Mdm35p imports Ups proteins into the mitochondrial intermembrane space by functional complex formation

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Ups1p, Ups2p, and Ups3p are three homologous proteins that control phospholipid metabolism in the mitochondrial intermembrane space (IMS). The Ups proteins are atypical IMS proteins in that they lack the two major IMS-targeting signals, bipartite presequences and cysteine motifs. Here, we show that Ups protein import is mediated by another IMS protein, Mdm35p. *In vitro* import assays show that import of Ups proteins requires Mdm35p. Loss of Mdm35p led to a decrease in steady state levels of Ups proteins in mitochondria. In addition, *mdm35Δ* cells displayed a similar phenotype to *ups1Δups2Δups3Δ* cells. Interestingly, unlike typical import machineries, Mdm35p associated stably with Ups proteins at a steady state after import. Demonstrating that Mdm35p is a functional component of Ups–Mdm35p complexes, restoration of Ups protein levels in *mdm35Δ* mitochondria failed to restore phospholipid metabolism. These findings provide a novel mechanism in which the formation of functional protein complexes drives mitochondrial protein import.

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Introduction

The majority of mitochondrial proteins are encoded in the nuclear genome. Therefore, they are synthesized in the cytosol and imported into one of four compartments in mitochondria, namely the outer membrane (OM), the inner membrane (IM), the intermembrane space (IMS), and the matrix. Earlier studies have revealed elaborate molecular mechanisms by which precursor proteins are delivered to specific compartments (Dolezal *et al.*, 2006; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009; Endo and Yamano, 2009). For instance, translocase of the OM, TOM, mediates translocation of virtually all mitochondrial proteins across the OM. Two translocases of the IM, TIM23 and TIM22, exist

within the IM. TIM23, which recognizes matrix-targeting signals, mediates insertion of IM proteins and translocation of matrix proteins. In contrast, TIM22 inserts polytopic IM proteins lacking a matrix-targeting signal.

Two major IMS protein import mechanisms, the stop-transfer system (Glick *et al.*, 1992; Esaki *et al.*, 1999; Meier *et al.*, 2005) and the disulphide relay system (also called the mitochondrial IMS assembly (MIA) system), have been studied extensively (Bihlmaier *et al.*, 2007; Chacinska *et al.*, 2008; Mesecke *et al.*, 2008; Stojanovski *et al.*, 2008; Banci *et al.*, 2009; Kawano *et al.*, 2009; Terziyska *et al.*, 2009; Tienison *et al.*, 2009). Both systems function after precursor proteins across the OM through TOM. The stop-transfer system imports IMS proteins that contain a bipartite presequence consisting of matrix-targeting and sorting signals. Bipartite presequences are inserted into the IM through TIM23 and cleaved by IM peptidase or rhomboid protease, releasing mature proteins into the IMS (Esser *et al.*, 2002; Gakh *et al.*, 2002). On the other hand, the disulphide relay system works to import IMS proteins that contain a cysteine motif (Gabriel *et al.*, 2007; Longen *et al.*, 2009; Milenkovic *et al.*, 2009; Sideris *et al.*, 2009). These motifs can form transient disulphide bonds with cysteine residues in Mia40p in the IMS to facilitate protein folding and production of mature forms of these proteins. The majority of IMS proteins are imported by these pathways.

However, some IMS proteins do not possess typical targeting signals. For example, Ups1p, Ups2p, and Ups3p, three homologous, evolutionarily conserved IMS proteins in the yeast *Saccharomyces cerevisiae*, lack a bipartite presequence and cysteine motif (Sesaki *et al.*, 2006; Tamura *et al.*, 2009a). In the IMS, these proteins form separate complexes and control the metabolism of two phospholipids, cardiolipin (CL) and phosphatidylethanolamine (PE), in mitochondria (Sesaki *et al.*, 2006; Osman *et al.*, 2009; Tamura *et al.*, 2009a). The Ups1p complex is required for the maintenance of CL, and *ups1Δ* cells contain a reduced amount of this phospholipid. In contrast, the Ups2p complex regulates CL metabolism in a manner that opposes the Ups1p complex as loss of Ups2p restores CL levels in *ups1Δ* cells. In addition, the Ups2p complex maintains PE levels in mitochondria. The function of Ups3p in phospholipid metabolism is currently unknown.

In this report, by identifying and characterizing a component of the Ups protein complexes, we discovered a novel mechanism for the import of these proteins. We show that Mdm35p, another IMS protein, associated stably with Ups proteins as a functional component of each complex. In addition, Mdm35p also mediated the import of Ups proteins into the IMS. Our data suggest that Mdm35p functionally interacts with Ups proteins and that these interactions facilitate efficient import and accumulation of Ups proteins into the IMS. This represents a novel mode of mitochondrial protein import in which the formation of functional Ups–Mdm35p complexes drives translocation of Ups proteins into the IMS.

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Results

Mdm35p can be crosslinked to Ups proteins

To identify components of the Ups protein complexes, we used a chemical crosslinking approach. Mitochondria were isolated from cells expressing Ups1pMyc, Ups2pMyc, or Ups3pMyc and incubated with increasing amounts of a chemical crosslinker, disuccinimidyl glutarate (DSG). Immunoblotting using anti-Myc antibodies showed cross-linked products of 62 and 65 kDa for Ups1pMyc, 72 kDa for Ups2pMyc, and 61 kDa for Ups3pMyc (Figure 1A). The amount of crosslinking increased in proportion to the concentration of DSG. In contrast, crosslinked products were not observed in the absence of DSG. As Ups1pMyc, Ups2pMyc, and Ups3pMyc migrated at 54, 59, and 53 kDa, respectively, in the absence of DSG, the crosslinked products contained an additional protein(s) of 8–13 kDa. By searching the *Saccharomyces Genome Database*

(<http://www.yeastgenome.org>), we found 54 mitochondrial proteins within this range of molecular weight. Among them, Mdm35p, similar to Ups proteins, had been shown to be located in the IMS and important for phospholipid metabolism and mitochondrial morphology in genome-wide studies (Dimmer *et al*, 2002; Gabriel *et al*, 2007; Longen *et al*, 2009; Osman *et al*, 2009). This prompted us to test whether the crosslinked products contained Mdm35p. We tagged Mdm35p and Ups proteins with the FLAG and Myc epitopes, respectively, and performed similar crosslinking experiments. The sizes of all crosslinked products increased in Mdm35pFLAG-expressing mitochondria (Figure 1A). Furthermore, these crosslinked products in Mdm35pFLAG-expressing mitochondria were immunoprecipitated by anti-FLAG antibodies (Figure 1A). These data clearly show that Mdm35p was crosslinked to Ups proteins by DSG and suggest that Mdm35p and Ups proteins associate in mitochondria.

Figure 1 Mdm35p interacts with Ups proteins. (A) (Left panel) *MDM35* (–) and *MDM35FLAG* (+) mitochondria expressing Ups1pMyc, Ups2pMyc, or Ups3pMyc were incubated with increasing amounts of DSG. Proteins were analysed by immunoblotting using anti-Myc antibodies. Arrowheads show crosslinked products. (Right panel) After incubation with DSG, mitochondria were lysed and subjected to immunoprecipitation using anti-FLAG antibodies. (B) *MDM35* (–) and *MDM35FLAG* (+) mitochondria expressing Ups2pGFP and Ups3pMyc were solubilized with digitonin and subjected to immunoprecipitation using anti-FLAG M2-agarose. The mitochondrial lysate (Control), the flow-through fraction (FT), and the eluate (Elute) are shown. Proteins were analysed by immunoblotting using the indicated antibodies. An asterisk indicates a non-specific band. (C) *MDM35FLAG* mitochondria expressing Ups2pGFP and Ups3pMyc were solubilized and analysed by glycerol density gradient centrifugation in the presence (+ FLAG) or absence (–FLAG) of anti-FLAG antibodies. (D) GST and GST-Mdm35p were incubated with radiolabelled Ups proteins and pelleted with glutathione-agarose beads; 10% of the input (control) and 100% of the bound fraction (GST and GST-Mdm35p) were resolved by SDS-PAGE, and analysed by coomassie brilliant blue staining (left panel) and autoradiography (right panel).

Mdm35p forms protein complexes with Ups proteins

To directly test whether Mdm35p and Ups proteins physically interact, we performed co-immunoprecipitation studies. Isolated mitochondria containing Mdm35pFLAG, Ups2pGFP, and Ups3pMyc were solubilized in the presence of 1.0% digitonin and incubated with anti-FLAG-agarose beads. We found that Ups proteins co-precipitated with Mdm35pFLAG (Figure 1B). In contrast, other mitochondrial proteins such as the OM protein Tom40p and the IM protein Tim23p did not co-precipitate with Mdm35pFLAG (Figure 1B). These results indicate that Mdm35p interacts specifically with Ups1p, Ups2p, and Ups3p.

As an independent confirmation of Ups–Mdm35p interaction, mitochondria expressing Mdm35pFLAG, Ups2pGFP, and Ups3pMyc were solubilized with digitonin and subjected to glycerol density gradient centrifugation. Consistent with an earlier publication (Tamura *et al*, 2009a), Ups1p and Ups3pMyc formed ~60-kDa complexes, whereas Ups2pGFP was found in ~100-kDa complexes (Figure 1C). The majority of Mdm35pFLAG co-migrated with Ups2p complexes with peaks at fractions six to eight (Figure 1C). A small fraction of Mdm35pFLAG also co-migrated with complexes containing Ups1p or Ups3p. To confirm that the Ups protein complexes contained Mdm35pFLAG, density gradient centrifugation was performed in the presence of anti-FLAG antibodies. Virtually, all the Ups protein complexes shifted to higher molecular weight fractions with the addition of anti-FLAG antibodies (Figure 1C). As a negative control, Tom40p was also examined; this protein was found to be unaffected. These results clearly show that Ups1p, Ups2p, and Ups3p form stable protein complexes with Mdm35p.

We also investigated whether Ups proteins interact directly with Mdm35p. We purified glutathione S-transferase (GST)-fused Mdm35p from *Escherichia coli* and performed pull down assays. Our data clearly show that *in vitro* translated, radiolabelled Ups protein binds GST-Mdm35p, but not GST alone, efficiently (Figure 1D). The control protein, pSu9-DHFR, did not bind GST-Mdm35p or GST. Finally, we examined the sizes of the protein complexes formed by recombinant Mdm35p and *in vitro* translated Ups proteins using glycerol density gradient centrifugation. In the absence of Mdm35p, Ups proteins were found in the heavier fractions, suggesting that they remain associated with components of the translation machinery or chaperones (Supplementary Figure S1). In contrast, when Mdm35p was added, a fraction of Ups proteins showed migration patterns similar to endogenous Mdm35p–Ups complexes. Our data suggest that Mdm35p and Ups proteins are the primary components of the Mdm35p–Ups complexes in mitochondria.

Deletion of MDM35 rescues defects in cell growth, mitochondrial protein import, and CL abundance in *ups1Δ* cells

The *ups1Δ* cells show defects in cell growth, CL metabolism, and protein import into the matrix (Sesaki *et al*, 2006; Tamura *et al*, 2009a). Additional loss of Ups2p can rescue these phenotypes in *ups1Δups2Δ* cells (Tamura *et al*, 2009a). As *ups2Δ* and *mdm35Δ* cells are similar phenotypically, showing a reduction in mitochondrial PE levels (Osman *et al*, 2009; Tamura *et al*, 2009a), we tested whether loss of Mdm35p could also rescue the *ups1Δ* phenotypes. We deleted the *MDM35* gene in combination with *UPS1* and/or *UPS2*, and

analysed cell growth, two matrix-targeting proteins Mdj1p and Hsp60p, and mitochondrial phospholipid compositions in the resultant mutants. As expected, *ups1Δ* cells showed reduced cell growth on YPD (Figure 2A). Strikingly, the deletion of *MDM35*, similar to the deletion of *UPS2*, rescued the growth defects of *ups1Δ* cells (Figure 2A). Although an earlier study reported that *mdm35Δ* cells show a reduced growth rate (Longen *et al*, 2009), we found that *mdm35Δ* cells grew normally on both fermentable (e.g. YPD) and non-fermentable (e.g. YPGE) carbon sources (Figure 2A). Similar to cell growth, accumulation of uncleaved precursor forms of Mdj1p and Hsp60p in *ups1Δ* cells was also rescued by additional deletion of *MDM35* or *UPS2* (Figure 2B). Finally, reduced levels of CL in *ups1Δ* cells were restored in *ups1Δmdm35Δ* and *ups1Δups2Δ* cells to similar extents (Figure 2C). These results clearly show that loss of Mdm35p rescues the phenotypes associated with loss of Ups1p.

Loss of Mdm35p leads to a decrease in the steady state levels of Ups proteins

To elucidate a mechanism for the suppression of the *ups1Δ* phenotypes with loss of Mdm35p, we examined the steady state levels of Ups2p. If Mdm35p is required for the maintenance of Ups2p levels, then loss of Mdm35p would lead to a reduction in Ups2p, thereby rescuing the *ups1Δ* phenotypes. Immunoblotting of whole cell extracts using wild-type and *mdm35Δ* cells expressing Ups1pFLAG, Ups2pFLAG, or Ups3pFLAG revealed that the levels of all three Ups proteins decreased with *MDM35* deletion (Figure 3A). We also found decreased levels of Ups proteins in mitochondria isolated from *mdm35Δ* cells (Figure 3B). When we examined another IMS protein, amounts of Tim10p were reduced slightly in *mdm35Δ* mitochondria. The OM proteins, Tom40p and Tom22p, and the IM protein Tim23p were unaffected by the loss of Mdm35p. These results show that Mdm35p is required for the maintenance of the steady state levels of Ups proteins and provide an explanation for how the *ups1Δ* phenotypes are rescued by additional loss of Mdm35p.

Our data also suggest that *mdm35Δ* cells mimic the phenotype of *ups1Δups2Δups3Δ* cells. Supporting this notion, both *mdm35Δ* and *ups1Δups2Δups3Δ* cells displayed normal cell growth, cardiolipin levels, and mitochondrial protein import into the matrix along with reduced levels of PE in mitochondria (Figure 2; Tamura *et al*, 2009a). Furthermore, a similar mitochondrial morphology was observed in *mdm35Δ* and *ups1Δups2Δups3Δ* cells (Figure 3C and D).

Mdm35p facilitates the import of Ups proteins into the IMS

To determine how the loss of Mdm35p leads to decreased levels of Ups proteins, we tested whether Mdm35p mediates Ups protein import into mitochondria in *in vitro* import assays. As *mdm35Δ* mitochondria contained decreased amounts of Tim10p, an IMS chaperone involved in protein import, we used mitochondria isolated from wild-type and Mdm35p-depleted cells (Figure 4A–C). To deplete Mdm35p, the glucose-repressible, galactose-inducible *GAL1* promoter was inserted in front of the open reading frame of *MDM35FLAG* and cells were grown in glucose-containing media. Immunoblotting confirmed the decrease

Figure 2 Loss of Mdm35p rescues defects in cell growth, mitochondrial protein import, and cardiolipin abundance in *ups1Δ* cells. (A) Serial dilutions of yeast cells were spotted onto YPD and YPGE plates and grown at 30°C for 2 and 4 days, respectively. (B) Whole cell extracts prepared from wild-type (*MDM35*) and *mdm35Δ* cells lacking *UPS1* (1Δ) and/or *UPS2* (2Δ) were analysed by immunoblotting (p, precursor form; m, mature form). (C) Analysis of mitochondrial phospholipids. Cells were grown in YPLac containing 0.4% sucrose in the presence of ³²Pi. Phospholipids were extracted from crude mitochondrial fractions and separated on thin-layer chromatography. CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine. Amounts of each lipid relative to total phospholipids were determined, and those detected in wild-type mitochondria were set to 100%. Values are mean ± s.e.m. (n = 3).

in Mdm35pFLAG in the Mdm35p-depleted mitochondria (Figure 4A, Mdm35↓). In contrast, the IMS protein Cyb2p and the IM protein Cyt1p, as well as components of mitochondrial import machineries such as Tim23p and Tim44p (TIM23), Tim22p and Tim18p (TIM22), Tim9p and Tim10p (IMS chaperones), Mia40p (MIA), Sam50p (SAM), and Tom40p (TOM) remained unaffected in the Mdm35p-depleted mitochondria. In addition, we found normal levels of PE in Mdm35p-depleted mitochondria (Figure 4B).

The isolated Mdm35p-depleted mitochondria were incubated with ³⁵S-labelled Ups proteins and treated with proteinase K to remove non-imported proteins. Remarkably, we found that import of Ups1p, Ups2p, and Ups3p was impaired in the Mdm35p-depleted mitochondria (Figure 4C). The effect of Mdm35p depletion varied among the Ups proteins. In particular, import of Ups1p, Ups2p, and Ups3p were decreased by ~35, ~65, and ~35%, respectively. These data indicate that Mdm35p facilitates the import of Ups proteins into mitochondria. These findings were unexpected as earlier studies had shown that mitochondrial protein import is, in principle, catalysed by transient protein–protein interactions and that imported proteins dissociate from their import machineries. However, our data suggest that Ups proteins are imported by Mdm35p and stably associate with Mdm35p after import.

As controls, we examined other proteins whose import is mediated by known mechanisms, namely Hsp60p (the TIM23 pathway), AAC (the TIM22 pathway and IMS chaperones), Tom40p (the SAM pathway and IMS chaperones) and Tim9p (the MIA pathway), and found their import to be normal in Mdm35p-depleted mitochondria (Figure 4C). In addition, cytochrome *c* heme lyase (CCHL), an IMS protein whose import mechanism is largely unknown (Steiner *et al*, 1995), is imported similarly in wild-type and Mdm35p-depleted mitochondria. These results show that Mdm35p specifically facilitates import of Ups proteins.

Furthermore, we examined the effect of Mdm35p overexpression on Ups protein import. Mitochondria were isolated from cells overexpressing Mdm35p from the galactose-inducible *GAL1* promoter. With this, Mdm35p expression was increased approximately four-fold (Figure 4D). We found that Ups proteins were similarly imported into wild-type and Mdm35p-overexpressing mitochondria (Figure 4D). Our data show that increasing Mdm35p levels does not enhance Ups protein import.

Ups proteins cross the OM through TOM

Next, we tested whether Ups proteins cross the OM through TOM. *In vitro* import assays were performed using mitochondria isolated from wild-type cells and temperature-sensitive

Figure 3 *mdm35Δ* mitochondria contain decreased amounts of Ups proteins. (A) Whole cell extracts prepared from wild-type (+) and *mdm35Δ* (–) cells expressing Ups1pFLAG, Ups2pFLAG, or Ups3pFLAG were subjected to immunoblotting using the indicated antibodies. (B) Isolated mitochondria were analysed by immunoblotting. Interestingly, Ups2p exhibited a doublet in *mdm35Δ* mitochondria. As shown in Supplementary Figure S4, this was independent of phosphorylation or an altered redox state of cysteine residues. (C) Mitochondria were visualized in wild-type, *mdm35Δ*, and *ups1Δups2Δups3Δ* cells using matrix-targeted Su9-GFP (Sesaki *et al*, 2006). Cells were grown to log phase in YPD, fixed in 3.7% formaldehyde, and then examined by differential interference contrast (DIC) and fluorescence (Su9-GFP) microscopy. Bars, 3 μm. (D) Quantitation of mitochondrial morphology. Cells containing tubular mitochondria were scored. Values are mean ± s.e.m. (*n* = 3). At least 300 cells were examined in each experiment.

tom40-4 mutants (Krimmer *et al*, 2001). We found that import of Ups proteins was inhibited in *tom40-4* mitochondria (Figure 5A), suggesting that TOM is required for import of Ups proteins.

Then, we sought to determine the order of events during Ups proteins import. If Ups proteins move through TOM and then interact with Mdm35p, then Ups proteins would be arrested at TOM in the absence of Mdm35p. To test this

Figure 4 Mdm35p facilitates efficient import of Ups proteins into mitochondria. **(A)** Cells carrying *MDM35FLAG* (WT) or *GAL1-MDM35FLAG* (Mdm35 Δ) were grown in YPLac containing 0.4% glucose for 12 h. Mitochondria were isolated from these cells and analysed by immunoblotting using the indicated antibodies. **(B)** Phospholipids were extracted from wild-type, Mdm35 Δ , and *psd1 Δ* mitochondria, and separated on thin-layer chromatography. Phospholipids were visualized by molybdenum blue (Sigma). *psd1 Δ* mitochondria were used as a control. Psd1p is a mitochondrial phosphatidylserine decarboxylase that is required for the production of PE in this organelle (Clancey *et al*, 1993). **(C)** Mitochondria were incubated with radiolabelled precursors for the indicated times then treated with proteinase K followed by SDS-PAGE and autoradiography. Import into wild-type mitochondria after 10 min was set as 100%. C, control (10% of precursor proteins). A bar graph shows amounts of imported proteins at 4 min. Values are mean $\pm$ s.e.m. ($n = 3$). **(D)** *In vitro* import assays using wild-type and Mdm35p-overexpressing mitochondria were performed and analysed as described in **(C)**. Immunoblotting of mitochondria using the indicated antibodies are shown.

model, we incubated 35 S-labelled Ups2p with wild-type, *MDM35FLAG*, and *mdm35 Δ* mitochondria. After removing unbound Ups2p by washing, mitochondria were incubated with the chemical crosslinker DSG. Consistent with our earlier data (Figure 1A), we found that newly imported Ups2p was crosslinked to Mdm35p (Figure 5B, lane 2; arrowhead) and Mdm35pFLAG (Figure 5B, lane 4; arrowhead). More importantly, a crosslinked product of ~ 46 kDa was detected in *mdm35 Δ* , but not wild-type, mitochondria. (Figure 5B,

lane 6; closed circle). As Ups2p migrates at 24 kDa, the crosslinked product contains an ~ 22 -kDa protein. We suspected that Ups2p was crosslinked to receptors, Tom20p or Tom22p, in TOM. Indeed, the crosslinked products were immunoprecipitated by antibodies against Tom20p (Figure 5C, lane 3) and Tom22p (Figure 5C, lane 4). Taken together, our data strongly suggest that, during import, Ups proteins are recruited to the Tom20p and Tom22p receptors on the surface of mitochondria, translocated across the OM

Figure 5 Ups protein import requires TOM. (A) After incubation at 37°C for 10 min, wild-type and *tom40-4* mitochondria were mixed with radiolabelled Ups proteins for the indicated times then treated with proteinase K followed by SDS-PAGE and autoradiography. Import into wild-type mitochondria after 10 min was set as 100%. C, control (10% of precursor proteins). A bar graph shows amounts of imported proteins at 4 min. Values are mean $\pm$ s.e.m. ($n = 3$). (B) Radiolabelled Ups2p was added to MDM35 (WT), MDM35FLAG, and *mdm35Δ* mitochondria. After incubation in the presence (+) or absence (–) of 300 μ M DSG, mitochondria were subjected to SDS-PAGE and autoradiography. (C) *mdm35Δ* mitochondria were incubated with radiolabelled Ups2p and treated with 300 μ M DSG. Mitochondria were solubilized and subjected immunoprecipitation using the indicated antibodies. The immunoprecipitates were analysed by SDS-PAGE and autoradiography.

through the TOM channel, and then captured by Mdm35p in the IMS. In addition to the TOM receptors, Ups2p was also crosslinked to ~30-kDa protein only in the presence of Mdm35p (Figure 5B, lanes 2, 4, and 6), further suggesting a function of Mdm35p in the biogenesis of Ups proteins.

Ups protein import does not require the TIM23 and MIA pathways

We found that Ups protein import does not involve the TIM23 and MIA mechanisms or IMS chaperones. First, consistent with the lack of a targeting signal recognizable by TIM23 (e.g. bipartite presequences), Ups proteins are normally imported into temperature-sensitive *tim50-279*, *282*, *286* (*tim50-ts*) mitochondria (Figure 6A), which are defective in the TIM23 pathway (Tamura *et al*, 2009b). In contrast, the import of pSu9-DHFR, a presequence-containing protein, was inhibited strongly in *tim50-ts* mitochondria (Figure 6A).

Second, the MIA pathway recognizes cysteine motifs that consist of four cysteines. Ups1p and Ups3p have none and only two cysteines, respectively. Although Ups2p contains four cysteines, they appear not to form a cysteine motif. To confirm that the four cysteines in Ups2p are not required for its import, we substituted all of them with serines (cys-null Ups2p). Cys-null Ups2p did not display defective import *in vitro* (Figure 6B) and restored normal PE levels in *ups2Δ* cells (Figure 6C).

Finally, the import of Ups proteins does not require the IMS-located chaperones, Tim10p, Tim8p, and Tim13p, which mediate protein import and assembly into the OM and IM (Koehler *et al*, 1998; Paschen *et al*, 2000; Wiedemann *et al*, 2004). Tim10p-depleted mitochondria normally imported Ups proteins (Figure 6D). In contrast, import of AAC, a known substrate for Tim10p, was inhibited in Tim10p-depleted mitochondria (Figure 6D). Similarly, *tim8Δtim13Δ* mitochondria also imported Ups proteins at normal rates (Figure 6E).

Mdm35p is not required for the maintenance of Ups proteins in mitochondria

In addition to Ups protein import, Mdm35p may be critical for maintenance of these proteins in mitochondria. It is possible that in the absence of Mdm35p, Ups proteins are released from mitochondria or undergo degradation after import. To test this hypothesis, we expressed Ups2p from the inducible *GAL1* promoter in wild-type and *mdm35Δ* cells and isolated mitochondria. On induction, Ups2p accumulated to a similar extent in wild-type and *mdm35Δ* mitochondria (Supplementary Figure S2A). This accumulation is consistent with our observations that Ups proteins can be imported into mitochondria in *mdm35Δ* cells at reduced levels (Figure 3B). We then investigated whether Ups2p is released from mitochondria. We found that the majority of Ups2p were not

Figure 6 Ups protein import does not require TIM23 and MIA. (A) *In vitro* import assays were performed using wild-type and *tim50-ts* mitochondria. m, mature form. Import into wild-type mitochondria after 10 min was set as 100%. A bar graph shows amounts of imported proteins at 4 min. Values are mean $\pm$ s.e.m. ($n = 3$). (B) Wild-type and cys-null Ups2p were imported into wild-type mitochondria *in vitro*. Amounts relative to the input are determined. The input is set as 100%. (C) *ups2Δ* cells carrying a control vector, wild-type *UPS2FLAG*, and cysteine-null *UPS2FLAG* were grown in SCD-Trp in the presence of 32 Pi. Mitochondrial phospholipids were analysed on thin-layer chromatography (upper left panel) and quantitated as described in Figure 2C. Whole cell extracts were subjected to immunoblotting using antibodies to FLAG and Tim23p (lower left panels). (D, E) *In vitro* import assays were performed using wild-type and Tim10p-depleted mitochondria (D), and wild-type and *tim8Δtim13Δ* mitochondria (E). Import into wild-type mitochondria after 10 min was set as 100%. C, control (10% of precursor proteins). Bar graphs show amounts of imported proteins at 4 min. Values are mean $\pm$ s.e.m. ($n = 3$).

released. Furthermore, degradation of Ups2p proteins was negligible in these mitochondria. These results indicate that Mdm35p is not involved in the maintenance of Ups proteins in mitochondria. Further supporting this conclusion, the stability of newly imported Ups2p was not decreased in *mdm35Δ* mitochondria (Supplementary Figure S2B).

Both Mdm35p and Ups2p are required for the maintenance of PE levels in mitochondria

Stable interactions between Mdm35p and Ups proteins at a steady state suggest that the function of Mdm35p is not solely for Ups protein import. To test this idea, we expressed Ups2p from the inducible *GAL1* promoter in wild-type and *mdm35Δ* cells, and examined mitochondrial PE levels. We found that

Figure 7 Mdm35p and Ups2p are required for phospholipid metabolism. (A) *UPS2FLAG* (WT Ups2p), *GAL1-UPS2FLAG* (WT Ups2p↑), and *mdm35ΔGAL1-UPS2FLAG* (*mdm35Δ* Ups2p↑) cells were grown in YPLac containing 0.4% galactose in the presence of ^{32}P i. Phospholipids were extracted from crude mitochondrial fractions and separated on thin-layer chromatography (left panel). Amounts PE relative to total phospholipids were determined, and those detected in wild-type (WT) mitochondria were set to 100%. Values are mean $\pm$ s.e.m. ($n = 3$). Expression of Ups2pFLAG was confirmed by immunoblotting of isolated mitochondria, with (+) and without (–) proteinase K treatments (PK) (upper right panels). The OM protein Tom22p and the IM protein Tim50p are shown as controls. (B) Wild-type, *ups1Δ* and *ups2Δ* cells carrying *MDM35FLAG* (Mdm35p) or *GAL1-MDM35FLAG* (Mdm35p↑) were grown in YPLac containing 0.4% galactose in the presence of ^{32}P i. Phospholipids were extracted from crude mitochondrial fractions and separated on thin-layer chromatography (upper left panel). Amounts of CL and PE were quantitated as described in (A). Values are mean $\pm$ s.e.m. ($n = 3$). Overexpression of *MDM35FLAG* was confirmed by immunoblotting of whole cell extracts (lower left panels). Tom40p is shown as a control. (C) A model for Ups protein import.

restoration of Ups2p levels in mitochondria was unable to restore PE to normal levels in *mdm35Δ* mitochondria (Figure 7A). These results indicate that, in addition to Ups2p import, Mdm35p likely has a function in PE metabolism. Similarly, Mdm35p overexpression did not restore CL and PE to normal levels in *ups1Δ* and *ups2Δ* cells, respectively (Figure 7B). Therefore, Mdm35p is unable to substitute for Ups1p and Ups2p. Taken together, our data suggest that both Mdm35p and Ups proteins are functionally important for phospholipid metabolism in mitochondria.

Discussion

Earlier genome-wide studies have shown that Mdm35p, similar to Ups proteins, is located in the IMS and required

for mitochondrial shape and PE metabolism (Dimmer *et al*, 2002; Gabriel *et al*, 2007; Longen *et al*, 2009; Osman *et al*, 2009). However, the function of Mdm35p is poorly understood. In this study, we found that Mdm35p directly interacts with Ups proteins. In mitochondria, Ups1p, Ups2p, and Ups3p form separate protein complexes (Tamura *et al*, 2009a), each of which contains Mdm35p. The Ups–Mdm35p complexes are functionally important for phospholipid metabolism. We conclude that the formation of the functional protein complexes drives the import of Ups proteins into the IMS.

We propose a model for Mdm35p-mediated Ups protein import (Figure 7C). During import, Ups proteins are first recognized and recruited by the two TOM receptors on the surface of mitochondria, Tom20p and Tom22p.

These receptors guide Ups proteins to the TOM channel. Then, Mdm35p ensures that Ups proteins move forwards through the TOM channel as a receptor by directly binding to incoming Ups proteins in the IMS. Association with Mdm35p could prevent Ups proteins from moving backwards, thereby facilitating unidirectional movement. In support of this function, Mdm35p deletion led to Ups protein arrest at the TOM receptors and failure to stably pass through the TOM channel (Figure 5C). In addition, Mdm35p may function as a chaperone to assist in the folding of Ups proteins during import. Moreover, the Mdm35p-mediated pathway is likely to be specific to Ups protein import as Mdm35p is not required for the import of all the other precursor proteins examined, including Tim9p (a cysteine-motif-containing IMS protein), CCHL (an IMS protein), Hsp60p (a presequence-containing matrix protein), AAC (a polytopic IM protein), and Tom40 (an OM protein) (Figure 5B). Finally, our model predicts that Mdm35p is more abundant than Ups proteins. Supporting this prediction, we found that the amount of Mdm35p exceeds the total amount of Ups proteins (Supplementary Figure S3).

Mdm35p facilitates efficient import of Ups proteins into the IMS, but is not an absolute requirement for this process. Supporting this notion, we observed a small fraction of Ups proteins in *mdm35Δ* mitochondria (Figure 3B). In addition, our *in vitro* import assays show that, albeit at reduced rates, Ups proteins can be imported into Mdm35p-depleted (Figure 4C) and *mdm35Δ* mitochondria (data not shown). These results suggest a functionally overlapping targeting mechanism for Ups proteins to the IMS. Once they are targeted to the IMS, their intrinsic properties may allow them to fold spontaneously to prevent Ups protein release from the compartment. Moreover, we tested whether Ups protein import requires Emi1p, a cysteine-motif-containing protein that is structurally related to Mdm35p (Longen *et al*, 2009). However, we found that Emi1p does not interact with Ups proteins and that Ups protein levels were unaffected in *emi1Δ* mitochondria (Supplementary Figure S5). These results suggest that a redundant mechanism involving Emi1p is unlikely.

The Mdm35p-mediated mechanism represents a new pathway for protein import into the IMS. Earlier proteomic studies have identified ~1000 proteins in yeast mitochondria (Sickmann *et al*, 2003). According to the Saccharomyces Genome Database, ~40 proteins are known to be located in the IMS (Supplementary Table S1). Most of them are imported by either the TIM23 (10 proteins) or MIA pathway (20 proteins) (Supplementary Table S1). However, Ups protein import is independent of both these pathways. First, Ups proteins lack a bipartite presequence recognizable by the TIM23 mechanisms. Second, we show that Ups protein import is normal in *tim50-ts* mitochondria (Figure 6A), which are defective in the TIM23 pathway (Tamura *et al*, 2009b). Third, the membrane potential across the IM, which is essential for TIM23-mediated protein import, was also not required for Ups protein import (data not shown). Finally, cysteine motifs for the MIA pathway consist of four cysteine residues; however, Ups1p and Ups3p contain none and two cysteines, respectively. Furthermore, serine substitution of the four cysteines in Ups2p did not affect its import and function (Figure 6B and C).

There are a few examples of IMS proteins that are imported into the compartment independent of TIM23 and MIA (Supplementary Table S1). Their unconventional import mechanisms are also different from those for Ups proteins. For example, during import, apocytochrome *c* is trapped by CCHL in the IMS (Dumont *et al*, 1991; Mayer *et al*, 1995). CCHL then conjugates heme to apocytochrome *c* and releases mature holocytochrome *c* into the IMS. After conjugation, CCHL is no longer required for the localization or function of holocytochrome *c*. Interestingly, import of CCHL itself is also mediated by an unconventional mechanism. CCHL contains atypical targeting sequence in the third quarter of the molecule (Diekert *et al*, 1999). It has been proposed that high-affinity interactions of CCHL with the IMS side of TOM drive import of this protein into the IMS (Diekert *et al*, 1999). In addition, Sod1p is trapped and folded by Ccs1p in the IMS through the formation of covalent, disulphide bonds between Sod1p and Ccs1p (Reddehase *et al*, 2009). After completion of import and folding, mature Sod1p dissociates from Ccs1p. Finally, there are other IMS proteins whose import mechanisms are largely unknown, including Rib3p (Jin *et al*, 2003) and two kinases, Adk1p and Ynk1p (Schricker *et al*, 2002; Amutha and Pain, 2003). It would be interesting to test whether these proteins are imported by stable associations with their functional partners, similar to the Ups–Mdm35p interactions described here.

Most mitochondrial precursor proteins are imported by transient interactions with import machineries (Dolezal *et al*, 2006; Neupert and Herrmann, 2007; Chacinska *et al*, 2009; Endo and Yamano, 2009). For example, IMS proteins are mediated by transient interaction with TOM, TIM23, MIA, CCHL, or Ccs1p. Once delivered to the IMS, the imported proteins no longer associate with these import machineries. Similarly, short-lived protein–protein interactions mediate import of other mitochondrial proteins that are targeted to the OM, IM, and matrix. In contrast, Mdm35p-mediated Ups protein import is driven mainly by the formation of stable, functional protein complexes. This mechanism likely allows an efficient coupling between protein import and assembly of protein complexes.

In the Ups–Mdm35p complexes, Mdm35p and Ups proteins are functional components. Supporting this conclusion, restoration of Ups2p levels failed to restore mitochondrial PE to normal levels in *mdm35Δ* cells (Figure 7A). Similarly, Mdm35p overexpression did not rescue decreased levels of CL and PE in *ups1Δ* and *ups2Δ* cells, respectively (Figure 7B). This conclusion also accounts for the observation that the phenotype of *mdm35Δ* cells is similar to *ups1Δups2Δups3Δ* cells. Without Mdm35p, the Ups protein complexes lose their biological function.

Our findings also suggest that Mdm35p has a function in CL metabolism. Mdm35p interacts with Ups1p and Ups2p, which regulate CL metabolism in an opposing manner (Tamura *et al*, 2009a). Therefore, Mdm35p likely functions in CL metabolism in positive and negative ways as a functional component of Ups1p–Mdm35p and Ups2p–Mdm35p complexes, respectively. However, in contrast to *ups1Δ* cells, which contain lower levels of CL in mitochondria, *mdm35Δ* cells have normal levels of CL. The requirement for Mdm35p is probably masked in *mdm35Δ* cells by simultaneous loss of Ups1p–Mdm35p and Ups2p–Mdm35p complex function. Several functions have been suggested for Ups proteins

(Sesaki *et al*, 2006; Gohil and Greenberg, 2009; Osman *et al*, 2009; Tamura *et al*, 2009a), including regulation of the catalytic activity of the enzymes involved in CL biosynthesis such as Crd1p, phospholipid-transfer between mitochondria and the ER, transport of phospholipids in mitochondria, and stability of phospholipids in the organelle. It would be of great interest to understand the exact functions of each Ups-Mdm35p complex in future studies.

Materials and methods

Strains, plasmids, media, and genetic methods

Yeast strains, plasmids, and primers used in this study are summarized in Supplementary data (Supplementary Tables S2, S3, and S4). Complete disruptions of the *UPS1*, *UPS2*, *MDM35*, and *PSD1* genes were constructed by PCR-mediated gene replacement (Brachmann *et al*, 1998). The FLAG-, Myc-, and GFP-tags were inserted proximal to the stop codon of *UPS1*, *UPS2*, *UPS3*, and *MDM35* by homologous recombination using appropriate gene cassettes from p3FLAG-KanMX (Gelbart *et al*, 2001), pYTE247 (Yoshihisa *et al*, 2003), pFA6a-FLAG-kanMX6 (see below), pFA6a-13Myc-His3MX6, pFA6a-13Myc-His3MX6, and pFA6a-GFP(S65T)-TRP1 (Longtine *et al*, 1998). The endogenous promoter of *MDM35* was replaced by the *GAL1* promoter using pFA6a-His3MX6-PGAL1 (Longtine *et al*, 1998). For *in vitro* translation, the *UPS1*, *UPS2*, *UPS3*, *CCHL*, and *TOM40* genes were amplified by PCR and cloned into pSP65 vector (Promega). Introduction of the point mutations into the *UPS2* gene was performed by the overlap extension method using two pairs of primers (Sambrook and Russell, 2001). As template for the PCR, the plasmid pRS314-Ups2FLAG was used. For expression of GST-fused Mdm35p in *E. coli* cells, a DNA fragment coding the *MDM35* gene was inserted into pGEX-2T vector (GE healthcare). To construct pFA6a-FLAG-kanMX6, a DNA fragment coding three copies of the FLAG epitope sequence followed by a termination codon, *ADH1* terminator, and *kanMX6* was amplified by PCR and inserted PacI/PmeI sites in pFA6a-3HA-kanMX6 (Longtine *et al*, 1998). Cells were grown in YPD (1% yeast extract, 2% polypeptone, and 2% glucose), YPGA (1% yeast extract, 2% polypeptone, 2% glycerol, and 3.2% ethanol), SCD (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid, and 2% glucose), and YPLac (1% yeast extract, 2% polypeptone, and 3% lactic acid, pH 5.6). Standard genetic techniques were used (Adams *et al*, 1997).

Chemical crosslinking

Mitochondria were incubated with the chemical crosslinker DSG in SEM buffer (250 mM sucrose; 1 mM EDTA; and 10 mM MOPS-KOH, pH 7.2) for 30 min. The crosslinking reaction was stopped by adding Tris-HCl pH 7.5 to final concentration of 50 mM. Mitochondria were washed once with SEM buffer and analysed by SDS-PAGE and immunoblotting. For immunoblotting, proteins were visualized by fluorophores conjugated with secondary antibodies (ZyMax Goat Anti-Rabbit IgG (H + L) Cy 5 conjugate and/or Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen)) and analysed using a PharosFX Plus Molecular Imager (Bio-Rad), and Quantity One (Bio-Rad) and Photoshop (Adobe) software.

Co-immunoprecipitation

A total of 500 µg of mitochondria were solubilized at 2 mg protein/ml in digitonin buffer (1% digitonin; 20 mM Tris-HCl, pH 7.5; 50 mM NaCl; 10% (v/v) glycerol; 0.1 mM EDTA; and 1 mM phenylmethylsulfonylfluoride (PMSF)). After centrifugation at 16100 g for 15 min, 200 µl of the supernatant was diluted with 800 µl co-ip buffer (20 mM Tris-HCl, pH 7.5; 50 mM NaCl; 10% (v/v) glycerol; 0.1 mM EDTA) containing 10 µl of anti-FLAG M2-agarose (Sigma). The samples were gently rotated for 2 h at 4°C. The agarose resin was washed three times with 1 ml of wash buffer (0.05% digitonin; 20 mM Tris-HCl, pH 7.4; 50 mM NaCl; 0.1 mM EDTA; 10% glycerol), and then the bound proteins were eluted by boiling in SDS-PAGE sample buffer. The eluted proteins were analysed by SDS-PAGE and immunoblotting.

Density gradient centrifugation

Mitochondria were solubilized at 2 mg protein/ml in digitonin buffer for 20 min on ice, and then centrifuged at 16100 g for 15 min. The supernatant (200 µl) was placed onto 5 ml glycerol gradient (20–40%) in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM 6-aminohexanoic acid, 0.1 mM EDTA, 0.1% digitonin, and protease inhibitor cocktail (Sigma), and then centrifuged at 45000 r.p.m. for 15 h in a Beckman SW55Ti rotor at 4°C (Tamura *et al*, 2009a). After centrifugation, 270 µl fractions were collected from the top. Proteins were precipitated with 10% TCA, then analysed by SDS-PAGE and immunoblotting.

Pull down assay

Ups1p, Ups2p, or Ups3p was synthesized *in vitro* in the presence of ³⁵S-methionine and incubated with ~20 µg of GST or GST-Mdm35p in PBS (1.86 mM NaH₂PO₄; 8.39 mM Na₂HPO₄; and 150 mM NaCl, pH 7.5) containing 10% glycerol at 4°C for 1 h. The samples were further incubated with 10 µl of glutathione-agarose beads (Sigma) at 4°C for 1 h. The beads were washed four times with 1 ml of 0.5% Triton buffer (0.5% Triton X-100; 20 mM MOPS-KOH, pH 7.2; 150 mM NaCl; and 10% glycerol). The bound proteins were eluted by boiling in SDS sample buffer and analysed by SDS-PAGE and autoradiography using a PharosFX Plus Molecular Imager (Bio-Rad) and Quantity One (Bio-Rad) and Photoshop (Adobe) software.

Microscopy

Cells were viewed on an Olympus BX61 upright microscope equipped with Olympus UIS2 100 × /1.3 objective. Fluorescence and differential interference contrast images were captured with a Roper Photometrics CoolSnap HQ CCD camera using SlideBook (3i) and processed with Photoshop (Adobe).

Phospholipid analysis

Yeast cells were grown in the presence of 10 µCi/ml ³²Pi (Tamura *et al*, 2009a). Phospholipids were extracted from crude mitochondrial fractions and separated by thin-layer chromatography as earlier described (Vaden *et al*, 2005; Claypool *et al*, 2006; Tamura *et al*, 2009a).

In vitro protein import

Radiolabelled precursor proteins were synthesized using rabbit reticulocyte lysate by coupled transcription/translation in the presence of ³⁵S-methionine (Promega) (Sesaki *et al*, 2006; Tamura *et al*, 2009a). Isolated mitochondria were incubated with radiolabelled precursor proteins in 100 µl of import buffer (250 mM sucrose; 10 mM MOPS-KOH, pH 7.2; 80 mM KCl; 2 mM KPi; 2 mM methionine; 5 mM dithiothreitol; 5 mM MgCl₂; 2 mM ATP; 2 mM NADH; 1% BSA; 0.5 mM creatin phosphate; and 0.1 mg/ml creatin kinase) at 25°C. The import reaction was stopped by addition of 100 µl of ice-cold SEM buffer containing 100 µg/ml proteinase K and 20 µg/ml valinomycin and incubated for 15 min on ice. Proteinase K was then inactivated by addition of 1.5 mM PMSF. Mitochondria were reisolated by centrifugation, and the imported proteins were analysed by SDS-PAGE and autoradiography.

To chemically crosslink newly imported Ups2p, radiolabelled Ups2p were incubated with mitochondria for 20 min at 25°C. Mitochondria were collected by centrifugation and washed with SEM buffer. Mitochondria were then resuspended in SEM buffer and incubated with 300 µM DSG for 30 min. The crosslinker was quenched with 50 mM Tris-HCl pH 7.5. Mitochondria were boiled in 50 µl of 1% SDS buffer (1% SDS; 10 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 1 mM PMSF) and diluted with 950 µl of 0.5% Triton buffer. The samples were incubated with antibodies against Tom20p or Tom22p overnight at 4°C, and then with 10 µl of protein A-sepharose beads for 2 h at room temperature. The beads were washed with 1 ml of 0.5% Triton buffer four times. The immunoprecipitated proteins were eluted by boiling in SDS sample buffer and analysed by SDS-PAGE and autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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