Mdm32 are required for localization of Mmm1 foci next to mtDNA.

Mdm31 and Mdm32 are present in distinct complexes in the mitochondrial membranes

We determined whether or not Mdm31 and Mdm32 are subunits of larger protein complexes. Isolated wild-type mitochondria were solubilized with a mild detergent, digitonin. Protein complexes were separated by gel filtration and analyzed by Western blotting. Interestingly, Mdm31 and Mdm32 reside in separate complexes. Mdm31 was eluted at ~600 kD, which was clearly larger than the size of the Mdm32 complex at ~175 kD (Fig. 7 A). The size of the Mdm31 complex was not changed in the absence of Mdm32, and vice versa (Fig. 7 B).

We considered the possibility that Mmm1 forms a double membrane-spanning protein complex together with Mdm31 or Mdm32. To test this possibility, we asked if Mmm1 cofractionates with Mdm31 or Mdm32 in gel filtration. Consistent with a previous study (Youngman et al., 2004), Mmm1 was found in a complex slightly larger than 600 kD, the size of which was similar to the Mdm31 complex (Fig. 7 A). If Mdm31 and Mmm1 were subunits of the same complex, it could be expected that the size of this complex would change in the absence of one of the subunits. Therefore, we performed gel filtration experiments with mitochondria isolated from *Δmdm31*, *Δmdm32*, *Δmdm31/Δmdm32*, and *Δmmm1* mutant cells. Neither deletion had a significant effect on the size of the other complexes (Fig. 7 B). We also noticed that the peak fractions of Mdm31 and Mmm1 were sometimes shifted by one fraction, with the Mdm31 complex being slightly larger (Fig. 7 B). Furthermore, we could not detect a direct interaction of Mdm31 or Mdm32 with Mmm1 in coimmunoprecipitation and cross-linking experiments. Thus, Mdm31, Mdm32, and Mmm1 are subunits of separate complexes in the mitochondrial membranes.

The structural and functional similarities of Mdm31 and Mdm32 point to a close collaboration of these proteins. Even

though they assemble into separate complexes, they might still interact in a weak or transient manner. To test this possibility, we imported radiolabeled Mdm32 into mitochondria and performed coimmunoprecipitation experiments with specific antibodies directed against endogenous Mdm31. Upon translation in vitro of Mdm32 in the presence of [^{35}S]methionine, SDS-PAGE, and autoradiography, a single band corresponding to the size of the precursor protein was observed (Fig. 7 C, lane 1). Upon incubation with isolated mitochondria, a slightly smaller form was generated by processing of the presequence by the matrix processing peptidase (Fig. 7 C, lanes 2 and 6). After import into wild-type mitochondria, a fraction of matured Mdm32 could be coimmunoprecipitated with Mdm31 antibodies (Fig. 7 C, lane 3). No precursor protein was found associated with Mdm31, demonstrating that the reaction was specific for the imported protein. Furthermore, no signal was obtained with mitochondria lacking Mdm31 (Fig. 7 C, lane 5) when preimmune serum was used (Fig. 7 C, lane 7) or when nonrelated inner membrane proteins were imported (not depicted). Thus, Mdm31 and Mdm32 interact with each other in a specific manner. The observation that only a small fraction of imported Mdm32 was coimmunoprecipitated with Mdm31 is consistent with a rather weak or transient interaction. We propose that Mdm31 and Mdm32 are subunits of two distinct protein complexes in the inner membrane that cooperate in establishing mitochondrial distribution and morphology.

Discussion

Mutants lacking the inner membrane proteins Mdm31 and Mdm32 display phenotypes that are strikingly similar to mutants lacking either one of the outer membrane proteins Mmm1, Mmm2, Mdm10, and Mdm12 (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998, 2003; Aiken Hobbs et al., 2001; Youngman et al., 2004). First, mutant cells harbor giant spherical mitochondria; second, aberrant mitochondria are largely immotile; third, the internal structure of mitochondria is dramatically altered; fourth, mtDNA is unstable; and fifth, mtDNA nucleoids are disorganized. Deletion of either one of the *MDM31* and *MDM32* genes is synthetically lethal with deletion of either one of the *MMM1*, *MMM2*, *MDM10*, and *MDM12* genes, suggesting that the gene products are required for the same cellular processes. We propose that Mdm31 and Mdm32 cooperate with Mmm1, Mmm2, Mdm10, and Mdm12 in maintaining mitochondrial morphology.

What might be the role of Mdm31 and Mdm32 in mitochondrial biogenesis? It has been proposed that Mmm1, Mmm2, Mdm10, and Mdm12 are involved in the attachment of mtDNA to the mitochondrial membranes and provide a link to a segregation machinery on the cytosolic side of the organelle. This hypothesis is based mainly on two findings. First, disordered nucleoids are seen in Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* mutants (Boldogh et al., 2003; Youngman et al., 2004). Similar structures are also found in the Δ*abf2* mutant, which lacks a mitochondrial member of the nonhistone high mobility group protein family (Newman et al., 1996). Thus, disordered nucleoids are indicative of a defect of mtDNA packaging and/or attachment to the membrane. Second, GFP fusion proteins of Mmm1, Mmm2, Mdm10, and Mdm12 form foci, a subset of which is located next to a subset of mtDNA nucleoids (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003; Youngman et al., 2004). However, the identity of inner membrane proteins that might link matrix-localized nucleoids to the putative segregation machinery in the outer membrane remained obscure. Here, we show that steady-state levels of Mmm1 in mitochondria, Mmm1 foci formation, and assembly of Mmm1 into a high molecular weight complex are not affected in mutants lacking Mdm31 and Mdm32. However, Mmm1-containing complexes lose their ability to interact with mtDNA nucleoids in Δ*mdm31* and Δ*mdm32* mutants. We propose that Mdm31 and Mdm32 are required to link mtDNA nucleoids to an Mmm1-containing segregation machinery in the mitochondrial outer membrane.

Respiratory functions of mitochondria are dispensable in *S. cerevisiae* when cells are grown on fermentable carbon sources. Hence, a defect in mtDNA inheritance is not sufficient to explain the observed synthetic lethal phenotypes of mdm mutants. Besides their role in respiration, mitochondria execute a variety of different metabolic functions, including biogenesis of iron sulfur clusters, which are essential for life (Lill and Kispal, 2000). Thus, the inheritance of the organelle is an essential process. We observed that mitochondria lacking Mdm31 and Mdm32 are almost immotile, similar to mitochondria lacking Mmm1, Mdm10, and Mdm12. Consequently, compromised mitochondrial motility leads to the appearance of mitochondria-free buds in the mutants. It is conceivable that a combination of the defects in double mutants lacking Mmm1 (or Mmm2, or Mdm10, or Mdm12) and Mdm31 (or Mdm32) results in a complete block of mitochondrial transport, and thus causes inviability of daughter cells.

We consider it unlikely that deletion of *MDM31* and *MDM32* directly influences the ability of mitochondria to bind to the actin-dependent transport machinery because mitochondria lacking Mdm31 and Mdm32 were found to be able to interact with actin in an ATP-dependent manner in vitro. It has been suggested that Mmm1 is required for coupling of mitochondria to the actin cytoskeleton (Boldogh et al., 1998). However, several lines of evidence suggest that Mmm1 is not directly acting as a receptor for actin-dependent motility factors. The function of Mmm1 has been conserved in the filamentous fungus *N. crassa*, which uses microtubules for mitochondrial transport (Prokisch et al., 2000; Westermann and Prokisch, 2002). Mitochondria isolated from loss-of-function mutants in *N. crassa* are still able to bind to the cytoskeleton (Fuchs et al., 2002), and the *N. crassa* protein complements the yeast mutant (Kondo-Okamoto et al., 2003). Similarly, homologues of Mdm31 are found in organisms that rely on microtubules for mitochondrial transport, such as *N. crassa* and *S. pombe*. This finding suggests that the main function of Mdm31, Mdm32, and Mmm1 is independent of the cytoskeletal system used by the cell for mitochondrial motility.

Mitochondria lacking Mdm31 and Mdm32 show dramatic changes in the organization of their internal membranes. This is not merely due to a defect in cristae formation, because

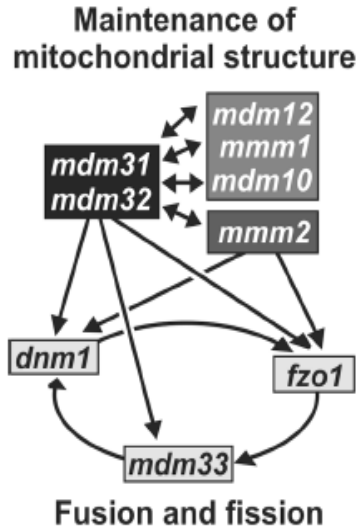


Figure 8. **Genetic relationships of components involved in mitochondrial dynamics.** Arrows indicate epistasis of mutant alleles, and double-headed arrows indicate synthetic lethality of mutations. Mutations that do not produce synthetic phenotypes are in the same box. A cycle of epistatic relationships between Δ*fzo1*, Δ*dnm1*, and Δ*mdm33* has been proposed by Messerschmitt et al. (2003). See text for further details.

some cristae are formed in glucose-grown cells (Fig. 4 E), and cristae are quite numerous in glycerol-grown cells (unpublished data). Interestingly, Aiken Hobbs et al. (2001) reported a similar phenotype for Δ*mmm1* mitochondria. Also in this mutant, cristae were lost and large extended or ring-shaped membrane inclusions were seen. These authors suggested that Mmm1 may be part of an internal scaffold-like structure required for normal mitochondrial shape and attachment of mtDNA. Our observations support a model in which Mdm31 and Mdm32 perform a similar function in the inner membrane. They may cooperate with Mmm1 in maintaining this scaffold-like structure and coordinate the behavior of the outer and inner membrane and provide anchoring sites for mtDNA nucleoids. When this function is lost, the internal structure of the organelle becomes disorganized, mitochondria lose their elongated shape, mtDNA nucleoids are destabilized, and mitochondrial motility is compromised as a consequence of aberrant mitochondrial shape.

Based on their genetic interactions and biochemical data, we can now propose at least three distinct functional entities involved in mitochondrial inheritance, the action of which is superior to the machineries of fusion and fission (summarized in Fig. 8). Mmm1, Mdm10, and Mdm12 have been proposed to be subunits of the same complex in the outer membrane (Boldogh et al., 2003). As combined deletion of their genes does not produce synthetic phenotypes (Berger et al., 1997; Hanekamp et al., 2002), these components likely share the same function. Mmm2 is a subunit of a separate complex in the outer membrane. Even though Δ*mmm1*/Δ*mmm2* double mutants are viable on fermentable carbon sources, conditional *mmm1* and *mmm2* alleles produce a synthetic lethal phenotype on nonfermentable carbon sources (Youngman et al., 2004). Hence, Mmm1 and Mmm2 act in functionally separable parallel pathways. Mdm31 and Mdm32 are functionally interdependent subunits of two novel complexes in the inner membrane, which might interact in a transient and dynamic manner. They are the first known inner membrane proteins that cooperate with the outer membrane proteins in inheritance of mitochondria and mtDNA. The functional characterization of novel components involved in these processes that were reported by Youngman et al. (2004) and herein revealed an unanticipated complexity of the machinery controlling mitochondrial behavior. It is a challenge for the future to reveal the precise molecular interactions of these complexes with components in the matrix and on the cytosolic face of the organelle that contribute to the complex process of mitochondrial inheritance.

Materials and methods

Plasmid and yeast strain constructions

Standard methods were used for cloning of DNA and growth and manipulation of yeast strains. Cloning procedures and strain constructions are described in detail in the online supplemental Materials and methods.

Microscopy

Mitochondria were labeled with mtGFP (Westermann and Neupert, 2000) or mitochondria-targeted DsRed (Mozdy et al., 2000). Staining of the actin cytoskeleton with rhodamine-phalloidin (Amberg, 1998) and DAPI staining of mtDNA in living cells (Aiken Hobbs et al., 2001) was performed according to published procedures. Staining of the vacuole with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (Molecular Probes) was performed according to the manufacturer's instructions. The ER was visualized with ER-targeted GFP (Prinz et al., 2000). Abf2-containing structures were labeled with a chimeric protein consisting of Abf2 and a GFP moiety derived from mtGFP. Mmm1-containing structures were stained with Mmm1 fused to DsRed.T4 (Bevis and Glick, 2002).

Epifluorescence microscopy was performed using a microscope (model Axioplan 2; Carl Zeiss MicroImaging, Inc.) equipped with a Plan-Neofluar 100×/1.30 Ph3 oil objective (Carl Zeiss MicroImaging, Inc.). Images were recorded either with a SPOT cooled color camera (Diagnostic Instruments) and processed with Lite Meta-Morph imaging software (Universal Imaging Corp.) or with an Evolution VF Mono Cooled monochrome camera (Intas) and processed with Image Pro Plus 5.0 and ScopePro 4.5 software (MediaCybernetics). Confocal images were taken with a confocal microscope (model TCS SP1; Leica) equipped with a 1.2 NA 63× water immersion lens (Leica; 63×, Planapo). For imaging, living cells were embedded in 1% low melting point agarose and observed at RT. Quantification of mitochondrial morphology defects was performed without prior reference to strain identity.

EM and immunocytochemistry were performed as described previously (Kärgel et al., 1996; Messerschmitt et al., 2003).

Analysis of mitochondria–actin interactions in vitro

Actin filaments were prepared by polymerizing nonmuscular human actin (tebu-bio GmbH) according to the manufacturer's instructions. Binding of filamentous actin (at a concentration of 100 μg/ml) to isolated mitochondria and cosedimentation of actin with mitochondria were performed as described previously (Lazzarino et al., 1994). Actin was detected by immunoblotting with monoclonal anti-actin antibodies (c4d6; Lessard, 1988).

Gel filtration analysis

Isolated mitochondria (1 mg) were pelleted by centrifugation for 10 min at 10,000 *g* and resuspended in 200 μl buffer A (1% digitonin, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 10 mM Tris-HCl, pH 7.4). After incubation for 1 h at 4°C under agitation, mitochondrial extracts were centrifuged for 30 min at 90,000 *g* in a rotor (model TLA45; Beckman Coulter) at 4°C. The supernatant was loaded on a Superose 6 gel filtration column (25-ml column volume; Amersham Biosciences) and chromatographed in buffer A with 0.05% digitonin (flow rate 0.5 ml/min). 0.5-ml fractions were collected, and proteins were precipitated with TCA and analyzed by SDS-PAGE and Western blotting. Calibration standards were as follows: thyroglobulin, 670 kD; apoferritin, 440 kD; alcohol dehydrogenase, 150 kD; carboanhydrase, 29 kD.

Miscellaneous

Antigens were expressed using the pQE system (QIAGEN) according to the manufacturer's instructions. Antisera were generated by injection of inclusion bodies into rabbits. Subfractionation of yeast cells and isolation, purification, and subfractionation of mitochondria were performed as described previously (Rowley et al., 1994). Mitochondrial fusion was examined according to published procedures (Nunnari et al., 1997; Fritz et al., 2003). Import of radiolabeled Mdm32 and coimmunoprecipitation was performed as described previously (Messerschmitt et al., 2003).

Online supplemental material

An alignment of Mdm31 protein family members is available as Fig. S1. Cloning procedures and yeast strain constructions are described in supplemental Materials and methods. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200410030/DC1.

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Teilarbeit D

Übersichtsartikel

Katrin Altmann, Mark Dürr und Benedikt Westermann (2007)

***Saccharomyces cerevisiae* as a model organism to study mitochondrial biology General considerations and basic procedures**

Methods in Molecular Biology Vol. 372: Mitochondria: Practical Protocols pp. 81-90

Darstellung des Eigenanteils

Mein Anteil an Teilarbeit D erstreckte sich auf Beiträge zum Text und die Abbildung 1.

Die Arbeit wurde hauptsächlich von Benedikt Westermann verfasst.

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Saccharomyces cerevisiae as a model organism to study mitochondrial biology

General considerations and basic procedures

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Budding yeast *Saccharomyces cerevisiae* is widely used to study mitochondrial biogenesis and function. In this chapter, we review some basic properties that make yeast an ideal model organism to investigate various aspects of mitochondrial biology. We discuss genetic features of commonly used yeast strains that are important for mitochondrial studies. Furthermore, this chapter provides protocols describing yeast culture conditions and procedures for isolation and purification of mitochondria.

1. Introduction

Budding yeast *Saccharomyces cerevisiae* has proven to be an excellent model organism to study a great variety of basic cellular functions that are conserved in eukaryotic cells. Several properties make yeast particularly suitable for genetic, biochemical and cell biological studies. For instance, *S. cerevisiae* can be cultured in an economic manner and has a short generation time (under optimal conditions less than 2 hours). This allows the isolation of biological material in amounts sufficient for further biochemical studies. Maybe most importantly, genetic engineering is highly efficient in yeast. *S. cerevisiae* is viable with numerous markers, a large selection of different kinds of plasmids and gene fusion cassettes is available, homologous recombination is very efficient, and laboratory yeast strains are stable both as haploid and diploid strains (1). The *S. cerevisiae* genome sequence is completely known since 1996. The genome has a size of ca. 12,000,000 base pairs, harbours about 6000 genes, and only about 4% of nuclear genes have introns (2). In recent years, several comprehensive genome-wide gene deletion and protein fusion libraries have been constructed. These are now available to the scientific community as a great resource for systematic studies (3-7).

The following databases provide good starting points to retrieve information about yeast genetics and biology: (i) Saccharomyces Genome Database, SGD (8), http://www.yeastgenome.org/; (ii) Yeast Virtual Library; http://www.yeastgenome.org/VL-yeast.html; (iii) Comprehensive Yeast Genome Database, CYGD (9), http://mips.gsf.de/genre/proj/ yeast/.

S. cerevisiae is a facultative anaerobic yeast capable of satisfying its energy requirements with ATP generated by fermentation. Thus, only relatively few mitochondrial proteins are essential for cell viability. These include a handful of factors essential for import and assembly of nuclear-encoded precursor proteins, iron/sulfur cluster assembly and flavin mononucleotide synthesis. The fact that many mitochondrial functions can be studied using viable knock-out mutants makes budding yeast an ideal organism for dissecting the molecular processes required for biogenesis of respiratory-competent mitochondria.

S. cerevisiae can live on a variety of carbon sources, but glucose and fructose are the preferred ones. On these carbon sources, most of the cellular ATP is generated in the cytosol by fermentation, and the expression of enzymes required for the utilization of other carbon sources is strongly reduced. This phenomenon is known as glucose repression or catabolite repression (10). Glucose repression affects the expression of many mitochondrial factors, including enzymes of the citric acid cycle and respiratory chain complexes. As synthesis of ATP by oxidative phosphorylation is a major function of mitochondria, mitochondrial size, volume and structure are adapted to the carbon source of the growth medium (11-14). Typically, wild type yeast cells growing logarithmically on glucose-containing medium display a relatively simple mitochondrial network that consists of few branched organelles (Fig. 1, top). In contrast, on non-fermentable carbon sources such as glycerol, mitochondria are much more numerous and form a highly branched interconnected network (Fig. 1, bottom).

The mitochondrial genome of *S. cerevisiae* is roughly 80,000 base pairs in size and encodes eight major proteins which are all essential for oxidative phosphorylation. These are cytochrome *b* (a subunit of the ubiquinol-cytochrome *c* oxidoreductase), Cox1, Cox2 and Cox3 (subunits of the cytochrome *c* oxidase), Atp6, Atp8 and Atp9

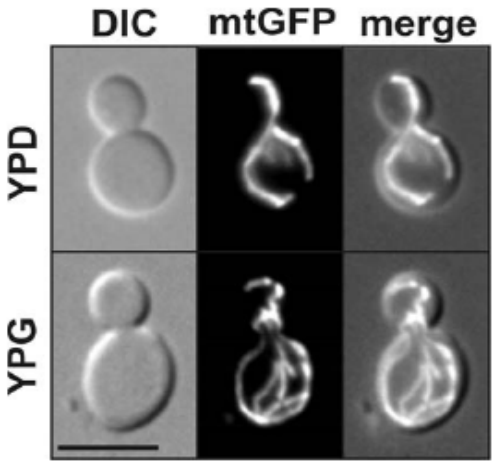

Fig. 1. Mitochondrial structure and volume depend on the carbon source of the medium. Wild type yeast cells (BY4741) expressing mitochondria-targeted green fluorescent protein (mtGFP) (32) were grown to logarithmic growth phase in glucose-containing medium (YPD; top) or glycerol-containing medium (YPG; bottom) and analyzed by differential interference contrast microscopy (DIC) and fluorescence microscopy (mtGFP). Bar, 5 µm.

(subunits of the F_o part of the ATP synthase), and Var1 (a component of the small subunit of the mitochondrial ribosome) (15). Many domesticated yeast strains produce high frequencies of mutants lacking intact mitochondrial genomes at rates of 2% or more (1). Strains harbouring an intact mitochondrial genome are designated ρ^+ (rho$^+$). Respiratory deficient strains harbouring a defective mitochondrial genome are ρ^- (rho$^-$), and strains completely lacking mitochondrial DNA are ρ^0 (rho^0). Despite the capacity of mitochondria to encode and synthesize proteins, more than 300 genes located in the nucleus are required for respiratory competence (16). Mutants in these genes are commonly referred to as nuclear *petite* or *pet* mutants (17).

The mitochondrial proteome of *S. cerevisiae* has been extensively characterized by analysis of purified mitochondria by mass spectrometry (18, 19). These approaches have led to the identification of most of the estimated 700-800 proteins that constitute the yeast mitochondrial proteome (19, 20).

Mitochondrial research using *S. cerevisiae* as a model organism has been instrumental in elucidating the biogenesis and biological function of this organelle (21). It is safe to predict that the knowledge of the mitochondrial proteome combined with the availability of comprehensive mutant collections will give mitochondrial research with yeast another boost in the coming years.

2. Materials

2.1. Commonly used yeast strains

A selection of commonly used yeast strains together with their genotypes and sources where they can be obtained is presented in Table 1.

Table 1. Commonly used yeast strains.

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF:Y00000
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF:Y10000
BY4743	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*	EUROSCARF:Y20000
D273-10B	*MATα mal*	ATCC:24657
S288C	*MATα SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*	ATCC:204508
W303 MATa	*MATa ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*	EUROSCARF:20000A
YPH499	*MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*	ATCC:204679
YPH500	*MATα ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*	ATCC:204680
YPH501	*MATa/MATα ura3-52/ura3-52 lys2-801_amber/lys2-801_amber ade2-101_ochre/ade2-101_ochre trp1-Δ63/trp1-a63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*	ATCC:204681

Strains can be obtained from the Yeast Genetics Stock Culture Center of the American Type Culture Collection, ATCC (http://www.atcc.org/SearchCatalogs/YeastGeneticStock.cfm) or EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html)

2.2. Yeast Culture

Amounts are per liter medium; for liquid medium omit agar (*see* **Note 1**).

1. YPD (yeast extract/peptone/dextrose medium): 10 g Bacto-yeast extract, 20 g Bacto-peptone, 20 g glucose (100 ml of a 20% stock solution), 20 g Bacto-agar, distilled water ad 1000 ml.
2. YPG (yeast extract/peptone/glycerol medium): 10 g Bacto-yeast extract, 20 g Bacto-peptone, 3% (v/v) glycerol (100 ml of a 30% stock solution), 20 g Bacto-agar, distilled water ad 1000 ml.
3. YPGal (yeast extract/peptone/galactose medium): 10 g Bacto-yeast extract, 20 g Bacto-peptone, 20 g galactose (100 ml of a 20% stock solution), 20 g Bacto-agar, distilled water ad 1000 ml.
4. YPRaffinose (yeast extract/peptone/raffinose medium): 10 g Bacto-yeast extract, 20 g Bacto-peptone, 20 g raffinose (100 ml of a 20% stock solution), 20 g Bacto-agar, distilled water ad 1000 ml.
5. SD (synthetic minimal medium, dextrose): 6,7 g yeast nitrogen base (including ammonium sulfate), 2% glucose (100 ml of a 20% stock solution), 20 g Bacto-agar, distilled water ad 1000 ml. Depending on the auxotrophic markers of the yeast strain, supplements may have to be added (the amounts are as indicated below for SC medium).
6. SC (synthetic complete medium, dextrose): SD medium supplemented with adenine sulfate (20 mg/l), uracil (20 mg/l), L-tryptophan (20 mg/l), L-histidine-HCl (20 mg/l), L-arginine-HCl (20 mg/l), L-methionine (20 mg/l), L-tyrosine (30 mg/l), L-leucine (30 mg/l), L-isoleucine (30 mg/l), L-lysine-HCl (30 mg/l), L-phenylalanine (50 mg/l), L-glutamic acid (100 mg/l), L-aspartic acid (100 mg/l), L-valine (150 mg/l), L-threonine (200 mg/l), L-serine (400 mg/l). For selection on auxotrophic markers, relevant supplements are omitted.
7. Lactate-medium: 3 g Bacto-yeast extract, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.5 g NaCl, 0.6 g $MgSO_4 \cdot H_2O$, 3 mg $FeCl_3$, 2% (v/v) lactate, distilled water ad 1000ml. Adjust pH to 5.5 (ca. 7.5 g/l NaOH pellets).

2.3. Isolation of mitochondria by differential centrifugation

Amounts are for 2 liters yeast culture at OD_{600} ≈1.2 (approx. 10 g wet weight of cells).

1. Tris-SO_4 buffer (30 ml): 100 mM Tris-SO_4, pH 9.4, 10 mM DTT (DTT: dithiothreitol, add just before use from freshly prepared 1 M stock solution).
2. Sorbitol buffer (80 ml): 1.2 M sorbitol, 20 mM KPi, pH 7.4 (chill 40 ml on ice).
3. Zymolyase 20T (approx. 30 mg).
4. Buffer A (60 ml): 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 1 mM PMSF; chill buffer on ice before use (*see* **Note 2**).
5. Buffer B (40 ml): 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4; chill buffer on ice before use.
6. BSA solution (50 µl): 75 mg/ml fatty acid-free bovine serum albumin (BSA), 0.6 M sorbitol.
7. Liquid nitrogen.

2.4. Purification of mitochondria by sucrose gradient purification

1. SEM buffer: 250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2.
2. Sucrose step gradient: 20%, 30%, 40%, 50%, and 60% sucrose (w/w) in 100 mM KCl, 1 mM EDTA, 1 mM PMSF, 10 mM MOPS-KOH, pH 7.2 (*see* **Note 2**).

3. Methods

3.1. Choice of suitable yeast strains

Commonly used laboratory yeast strains are not truly wild type *Saccharomyces cerevisiae* strains. Many laboratory stocks have been inbred with related *Saccharomyces* species. Thus, genetic backgrounds and growth properties of different yeast strains might differ considerably (1). Therefore, care should be taken in choosing strains for research on mitochondria (*see* **Note 3**).

S288C (22) is often used as a normal standard, because it was used to sequence the yeast genome (2). Furthermore, BY wild type strains, BY4741, BY4742 and BY4743 (23), that were used in the world wide gene deletion project (3) are derived from S288C. Unfortunately, S288C and its derivatives carry an insertion of a defective Ty1 retrotransposon element in the coding region of the *HAP1* gene (24). *HAP1* encodes a transcriptional regulator which is involved in the regulation of a variety of genes involved in electron transfer reactions, sterol metabolism and protein synthesis. As Hap1 function is compromised in S288C and its derivatives, these are not the best strains for mitochondrial research.

W303 is widely used for studies on mitochondrial biology. This strain contains the *ybp1-1* mutation, which makes it more sensitive to oxidative stress (25). W303 also contains a *bud4* mutation that causes defects in the budding pattern of haploid cells. In addition, W303 strains contain the *rad5-535* allele (Saccharomyces Genome Database).

D273-10B has been used for mitochondrial studies in numerous laboratories. It has normal cytochrome content and respiration, shows a low frequency of spontaneous ρ^- generation and is relatively resistant to glucose repression (26).

Strains YPH499, YPH500 and YPH501 contain six nonrevertible auxotrophic mutations that can be conveniently used for selection of vectors (27).

3.2. Yeast culture

Yeasts are grown either on agar plates or in Erlenmeyer flasks in liquid cultures under constant agitation (ca. 140 rpm). The optimal growth temperature for *S. cerevisiae* is 30°C. For long term preservation of strains, yeast cells are resuspended in 15% (v/v) glycerol and stored at -80°C (1).

YPD is a glucose-containing rich medium for routine growth. It supports growth of respiratory-deficient mutants. However, mitochondrial functions may be reduced due to glucose repression. YPG is a complex medium containing a non-fermentable carbon source (glycerol). It does not allow growth of respiratory-deficient (ρ^- or *petite*) mutants. Mitochondrial functions are induced on glycerol (compare Fig. 1). YPGal contains galactose as a carbon source. This medium is often used to induce genes that have been placed under control of the *GAL* promoter. Galactose is a fermentable carbon source that allows growth of ρ^- or *petite* mutants and does not induce glucose repression. YPRaffinose similarly supports growth of respiratory deficient mutants without causing glucose repression, but it does not induce the *GAL* promoter. It should be noted that the *GAL* promoter is repressed in the presence of even minor amounts of glucose.

Synthetic dextrose minimal medium (SD) is used for selection on auxotrophic markers. It is a synthetic minimal medium containing salts, trace elements, vitamins, a nitrogen source, and glucose. Depending on the auxotrophic markers, certain supplements may have to be added. Synthetic dextrose complete medium (SC) contains all possible supplements, except those which have been omitted to select on auxotrophic markers. Depending on the type of experiment, glucose can be replaced by any other carbon source in both types of minimal media.

Lactate medium is a semi-synthetic medium that is often used to grow yeast cultures for preparation of mitochondria (28). In order to have an optimal induction of mitochondrial functions, yeasts are precultured for several generations in lactate medium (*see* **Note 4**).

3.3. Isolation of mitochondria by differential centrifugation

Yeast mitochondria can be conveniently isolated by differential centrifugation (28-30). The following protocol is outlined for 2 liters of culture of an OD_{600} of 1-2, corresponding to ca. 10 g of wet weight of cells (*see* **Note 5**).

1. Collect the cells at 2000 x g for 5 min and determine wet weight.
2. Resuspend the pellet in 100 ml dH_2O and centrifuge at 2000 x g for 5 min (*see* **Note 6**).
3. Resuspend the cells in 30 ml Tris-SO_4 buffer and incubate the suspension for 10 min at 30°C under agitation (ca. 140 rpm).
4. Collect the cells by centrifugation at 2000 x g for 5 min and resuspend them in 40 ml sorbitol buffer. Add Zymolyase 20T (2 mg/g cells) to the suspension and incubate under gentle agitation for 20-40 min at 30°C until spheroplasts have formed (*see* **Note 7**).
5. All following steps will be performed on ice using ice-cold buffers, and centrifugation steps are performed at 4°C. Harvest the spheroplasts by centrifugation at 2000 x g for 5 min.
6. Resuspend the pellet in 40 ml sorbitol buffer (gently shaking or stirring with a pipette) and spin at 2000 x g for 5 min.
7. Resuspend the spheroplasts carefully in 30 ml buffer A, transfer the suspension to a 50 ml dounce homogenizer (tight-fitting glass pistil) and homogenize with 15 strokes.
8. Centrifuge the homogenate at 2000 x g for 5 min and keep the supernatant.
9. Resuspend the pellet carefully in 30 ml Buffer A, homogenize with 15 strokes and spin at 2000 x g for 5 min.
10. Combine the supernatants and centrifuge at 12,000 x g for 10 min.
11. Resuspend the pellets in 1 ml Buffer B using cut pipette tips and fill up to 30 ml with Buffer B.
12. Spin down any remaining cell debris at 2000 x g for 5 min, transfer the supernatant to a fresh tube and centrifuge at 12,000 x g for 10 min.
13. Resuspend the pellet in 0.5 ml Buffer B.
14. Take an aliquot to determine protein concentration.
15. Add 35 µl of BSA solution (7 % v/v).
16. Make aliquots of 30-50 µl, immediately snap-freeze in liquid nitrogen. Store at -80°C (*see* **Note 8**).

3.4. Purification of mitochondria by sucrose gradient purification

Mitochondria isolated by differential centrifugation can be further purified by centrifugation on a sucrose step gradient (31).

1. Load mitochondrial suspension on top of a gradient consisting of 20%, 30%, 40%, 50%, and 60% sucrose buffer (w/w) in a Beckman SW41 centrifuge tube (*see* **Note 9**).
2. Centrifuge 15 min at 240,000 x g at 4°C.
3. Collect mitochondria from the band between the 40% and 50% sucrose layer.
4. Concentrate mitochondria by centrifugation in a microfuge tube at 12,000 x g at 4°C.

5. Wash pellet with SEM and resuspend purified mitochondria in SEM.

4. Notes

1. One liter medium is sufficient for ca. 30 plates. To avoid hydrolysis of the agar, caramelized glucose and mushy plates, it is recommended to autoclave the components of the medium separately; e.g. 20 g agar in 500 ml H_2O, carbon source in 100 ml H_2O, and the other components in 400 ml H_2O. Combine the solutions directly after autoclaving (15 min at 120°C, 1 atm pressure), mix thoroughly and pour plates. For safety reasons, melting solidified agar in a microwave oven must be avoided!
2. PMSF (phenylmethylsulfonyl fluoride) is dissolved at 200 mM in EtOH. Prepare freshly before use. PMSF is a protease inhibitor. It works fine for many applications. However, some assays using isolated mitochondria might be inhibited by the presence of PMSF during mitochondria isolation. In this case, other protease inhibitor cocktails might be tried.
3. Several laboratory yeast strains (e.g. W303, YPH499, YPH500, YPH501) carry the *ade2* marker. When adenine in the growth medium becomes limiting, these strains accumulate a pink pigment as an intermediate during the purine nucleotide biosynthetic pathway and form red colonies. As formation of the pigment is dependent on oxidative metabolism, the presence of the *ade2* marker is often useful to judge respiratory activity of mutants with defective oxidative phosphorylation.
4. A small amount of glucose (0.05%) may be added to the first culture to help the cells adapt to the culture conditions. However, the addition of glucose to later cultures should be avoided to prevent glucose repression. The incubation time depends markedly on the yeast strain. Therefore, it is recommended to measure the growth rate of a preculture before inoculating the big culture that will be used for mitochondria isolation. Precultures should be always kept in the logarithmic growth phase (i.e. $OD_{600} < 2.0$).
5. To obtain a high yield of mitochondria, cultures are usually grown in lactate medium (see 3.2. and Note 4). Alternatively, YPG or minimal media may be used. For respiratory-deficient mutants, fermentable non-repressing carbon sources such as galactose or raffinose are recommended.
6. When working with large amounts of culture, it is convenient to pool the cells.
7. To test for spheroplast formation, add 50 µl cells to 2 ml H_2O and sorbitol buffer. When the suspension in H_2O clears up, stop the incubation and proceed with the next step.
8. Frozen mitochondria are good for many applications. However, some in vitro assays might require that mitochondria are prepared freshly.
9. Use 14x89 mm ultraclear centrifuge tubes (e.g. Beckman No. 344059) for a Beckman SW41 ultracentrifuge swing-out rotor, or equivalent equipment. The gradient should have a total volume of 10 ml and is overlaid with 1 ml mitochondria suspension containing not more than 50-100 mg mitochondrial protein in SEM.

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Teilarbeit E

Übersichtsartikel

Sandra Merz, Miriam Hammermeister, Katrin Altmann, Mark Dürr und Benedikt Westermann (2007)

Molecular machinery of mitochondrial dynamics in yeast

Biological Chemistry 388, 917–926.

Darstellung des Eigenanteils

Mein Anteil an Teilarbeit E erstreckte sich auf Beiträge zum Abschnitt „Mitochondrial Motility“. In Abbildung 1 stammen das Bild der Wildtypzelle und der Bewegungsmutante von mir.

Verfasst wurde diese Teilarbeit von Benedikt Westermann mit Beiträgen der anderen Co-Autoren.

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Review

Molecular machinery of mitochondrial dynamics in yeast

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Abstract

Mitochondria are amazingly dynamic organelles. They continuously move along cytoskeletal tracks and frequently fuse and divide. These processes are important for maintenance of mitochondrial functions, for inheritance of the organelles upon cell division, for cellular differentiation and for apoptosis. As the machinery of mitochondrial behavior has been highly conserved during evolution, it can be studied in simple model organisms, such as yeast. During the past decade, several key components of mitochondrial dynamics have been identified and functionally characterized in *Saccharomyces cerevisiae*. These include the mitochondrial fusion and fission machineries and proteins required for maintenance of tubular shape and mitochondrial motility. Taken together, these findings reveal a comprehensive picture that shows the cellular processes and molecular components required for mitochondrial inheritance and morphogenesis in a simple eukaryotic cell.

Keywords: actin cytoskeleton; membrane division; membrane fusion; mitochondria; organelle biogenesis; *Saccharomyces cerevisiae*.

Introduction

Mitochondria are essential organelles of eukaryotic cells. They supply the cell with ATP generated by oxidative phosphorylation, they are the site of numerous metabolic pathways, they play a key role in the assembly of Fe/S clusters, and they are central regulators of programmed cell death. Given this multitude of different functions, it is not surprising that mitochondrial copy number, size, morphology and intracellular position constantly adapt to the cell's ever-changing physiological conditions. Thus, mitochondria are highly dynamic organelles that frequently fuse, divide and move around in many eukaryotic cell types (Bereiter-Hahn, 1990; Okamoto and Shaw, 2005). This dynamic behavior is important for a number of cellular processes, including cell development (Hales and Fuller, 1997; Honda and Hirose, 2003), transmission of calcium signals (Szabadkai et al., 2006), apoptosis (Heath-Engel and Shore, 2006; Parone and Martinou, 2006), and aging (Sato et al., 2006). The fundamental mechanisms controlling mitochondrial behavior are very similar in yeasts, fungi, worms, flies, mice and humans, and many of the proteins mediating mitochondrial behavior have been evolutionarily conserved across the fungal and animal kingdoms (Table 1). Cellular roles and molecular components of mitochondrial morphology and distribution in mammalian cells have been discussed in an excellent review published in a recent issue of *Biological Chemistry* (Frazier et al., 2006). Here we summarize our current knowledge about the molecular machinery of mitochondrial dynamics in budding yeast *Saccharomyces cerevisiae*.

Yeast has proven to be an excellent model organism to study the molecular machinery of mitochondrial dynamics, and most of the key components were first discovered and functionally characterized in *S. cerevisiae*. Mitochondria exhibit complex behavior even in a relatively simple unicellular organism such as yeast. In vegetatively growing wild-type cells they form a branched tubular network below the cell cortex (Hoffmann and Avers, 1973; Stevens, 1981; see Figure 1). Yeast cells growing logarithmically in glucose-containing medium show approximately 2.5 mitochondrial fusion and fission events per minute, resulting in complete remodeling of the mitochondrial network every few minutes (Nunnari et al., 1997; Jakobs et al., 2003). Mitochondrial mass and morphology depend on the metabolic activity of the cell, i.e., the mitochondrial network is much larger and more ramified on non-fermentable carbon sources (Visser et al., 1995; Egner et al., 2002). During mitotic cell division, mitochondria enter the bud via a polarized actin-dependent movement immediately after bud emergence. At the same time, a retention mechanism immobilizes some mitochondria at the opposite pole in the mother cell. Frequent anterograde and retrograde movements of mitochondria ensure equal distribution of the organelles before cytokinesis (Simon et al., 1997; Yang et al., 1999; Fehrenbacher et al., 2004). Diploid cells undergoing meiosis and sporulation show a number of stage-specific changes in mitochondrial distribution and morphology. When yeast cells enter stationary phase the mitochondrial network breaks down into numerous small fragments. During pre-meiotic S phase mitochondrial fragments rejoin to form an interconnected tubular network, which then moves to the cell center where it remains associated with the nucleus during meiosis II. Thus, faithful inheritance of mitochondria to all four developing spores is ensured. Mitochondria fragment again in mature tetrads and remain fragmented until the spores germinate (Miyakawa et al., 1984; Gorsich and Shaw, 2004). Mitochondrial morphology and

Table 1 Yeast and homologous mammalian proteins involved in mitochondrial dynamics.

Process	Yeast	Alternative names	Mammals	Location	Proposed function
Fusion	Fzo1	Ybr179c	Mfn1 and 2	OM ,	OM fusion
	Mdm30	Ylr368w	–	Cytosol, OM	Turn-over of Fzo1
	Mgm1	Yor211c	OPA1	IM, IMS	IM fusion
	Pcp1	Mdm37, Rbd1, Ygr101w	PARL	IM	Processing of Mgm1
	Ugo1	Ydr470c	–	OM	Connection of Fzo1 and Mgm1
	Ups1	Ylr193c	PREL1	IMS	Regulation of Pcp1-mediated processing of Mgm1
Division	Caf4	Ykr036c	–	Cytosol, OM	OM division
	Dnm1	Yll001w	Drp1 (Dlp1)	Cytosol, OM	OM division
	Fis1	Mdv2, Ydr065c	Fis1	OM	OM division
	Mdm33	She9, Ydr292w	–	IM	IM constriction and/or division
	Mdv1	Fis2, Gag3, Net2, Yjl112w	–	Cytosol, OM	OM division
	Num1	Ydr150w	–	Cell cortex, OM	Alternative Dnm1-containing complex
Tubulation	Mdm10	Yal010c	–	OM	Tubulation, mtDNA inheritance, assembly of β-barrel proteins
	Mdm12	Yol009c	–	OM	Tubulation, mtDNA inheritance, assembly of β-barrel proteins
	Mdm31	Yhr194w	–	IM	Tubulation, mtDNA inheritance
	Mdm32	Yor147w	–	IM	Tubulation, mtDNA inheritance
	Mmm1	Yll006w, Yme6	–	OM	Tubulation, mtDNA inheritance, assembly of β-barrel proteins
	Mmm2	Mdm34, Ygl219c	–	OM	Tubulation, mtDNA inheritance
Motility	Arp2/3 complex		Arp2/3 complex	Cytosol	Actin polymerization-driven mitochondrial motility
	Mlc1	Ygl106w	Myosin light chain	Cytosol	Essential light chain of Myo2
	Myo2	Yor326w	Myosin V	Cytosol	Organelle motor
Other	Gem1	Yal048c	Miro-1 and -2	OM	Maintenance of mitochondrial morphology
	Mfb1	Ydr219c	–	Cytosol, OM	Maintenance of mitochondrial morphology

IM, inner membrane; IMS, intermembrane space; mtDNA, mitochondrial DNA; OM, outer membrane.

dynamic behavior are determined by four basic processes: fusion, division, tubulation and motility. Characteristic mutants defective in any one of these processes are depicted in Figure 1, and the molecular components mediating these processes in yeast are summarized in Figure 2 and Table 1. In the following, we briefly review what is known about the molecular machineries mediating mitochondrial dynamics in yeast.

Mitochondrial fusion

Three proteins constitute the core machinery of mitochondrial fusion in yeast: Fzo1 and Ugo1 in the outer membrane, and Mgm1, which is associated with the inner membrane. Fzo1 is a large GTPase that assembles into a high-molecular-weight complex in the outer membrane (Hermann et al., 1998; Rapaport et al., 1998). Coiled-coil domains exposed to the cytosol are thought to mediate inter-mitochondrial interactions (Rojo et al., 2002; Koshiba et al., 2004). The two transmembrane domains of Fzo1 are connected by a short loop in the intermembrane space that is important in establishing contacts to the inner membrane (Fritz et al., 2001). Mutants lacking functional Fzo1 contain fragmented mitochondria because mitochondrial fission occurs in the absence of fusion. As a consequence, these mutants are defective in mitochondrial DNA (mtDNA) inheritance (Hermann et al., 1998; Rapaport et al., 1998). As mitochondria devoid of mtDNA show increased motility (Boldogh et al., 2003) it is conceivable that mitochondrial fragments lacking mtDNA are preferentially transmitted to the daughter cells during cell division in fusion-deficient mutants. Mitochondria lacking Fzo1 are unable to exchange their matrix contents both *in vivo* and *in vitro*. Thus, a block in fusion is clearly the primary defect in *fzo1* mutants (Hermann et al., 1998; Meeusen et al., 2004).

Mgm1 is a dynamin-related GTPase associated with the mitochondrial inner membrane facing the intermembrane space (Wong et al., 2000). Deletion mutants or conditional mutants lacking functional Mgm1 are defective in mitochondrial genome maintenance (Jones and Fangman, 1992), they contain aggregated or fragmented mitochondria (Shepard and Yaffe, 1999; Wong et al., 2000), and they are defective in matrix content mixing *in vivo* (Wong et al., 2000). The Mgm1 protein is present in two isoforms: a larger isoform, the N-terminal end of which is inserted in the inner membrane, and a smaller isoform located in the intermembrane space. Both isoforms are necessary for efficient fusion, because mutants lacking either of the isoforms contain fragmented mitochondria (Herlan et al., 2003). A direct role of Mgm1 in mitochondrial inner membrane fusion has recently been demonstrated using an *in vitro* mitochondrial fusion assay, which is based on mixing of matrix and membrane markers of isolated organelles. In this assay, conditional

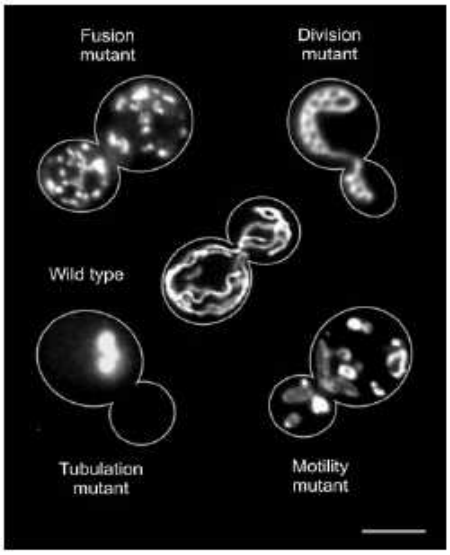

Figure 1 Mitochondrial morphology in *Saccharomyces cerevisiae*.
Wild-type and characteristic mutant cells defective in mitochondrial fusion (*Δugo1*), division (*Δmdv1*), tubulation (*Δmdm10*) and motility (*myo2*) were grown in glucose-containing medium, and mitochondria were visualized with mitochondria-targeted GFP (Westermann and Neupert, 2000). The shape of each cell is indicated by a gray line. Images of cells from separate cultures have been mounted into one illustration. Scale bar represents 5 μm.

mutant alleles allowed the elucidation of Mgm1 activities in inner membrane tethering, fusion and cristae maintenance (Meeusen et al., 2006).

Ugo1 is a multi-pass transmembrane protein of the outer membrane (Coonrod et al., 2007). Mutant cells lacking Ugo1 contain fragmented mitochondria, are devoid of mtDNA, and do not mix their matrix contents *in vivo*, similar to *fzo1* and *mgm1* mutants (Sesaki and

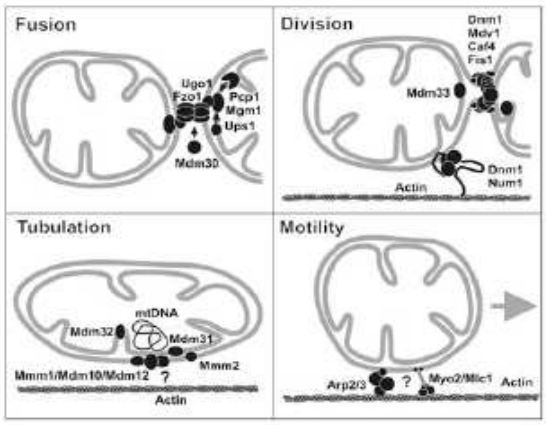

Figure 2 Cartoon depicting the core components of the molecular machineries mediating mitochondrial fusion, division, tubulation and motility.
See text for details.

Jensen, 2001). Ugo1 has been shown to interact physically with both Fzo1 and Mgm1. However, these interactions may be transient, and it is not known whether they constitute an integral part of the mitochondrial fusion machinery or whether they are just required to regulate Fzo1 and/or Mgm1 activity (Wong et al., 2003; Sesaki and Jensen, 2004).

How do Fzo1, Mgm1 and Ugo1 work together to mediate fusion? Mitochondrial fusion is a particularly complex process because it requires merging of four membranes in a coordinated manner. Fzo1 contains all the domains that would be expected for a fusogen of the outer membrane: coiled-coil domains that could draw together the membranes of opposing organelles, transmembrane domains that could transmit force to the membranes to initiate lipid bilayer mixing, and a GTPase that could provide energy to overcome the energy barrier of membrane fusion (Westermann, 2003). Consistent with this model of Fzo1 function, it has been demonstrated for the mammalian ortholog, Mfn1, that the coiled-coil domains form inter-molecular complexes that provide a mechanism for organelle tethering (Koshiba et al., 2004). While these data underscore the central role of Fzo1 in mitochondrial fusion, it remains to be tested whether its activity is sufficient for lipid bilayer mixing of the mitochondrial outer membrane. The phenotype of *mgm1* mutants and the association of Mgm1 with the inner membrane make this dynamin-related protein the prime candidate for a fusogen of the inner membrane. Interestingly, fusion of the outer and inner membranes can be uncoupled *in vitro* when content and membrane mixing of isolated organelles are monitored (Meeusen et al., 2004; Meeusen et al., 2006). However, it appears that fusion of the mitochondrial membranes is tightly coupled *in vivo*, because *fzo1* mutants that cannot establish contacts between the Fzo1 complex and the inner membrane show lower mitochondrial fusion activity (Fritz et al., 2001). Fusion-promoting contact sites connecting the mitochondrial outer and inner membranes might be established by direct interactions of the intermembrane space loop of Fzo1 with Mgm1, or by Ugo1, which might act as a linker between Fzo1 and Mgm1.

Mitochondrial fusion and fission must be tightly balanced to maintain a tubular network in vegetatively growing cells. During developmental processes, or in response to environmental challenges, a shift of this balance towards fusion or fission adapts organellar morphology to the physiological conditions of the cell. Thus, complex mechanisms must exist to regulate mitochondrial fusion and fission activities. To date, three regulatory factors of mitochondrial fusion have been identified in yeast: Mdm30 acting on Fzo1, and Pcp1 and Ups1, both acting on Mgm1. Mdm30 belongs to the family of F-box proteins that are subunits of Skp1-Cullin-F-box (SCF) ubiquitin ligases and non-SCF complexes, which regulate a large number of cellular processes (Petroski and Deshaies, 2005). Mdm30 is a cytosolic protein for which a portion is associated with the mitochondrial outer membrane (Fritz et al., 2003). Cells lacking Mdm30 contain fragmented and aggregated mitochondria because turnover of Fzo1 is lower and apparently unproductive

Fzo1 complexes accumulate (Dimmer et al., 2002; Fritz et al., 2003; Dürr et al., 2006). Interestingly, Mdm30-dependent turnover of Fzo1 in vegetatively growing cells is independent of ubiquitinylation, SCF complexes, and 26S proteasomes (Escobar-Henriques et al., 2006), while degradation of Fzo1 in α factor-arrested yeast cells is not regulated by Mdm30, but involves the 26S proteasome (Neutzner and Youle, 2005; Escobar-Henriques et al., 2006). Pcp1 belongs to the family of rhomboid-related proteases. It cleaves off the inner membrane domain of newly imported Mgm1 and thereby generates the small isoform, which is located in the intermembrane space (Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003). It has been speculated that a shift in the balance between both Mgm1 isoforms by Pcp1 processing may adapt mitochondrial fusion activity to the energy demands of the cell (Herlan et al., 2004). Ups1 is an intermembrane space protein that regulates Pcp1-dependent processing of newly synthesized Mgm1 during its import into mitochondria. Intriguingly, Ups1 activity is only required on fermentable carbon sources, suggesting that Ups1 provides a means to adapt the mitochondrial fusion rate to the metabolic activity of the cell (Sesaki et al., 2006).

Mitochondrial division

Three proteins build the core machinery of mitochondrial outer membrane division in yeast: Dnm1, Fis1 and Mdv1. Dnm1 is a conserved dynamin-related GTPase located in the cytosol. Mutants lacking Dnm1 contain interconnected mitochondria resembling fishing nets (Otsuga et al., 1998). These aberrant organelles are formed because ongoing fusion continues to concatenate mitochondrial tubules, while the meshes of the net cannot be resolved owing to blockage of mitochondrial fission (Bleazard et al., 1999; Sesaki and Jensen, 1999). Purified Dnm1 assembles with Fis1 and Mdv1 into a ternary complex that mediates mitochondrial outer membrane division (Tieu et al., 2002; Cerveny and Jensen, 2003; Naylor et al., 2006). Fis1 is an evolutionarily conserved integral membrane protein that is evenly distributed over the mitochondrial surface. Its C-terminus is anchored in the outer membrane, while its N-terminus is exposed to the cytosol (Mozdy et al., 2000). Mdv1 is a cytosolic WD40 repeat protein that assembles together with Dnm1 in punctate structures on the mitochondrial surface (Fekkes et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001; Tieu et al., 2002). Cells lacking Mdv1 or Fis1 contain net-like mitochondria very similar to those observed in Δ*dnm1* mutants (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001).

Mitochondrial tubules have a diameter of approximately 300–400 nm (Egner et al., 2002). How is division of such a large double-membrane-bounded organelle achieved? Three-dimensional time-lapse imaging of live yeast cells revealed that Dnm1 patches assemble on hot spots on the mitochondrial surface. At the same time, constriction of mitochondrial tubules is observed at sites that may or may not overlap with Dnm1 assembly sites. A successful division event only occurs when Dnm1 assembly and mitochondrial constriction coincide (Legesse-Miller et al., 2003). Purified Dnm1 self-assembles into rings and extended spirals with a diameter of approximately 100 nm *in vitro*. This diameter fits remarkably well to the dimensions that can be expected for a constricted mitochondrion (Ingerman et al., 2005). These spirals might correspond to assemblies containing 100–500 Dnm1 molecules surrounding a mitochondrial constriction site that can be detected *in vivo* (Schauss et al., 2006). Thus, it is conceivable that Dnm1 spirals sever the mitochondrion in a manner similar to other dynamin family members that are involved in various intracellular membrane scission events (Praefcke and McMahon, 2004). In this scenario, Fis1 and Mdv1 would be primarily required to trigger self-assembly of Dnm1 on the mitochondrial surface (Ingerman et al., 2005).

Several recent reports showed that Dnm1 may also interact with alternative partner proteins to control mitochondrial morphology. The WD40 repeat protein Caf4 resembles Mdv1 in many respects: it has a similar domain structure, it assembles in punctate structures on the mitochondrial surface, and it interacts with Fis1, Dnm1 and Mdv1. Deletion of the *CAF4* gene does not produce any obvious phenotype. However, the residual division activity that is detectable in Δ*mdv1* mutants is completely abolished in the absence of Caf4, indicating redundant activities of Caf4 and Mdv1 (Griffin et al., 2005). Sometimes, Dnm1 complexes are formed that do not surround the mitochondrial tubule in its entirety. These complexes are mostly oriented to the cell cortex. As this asymmetric orientation is dependent on Fis1 and Caf4, but not on Mdv1, Dnm1 may form complexes with alternative WD40 repeat proteins that might be functionally distinct (Schauss et al., 2006). However, the role of these cortex-oriented Dnm1 patches is currently unknown.

Num1 has recently been identified as another interaction partner of Dnm1 (Cerveny et al., 2007). Num1 is a large, 313-kDa cell cortex-associated protein (Farkasovsky and Küntzel, 1995). Mutants lacking Num1 show collapsed mitochondrial nets that resemble mitochondria in fission-defective mutants (Dimmer et al., 2002; Cerveny et al., 2007). Num1 acts independently of Mdv1 and Fis1 and provides an alternative mitochondrial attachment site for Dnm1. It has been proposed that Num1 coordinates the interplay of mitochondrial division, mitochondrial inheritance and actin-dependent association of mitochondria with the cell cortex (Cerveny et al., 2007). In sum, these activities contribute to faithful partitioning of the organelles during cell division.

While intense research in recent years has revealed many details of outer membrane division, much less is known about the mechanisms dividing the inner membrane. Several lines of evidence indicate that division of the outer and inner membranes are separate events. First, assembly of a Dnm1 spiral around a mitochondrion requires a prior constriction event (Legesse-Miller et al., 2003; Ingerman et al., 2005; Schauss et al., 2006). Second, after separation of the matrix compartment, mitochondria often immediately re-fuse at the same site, presumably because they remain connected by an outer membrane tubule (Jakobs et al., 2003;

Legesse-Miller et al., 2003). Third, research in *Caenorhabditis elegans* revealed that in dominant-negative mutants of *drp-1*, encoding the worm ortholog of Dnm1, the mitochondrial matrix retracts into large blebs that are connected by thin tubules of the outer membrane. This indicates that scission of the inner membrane continues while scission of the outer membrane is blocked (Labrousse et al., 1999). It has been suggested that Mdm33 plays an important role in inner membrane fission and/or mitochondrial constriction. Mdm33 is a protein of the mitochondrial inner membrane and possesses extensive coiled-coil domains exposed to the matrix. Δ*mdm33* mutants harbor giant extended ring-shaped mitochondria. Intriguingly, overexpression of Mdm33 induces septation and vesiculation of the inner membrane, which can be explained by enhanced inner-membrane fission activity. A hypothetical model predicts that homotypic Mdm33 protein interactions on the matrix side of opposing inner membranes could mediate constriction and/or fission of the inner membrane (Messerschmitt et al., 2003). This step might well correspond to the constriction and matrix separation events observed by time-lapse microscopy (Jakobs et al., 2003; Legesse-Miller et al., 2003) and might be a prerequisite for assembly of a 100-nm Dnm1 spiral surrounding the organelle (Ingerman et al., 2005).

Mitochondrial tubulation

Six proteins are thought to function primarily in maintenance of the tubular shape of mitochondria: Mdm10, Mdm12, Mmm1 and Mmm2 in the outer membrane, and Mdm31 and Mdm32 in the inner membrane. Mutants lacking any one of these components contain large spherical mitochondria instead of long tubular organelles. Mutant mitochondria are almost immotile, and buds devoid of mitochondria are generated at high frequency (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998, 2003; Youngman et al., 2004; Dimmer et al., 2005). Another hallmark of tubulation-defective mutants is a defect in the organization and inheritance of mtDNA. In yeast, mtDNA is organized in ca. 20 nucleoids per cell, large membrane-associated DNA-protein complexes that can be visualized as discrete structures by light microscopy (Chen and Butow, 2005). In tubulation-defective mutants, mtDNA is present in a single diffuse structure that can easily be lost upon cell division (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Youngman et al., 2004; Dimmer et al., 2005).

Mdm10, Mdm12 and Mmm1 assemble into a ternary complex in the outer membrane (Boldogh et al., 2003), which is called Mmm1 complex in the following. Mmm1 complexes form discrete foci in the outer membrane that are located next to a subset of mtDNA nucleoids in the matrix, suggesting that a physical link spanning both mitochondrial membranes exists. As mtDNA nucleoids are disorganized in mutants lacking Mdm10, Mdm12 or Mmm1, it is conceivable that the Mmm1 complex is directly required to connect mtDNA nucleoids with a segregation machinery in the cytosol (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003). Intriguingly, yeast Mmm1 spans both mitochondrial membranes (Kondo-Okamoto et al., 2003). However, the inner membrane-spanning segment does not exist in the homologous MMM1 protein of *Neurospora crassa* (Prokisch et al., 2000), and it is not required for maintenance of mtDNA nucleoids in yeast (Kondo-Okamoto et al., 2003). Therefore, it seems likely that the Mmm1 complex is connected to mtDNA by distinct proteins in the inner membrane.

Mmm2 is another outer membrane protein that plays a role in the tubulation pathway. Deletion of the *MMM2* gene causes typical phenotypes very similar to *mmm1*, *mdm10* and *mdm12* mutants (Dimmer et al., 2002; Youngman et al., 2004). Mmm2 forms discrete foci that partially co-localize with Mmm1 foci or mtDNA. However, Mmm2 does not stably associate with the Mmm1 complex. Rather, it seems to cooperate with Mmm1 in a dynamic manner to maintain mitochondrial shape and mtDNA nucleoids (Youngman et al., 2004).

Mdm31 and Mdm32 are related inner membrane proteins that assemble into two separate complexes and expose large domains to the intermembrane space. Similar to mutants lacking the outer membrane tubulation components, mutants lacking Mdm31 or Mdm32 contain large spherical organelles with aberrant inner membrane structure and disorganized mtDNA nucleoids. Furthermore, the connection of Mmm1 foci to mtDNA is lost in Δ*mdm31* and Δ*mdm32* mutants, suggesting that Mdm31 and Mdm32 are required to establish the interaction of mtDNA nucleoids with the cytosolic segregation machinery. Deletion of either of the *MDM31* or *MDM32* genes in combination with a deletion of any of the *MDM10*, *MDM12*, *MMM1* or *MMM2* genes produces a synthetic lethal phenotype demonstrating the essential nature of this pathway (Dimmer et al., 2005).

Taken together, four separate complexes cooperate in the maintenance of tubular mitochondrial shape: the Mmm1 complex containing Mdm10 and Mdm12, the Mmm2 complex, the Mdm31 complex and the Mdm32 complex. However, the function of these complexes is still a matter of debate. The actin cytoskeleton is essential for mitochondrial distribution and morphology in yeast (Boldogh and Pon, 2006), and it seems possible that the mitochondrial network collapses into a giant spherical organelle when its connection to the cytoskeleton is lost. It has been suggested that the Mmm1 complex plays an important role in this process by establishing mitochondrial interaction with filamentous actin (Boldogh et al., 1998). However, for several reasons we consider it unlikely that such activity is the main function of the tubulation complexes. First, genetic or drug-induced disruption of actin filaments does not promote the formation of spherical mitochondria (Drubin et al., 1993; Hermann et al., 1997; Boldogh et al., 1998; Altmann and Westermann, 2005). Second, the *Neurospora* homolog of Mmm1 clearly acts independently of the actin cytoskeleton (Prokisch et al., 2000; Fuchs et al., 2002). Third, at least in Δ*mdm31* and Δ*mdm32* mutants, mitochondrial actin-binding activity is not impaired (Dimmer et al., 2005). In our view the available data are best compatible with a scaffolding function. In this model, the tubulation complexes would provide anchoring points for cytoskeletal elements and/or intramitochondrial struc-

tures that would help to maintain the tubular shape. At the same time, these complexes could connect mtDNA nucleoids to the scaffold and provide a functional link between maintenance of mitochondrial structure and mtDNA inheritance.

Mdm10 has been shown to be a subunit of the SAM complex, which mediates sorting and assembly of β-barrel proteins in the outer membrane. In particular, it plays a specific role in assembly of the TOM complex, which constitutes the general insertion pore for import of cytosolically synthesized mitochondrial precursor proteins (Meisinger et al., 2004, 2006). More recently, it has also been proposed that Mdm12 and Mmm1 function in the β-barrel protein assembly pathway (Meisinger et al., 2007). As all components of the mitochondrial fusion, division and motility machineries have to be post-translationally imported into the organelle, it is conceivable that defects in the mitochondrial protein import and sorting complexes result in altered mitochondrial morphology (Westermann and Prokisch, 2002; Stojanovski et al., 2006). Consequently, a large number of mitochondrial protein import mutants show mitochondrial morphology defects (Dimmer et al., 2002; Meisinger et al., 2004; Altmann and Westermann, 2005). Thus, the question may be raised as to whether the primary function of tubulation components is protein import. In this case, the mitochondrial morphology phenotypes could be regarded as secondary effects of an altered protein composition in the mitochondrial membranes.

Interestingly, localization of Mdm10 to mitochondria does not require Mdm12 or Mmm1, whereas mitochondrial localization of Mdm12 and Mmm1 depends on Mdm10 (Boldogh et al., 2003). This observation, together with differential behavior of Mdm10 in co-purification experiments (Meisinger et al., 2007), indicates that Mdm10 is a subunit of two distinct complexes, the SAM complex and the Mmm1 complex, and thus might have a dual function. The evidence for a role of Mdm10, Mdm12 and Mmm1 in β-barrel protein assembly is compelling. However, physical interaction of Mdm10, Mdm12 or Mmm1 with unassembled β-barrel proteins has not been demonstrated yet. On the other hand, even though a direct function of Mmm1, Mdm10 and Mdm12 in tubulation is difficult to prove, the striking co-localization of tubulation complexes with mtDNA nucleoids cannot be easily explained by a role in β-barrel protein assembly. Thus, it remains to be demonstrated whether the primary function of tubulation components is in protein sorting and assembly, or maintenance of mitochondrial structure, or whether these components have a dual role in mitochondrial biogenesis.

Mitochondrial motility

Mitochondrial motility in *S. cerevisiae* strictly depends on the actin cytoskeleton (Drubin et al., 1993; Simon et al., 1995, 1997; Hermann et al., 1997; Fehrenbacher et al., 2004; Altmann and Westermann, 2005). In contrast to other fungi, such as *Schizosaccharomyces pombe* (Yaffe et al., 1996) and *Neurospora crassa* (Steinberg and Schliwa, 1993), microtubules are not involved. Studies using cell-free systems revealed a mitochondrial actin-binding activity that is ATP-sensitive, saturable, reversible and mediated by proteins on the mitochondrial surface (Lazzarino et al., 1994; Simon et al., 1995). In the following we describe two models that have been put forward to explain mitochondrial motility in yeast: actin polymerization-driven movement of mitochondria requiring the Arp2/3 complex, and movement of mitochondria based on myosin-related motor proteins.

The Arp2/3 complex is the most important initiator of actin filament polymerization in the cell (Mullins and Pollard, 1999). This system is exploited by several intracellular bacterial pathogens that recruit the Arp2/3 complex to their rear end, where it nucleates actin filament polymerization. Growing actin filaments, which are visible as 'comet tail structures' in the light microscope, then drive vigorous intracellular movement of the pathogens (Stevens et al., 2006). As Arp2/3 complex subunits were found on the mitochondrial surface, it was proposed that yeast mitochondria move by a very similar actin polymerization-dependent mechanism (Boldogh et al., 2001). If this is the case, a specific mechanism must exist that recruits the Arp2/3 complex to the mitochondrial surface. Recent evidence suggests that targeting of the Arp2/3 complex to mitochondria might be supported by Jsn1 and Puf3, two members of the pumilio family of RNA-binding proteins. These proteins can be found on the mitochondrial surface, where they co-localize with the Arp2/3 complex (Fehrenbacher et al., 2005; Garcia-Rodriguez et al., 2007). Mutations of genes encoding Arp2/3 complex subunits or mitochondria-associated pumilio proteins reduce mitochondrial velocity, suggesting that the encoded proteins contribute to mitochondrial motility (Boldogh et al., 2001; Fehrenbacher et al., 2005; Garcia-Rodriguez et al., 2007). However, cells lacking Jsn1 or Puf3 do not show complete blockage of mitochondrial movement, and Puf3 levels are lower under conditions that stimulate mitochondrial biogenesis (Fehrenbacher et al., 2005; Garcia-Rodriguez et al., 2007). Thus, pumilio-related proteins do not appear to be essential parts of the mitochondrial motility machinery. In addition to their proposed Arp2/3-dependent activities (Fehrenbacher et al., 2005; Garcia-Rodriguez et al., 2007), they may modulate mitochondrial behavior by stimulating the turnover of cytosolic mRNAs encoding mitochondrial proteins (Gerber et al., 2004; Garcia-Rodriguez et al., 2007).

Myosin motor proteins of the class V subgroup are involved in many membrane trafficking events (Reck-Peterson et al., 2000). Yeast contains two class V myosins: Myo2, an essential protein involved in organelle transport, and Myo4, a non-essential protein involved in mRNA localization and movement of the endoplasmic reticulum (ER) (Pruyne et al., 2004). Cells carrying certain *myo2* mutant alleles show defects in mitochondrial distribution towards the bud, suggesting that Myo2 is crucial for polarized distribution of mitochondria (Itoh et al., 2002; Boldogh et al., 2004). A rab type GTPase, Ypt11, and a mitochondrial outer membrane protein, Mmr1, were found to interact with Myo2. In the absence of Ypt11 and Mmr1 mitochondrial transfer to the bud is impaired, suggesting that they cooperate with Myo2 in

mitochondrial motility (Itoh et al., 2002, 2004). The finding that depletion of Myo2 or its associated light chain, Mlc1, induces severe mitochondrial distribution and morphology defects further supports an important role of these proteins in mitochondrial motility (Altmann and Westermann, 2005). However, as mitochondrial morphology can be compromised by many different mutations that affect the actin cytoskeleton (Drubin et al., 1993; Hermann et al., 1997; Boldogh et al., 2001; Altmann and Westermann, 2005) it is difficult to rule out one of the proposed mechanisms. Clearly, more work is required to determine whether mitochondria are moved by myosin motor proteins, by actin-polymerization, or by a combination of both.

Conclusions

Research during the past 15 years has identified approximately two dozen proteins that constitute the molecular machineries determining mitochondrial behavior in yeast. In addition to the components described above, a number of proteins have been characterized that may act in novel morphology pathways: Gem1 is a rho GTPase in the mitochondrial outer membrane that carries two calcium-binding EF hand motifs (Frederick et al., 2004), and Mfb1 is a mitochondria-associated F-box protein that acts independently of Mdm30 (Dürr et al., 2006; Kondo-Okamoto et al., 2006). Both proteins are not essential for fusion, fission, tubulation or motility of mitochondria, but they appear to affect mitochondrial morphology as regulatory factors. It is evident that the biogenesis of mitochondrial membranes depends on the uptake of ER-synthesized lipids, import of nuclear-encoded proteins and turnover of mitochondrial proteins. Consequently, a large number of proteins involved in biogenesis of the ER and the vesicular trafficking system, biosynthesis of ergosterol, mitochondrial protein import and the ubiquitin 26S proteasome system are essential for mitochondrial morphogenesis (Altmann and Westermann, 2005). Interestingly, it has recently been reported that mitochondrial fission is involved in the regulation of programmed cell death in yeast (Fannjiang et al., 2004; Pozniakovsky et al., 2005), and a decrease in mitochondrial fission increases the life span of yeast (Scheckhuber et al., 2007). These findings suggest that yeast will be a valuable model organism for studying the role of mitochondrial dynamics in apoptosis and aging. Clearly, mitochondria interact in many ways with the rest of the cell. Thus, it is safe to predict that the coming years will see the identification of many more proteins contributing to the dynamic behavior and cellular functions of mitochondria. Their identification and functional characterization remain a challenge for the future.

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Erklärung

Hiermit versichere ich, die vorliegende Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt zu haben.
Ferner erkläre ich, dass ich weder an der Universität Bayreuth noch an einer anderen Hochschule versucht habe, eine Dissertation einzureichen, oder mich einer Promotionsprüfung zu unterziehen.

Katrin Altmann
Bayreuth, 24.09.2007