Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria

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Cardiolipin, a unique phospholipid composed of four fatty acid chains, is located mainly in the mitochondrial inner membrane (IM). Cardiolipin is required for the integrity of several protein complexes in the IM, including the TIM23 translocase, a dynamic complex which mediates protein import into the mitochondria through interactions with the import motor presequence translocase–associated motor (PAM). In this study, we report that two homologous intermembrane space proteins, Ups1p and Ups2p, control cardiolipin metabolism and affect the assembly state of TIM23 and its association with PAM in an opposing manner. In *ups1Δ* mitochondria, cardiolipin levels were decreased, and the TIM23 translocase showed altered conformation and decreased association with PAM, leading to defects in mitochondrial protein import. Strikingly, loss of Ups2p restored normal cardiolipin levels and rescued TIM23 defects in *ups1Δ* mitochondria. Furthermore, we observed synthetic growth defects in *ups* mutants in combination with loss of Pam17p, which controls the integrity of PAM. Our findings provide a novel molecular mechanism for the regulation of cardiolipin metabolism.

Introduction

Most mitochondrial proteins are encoded in the nuclear genome. Newly synthesized mitochondrial proteins are translocated from the cytosol to the organelle and subsequently sorted into one of four submitochondrial compartments, namely the outer membrane (OM), intermembrane space (IMS), inner membrane (IM), or matrix. Different pathways are involved in importing different proteins to their final destination. Mitochondria contain multiple translocase complexes, including the translocase of the OM (TOM) in the OM and the two translocases of the IM, the TIM22 and TIM23 translocases, in the IM (Jensen and Johnson, 2001; Endo et al., 2003; Koehler, 2004; Mokranjac and Neupert, 2005; Dolezal et al., 2006; Neupert and Herrmann, 2007; Kutik et al., 2007). Although the TOM translocase is responsible for protein translocation across the OM, the TIM22 translocase inserts polytopic membrane proteins into the IM.

The TIM23 translocase consists of four integral IM proteins (Tim17p, Tim21p, Tim23p, and Tim50p) and mediates two distinct import pathways, translocation into the matrix and insertion into the IM. For translocation into the matrix, newly synthesized proteins carrying a matrix-targeting signal first pass the OM by way of the TOM translocase. These precursors are then recognized by the TIM23 translocase (Geissler et al., 2002; Yamamoto et al., 2002; Mokranjac et al., 2003a; Tamura et al., 2009) and partially translocated across the IM with aid from the mitochondrial membrane potential ($\Delta\psi$), which provides an electrophoretic motive force (Truscott et al., 2001; Martinez-Caballero et al., 2007). When precursor proteins enter the matrix partially, the presequences are cleaved by matrix processing proteases within this compartment. Further translocation is completed by the presequence translocase–associated motor (PAM), which is also called mtHsp70-associated motor and chaperone, consisting of mtHsp70 ATPase, Pam16p/Tim16p, Pam17p, Pam18p/Tim14p, Tim44p, and Zim17p/Tim15p (D'Silva et al., 2003; Mokranjac et al., 2003b, 2007; Truscott et al., 2003; Burri et al., 2004; Frazier et al., 2004; Kozany et al., 2004; Li et al., 2004; D'Silva et al., 2005; Sanjuán Szklarz et al., 2005; Yamamoto et al., 2005; Iosefson et al., 2007). The association of PAM with the TIM23 translocase requires Pam17p and Tim44p (van der Laan et al., 2005; D'Silva et al., 2008; Hutu et al., 2008; Schiller et al., 2008). Like matrix-targeted proteins,

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Abbreviations used in this paper: AAC, ATP/ADP carrier; BN, blue native; DHFR, dihydrofolate reductase; IM, inner membrane; IMS, intermembrane space; OM, outer membrane; PAM, presequence translocase–associated motor; PE, phosphatidylethanolamine; PiC, phosphate carrier; PRELI, protein of relevant evolutionary lymphoid interest; PS, phosphatidylserine; TOM, translocase of the OM.

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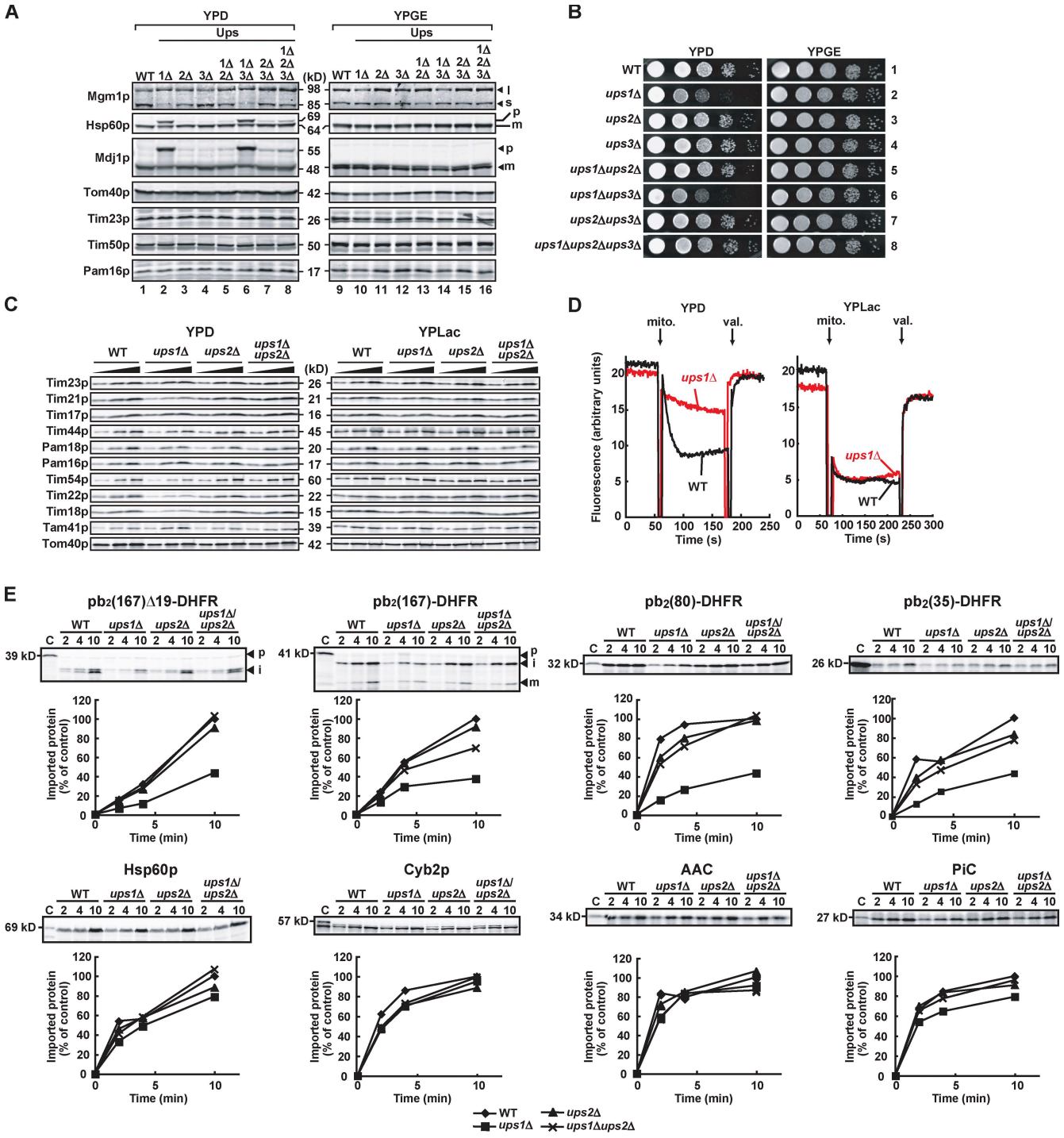
A
YPD
Ups
YPGE
Ups
WT 1Δ 2Δ 3Δ 1Δ 2Δ 1Δ 3Δ 2Δ 3Δ 1Δ 2Δ 3Δ (kD)
Mgm1p
Hsp60p
Mdj1p
Tom40p
Tim23p
Tim50p
Pam16p
l
s
p
m
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
YPD
YPGE
WT
ups1Δ
ups2Δ
ups3Δ
ups1Δups2Δ
ups1Δups3Δ
ups2Δups3Δ
ups1Δups2Δups3Δ
C
YPD
YPLac
WT
ups1Δ
ups2Δ
ups1Δ ups2Δ
(kD)
Tim23p
Tim21p
Tim17p
Tim44p
Pam18p
Pam16p
Tim54p
Tim22p
Tim18p
Tam41p
Tom40p
D
YPD
YPLac
mito.
val.
Fluorescence (arbitrary units)
ups1Δ
WT
Time (s)
E
pb2(167)Δ19-DHFR
pb2(167)-DHFR
pb2(80)-DHFR
pb2(35)-DHFR
Hsp60p
Cyb2p
AAC
PiC
Imported protein (% of control)
Time (min)
WT
ups1Δ
ups2Δ
ups1Δ/ups2Δ
F
Ups1p MVLLHKSTHIFPTDFASVSRAFFNRYPNPYSPHVLSIDTISRNVDQEG-NLRTTRLLKKS 59
Ups2p M-KLFQNSYDFNYPWDQVTAANWKKYPNEISTHVIAVDVLRRELKDQGKVLVTERLITVK 59
Ups3p M-KSFQKSYEFDYPWEKVTTANWMKYPNKISTHVIAVDVLRRELKEHGDVLLTERLITIR 59
Ups1p GKLPLWVKPFLRGITETWIIEVSVVNPANSTMKTYTRNLDHTGIMKVEEYTTYQFDSATS 119
Ups2p QGVPKWIMMMLGGTNMSHVREVSVVDLNKKSLTMRSCNLTMCNLLKVYETVTYSPHPDDS 119
Ups3p QNTPHWMSILVGNTNLAYVREVSTVDRRDRSLTMRSCNMTFPHILKCYETVRYVPHPKNP 119
Ups1p S--TIADSRVKFSSGFNMG-IKSKVEDWSRTKFDENVKKSRMGMAFVIQKLEEARNPQF- 175
Ups2p ANKTLFQQEAQITAYGSIRKLCNKMEDWSVQRFCENAKKGKMGFDAVLQVFSENWEKHVD 179
Ups3p SNVTLFKQDAKFLSGVPTKTFSEKVENWGVKRFSDNAVKGKVGFDSILAMFNDIWKN--- 176
Ups1p ----------
Ups2p DLSNQLVSKVNETMEDVKISAGTLLKGTERSGRTILQQNIDLFRDAYNHEN 230
Ups3p ---------ANE---------- 179

IM-targeted proteins that possess a hydrophobic-sorting signal in addition to a matrix-targeting signal also translocate across the OM through the TOM translocase and laterally inserted into the IM through the TIM23 translocase (Glick et al., 1992; Esaki et al., 1999). Insertion into the IM is less dependent on PAM than translocation into the matrix.

The function and stability of the TIM23 translocase depend on cardiolipin. Cardiolipin is a mitochondria-specific phospholipid that is located mainly in the IM (Li et al., 2007). Cardiolipin contains four fatty acid chains and stabilizes different protein complexes, including the ATP/ADP carrier (AAC) complex (Jiang et al., 2000), the electron transport chain supercomplex between complexes III and IV (Zhang et al., 2002), and the TIM23 translocase (van der Laan et al., 2007; Kutik et al., 2008). It has been shown that functional reconstitution of TIM23 using proteoliposomes requires cardiolipin (van der Laan et al., 2007). In addition, Tam41p/Mmp37p, a matrix protein required for TIM23–PAM association and TIM23-mediated protein import (Gallas et al., 2006; Tamura et al., 2006), plays a key role in the maintenance of cardiolipin levels in mitochondria (Kutik et al., 2008).

Mgm1p, a dynamin-related GTPase required for mitochondrial fusion, morphology, genome maintenance, and cristae structure (Shepard and Yaffe, 1999; Wong et al., 2000, 2003; Sesaki et al., 2003b; Meeusen et al., 2006), uses the TIM23 pathways and generates two protein isoforms that are targeted to distinct localizations, IM-integrated long form (l-Mgm1p) and IMS-located short form (s-Mgm1p; Herlan et al., 2004; Sesaki et al., 2006). Mgm1p is synthesized as a precursor containing a matrix-targeting signal followed by two hydrophobic regions that function as sorting signals (Shepard and Yaffe, 1999; Wong et al., 2000; Sesaki et al., 2003b). During translocation across the IM, approximately half of the Mgm1p molecules are arrested in the TIM23 translocase at the first sorting signal and inserted into the IM, generating l-Mgm1p (Herlan et al., 2004). This process depends on the $\Delta\psi$ but not PAM. Meanwhile, the remaining Mgm1p molecules are translocated further, allowing the second sorting signal to reach the TIM23 translocase. This additional translocation requires ATP and PAM (Herlan et al., 2004). Because the second hydrophobic region contains a recognition site for the IM-located rhomboid protease Pcp1p/Ugo2p, Mgm1p is cleaved by this enzyme and subsequently released into the IMS as a peripheral membrane protein as s-Mgm1p (Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003a). Therefore, Mgm1p import requires coordinated regulation of TIM23 and PAM activity.

In a screen for genes involved in Mgm1p biogenesis, the evolutionarily conserved IMS protein Ups1p was found to regulate production of s-Mgm1p (Sesaki et al., 2006). In the absence of Ups1p, cells generate only l-Mgm1p. Previous experiments show that Ups1p facilitates Mgm1p translocation from the first sorting signal to the second within the IM but not proteolytic cleavage (Sesaki et al., 2006). Reduction of hydrophobicity within the first sorting signal restores s-Mgm1p production in *ups1Δ* cells (Sesaki et al., 2006). Because generation of s-Mgm1p requires PAM (Herlan et al., 2004), one possible role for Ups1p is to regulate the TIM23 translocase and/or PAM. This model predicts that Ups1p plays important roles in import of other matrix- and IM-targeted proteins.

In this study, we investigated the role of Ups1p and its homologous proteins, Ups2p and Ups3p, in mitochondrial protein import and cardiolipin metabolism. We found that Ups1p and Ups2p antagonistically regulate cardiolipin levels in mitochondria. As a likely consequence of altered cardiolipin levels, loss of Ups1p and Ups2p affected protein import, TIM23–PAM association, and the integrity of the TIM23 translocase in an opposing manner. Our findings describe a novel mechanism for the control of cardiolipin metabolism in mitochondria

Results

Ups1p is important for protein import into mitochondria via the TIM23 translocase

Experiments have shown that Ups1p is required for the production of s-Mgm1p in fermentable carbon sources (Sesaki et al., 2006). To determine whether Ups1p plays additional roles in the biogenesis of other mitochondrial proteins, immunoblotting of whole cell extracts was performed using wild-type and *ups1Δ* cells grown in a fermentable carbon source (YPD) at 30°C (Fig. 1 A, lanes 1–8). As previously reported (Sesaki et al., 2006), *ups1Δ* cells displayed reduced levels of s-Mgm1p. In addition, accumulation of uncleaved precursor forms of two matrix-targeted proteins, Hsp60p and Mdj1p, was also observed (Fig. 1 A, lane 2). Protease digestion experiments using mitochondria isolated from *ups1Δ* cells indicate that these precursor forms were at least partially exposed to the cytosol (Fig. S1). This accumulation of precursors suggests that protein import into the matrix is defective in *ups1Δ* cells. We observed similar protein levels in the TIM23 translocase (Tim23p and Tim17p), the PAM proteins (Tim44p, Pam18p, and Pam16p), and the TOM translocase (Tom40p) in wild-type and *ups1Δ* mitochondria, suggesting that import defects are not simply caused by reduced levels of translocator proteins (Fig. 1 C, YPD). However, when mitochondria were incubated with a membrane potential–sensitive fluorescent dye, $DiSC_3(5)$ (Sims et al., 1974), we found that the membrane potential, which is required for mitochondrial protein import, was reduced in *ups1Δ* mitochondria (Fig. 1 D).

Figure 1. **Ups1p and Ups2p affect mitochondrial protein import.** (A) Whole cell extracts prepared from yeast cells lacking *UPS1* (1Δ), *UPS2* (2Δ), and/or *UPS3* (3Δ) were analyzed by immunoblotting. l, l-Mgm1p; s, s-Mgm1p; p, precursor form; m, mature form. (B) Serial dilutions of yeast cells were spotted onto YPD and YPGE and then grown at 30°C for 2 d and 4 d, respectively. (C) Mitochondria isolated from cells grown in YPD (left) or YPLac (right) at 30°C were analyzed by immunoblotting. (D) The $\Delta\Psi$ of wild-type (WT) and *ups1Δ* mitochondria was measured using a fluorescence-quenching assay using $DiSC_3(5)$. The addition of mitochondria (mito.) and valinomycin (val.) is indicated. $\Delta\Psi$ is shown as changes in fluorescence intensity after dissipation. (E) Mitochondria were incubated with radiolabeled precursors for the indicated times and 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; i, processed-intermediate form. (F) Alignment of Ups1p, Ups2p, and Ups3p amino acid sequences. Identical and similar amino acids are highlighted in black and gray, respectively.

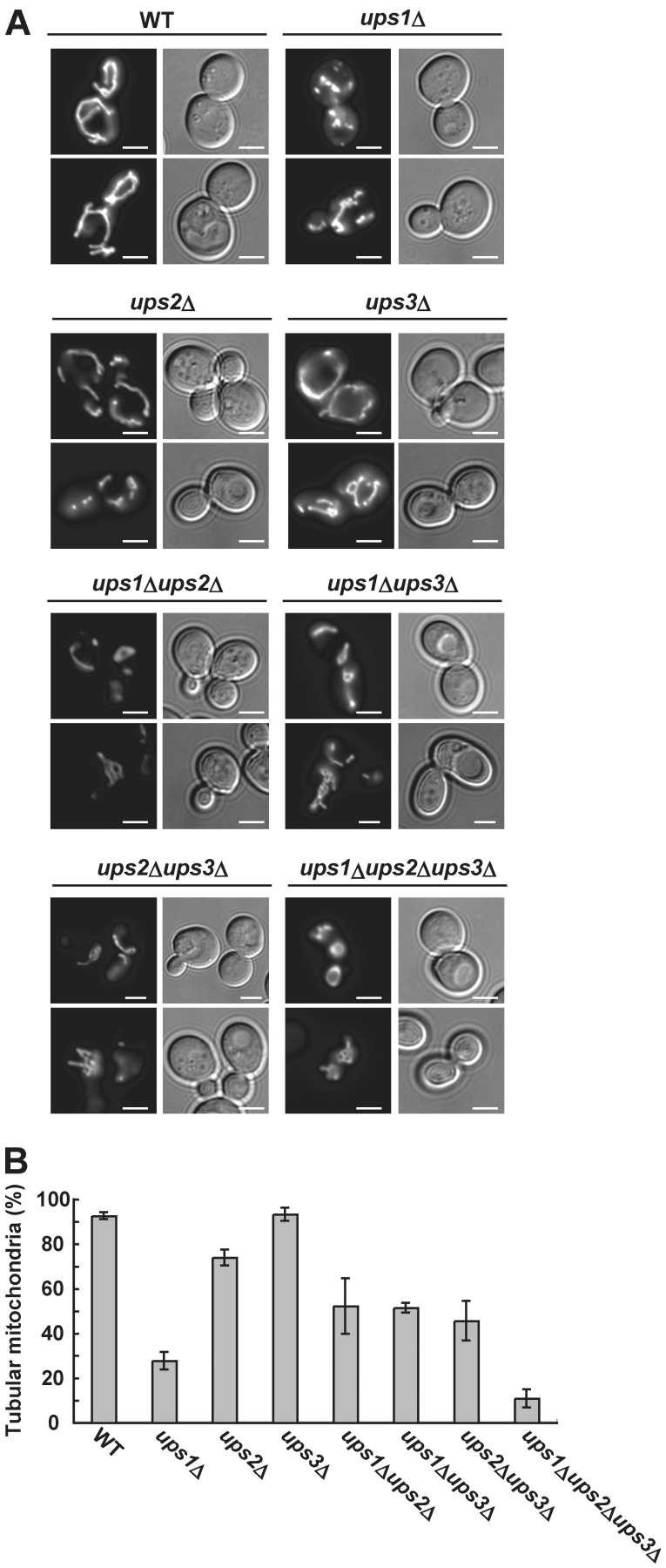

Figure 2. **Ups proteins are required for normal mitochondrial morphology.** (A) Mitochondria were visualized using mitochondria-targeted Su9-GFP (Sesaki et al., 2006). Cells were grown to log phase in YPD and then examined by differential interference contrast and fluorescence microscopy. (B) Quantitation of mitochondrial morphology. Cells containing tubular mitochondria were scored. Values are mean ± SD (n = 3). At least 300 cells were visualized in each experiment. WT, wild type. Bars, 3 µm.

However, when *ups1Δ* cells were grown in a nonfermentable carbon source (YPGE), wild-type levels of s-Mgm1p were detected as expected (Fig. 1 A, lanes 9–16; Sesaki et al., 2006). Similarly, precursor forms of Hsp60p or Mdj1p were not observed in YPGE-cultured *ups1Δ* cells. Similar levels of the TIM23 translocase, PAM, and the TOM translocase in addition to the TIM22 translocase (Tim54p, Tim22p, and Tim18p) were detected in mitochondria from wild-type and *ups1Δ* cells (Fig. 1 C, YPLac). In addition, levels of Tam41p/Mmp37p, a matrix protein required for TIM23-mediated protein import, were not affected in *ups1Δ* mitochondria (Gallas et al., 2006; Tamura et al., 2006). Furthermore, the membrane potential was normally maintained in *ups1Δ* mitochondria isolated from cells grown in YPLac (Fig. 1 D). Altogether, these results suggest that Ups1p is important for efficient protein import via the TIM23 translocase.

To further characterize the role of Ups1p in mitochondrial protein import, we examined protein import in mitochondria isolated from wild-type and *usp1Δ* cells. To exclude any secondary effects resulting from the accumulation of precursor proteins, decreased membrane potential, and a mitochondrial shape change (Sesaki et al., 2006), mitochondria were isolated from cells grown in YPLac. We initially analyzed import of pb_2(167)Δ19–dihydrofolate reductase (DHFR), pb_2(80)-DHFR, and pb_2(35)-DHFR preproteins with a matrix-targeting signal. Studies have shown that import of these proteins requires both the TIM23 translocase and PAM (Voos et al., 1993; Sato et al., 2005). As shown in Fig. 1 E, their mitochondrial import was slower in *ups1Δ* cells than in wild-type cells. We also analyzed pb_2(167)-DHFR, a precursor protein which possesses matrix-targeting and -sorting signals and is inserted into the IM via the TIM23 translocase but independent of PAM (Voos et al., 1993). Results demonstrate that pb_2(167)-DHFR import was also compromised in *ups1Δ* mitochondria. Interestingly, the import of other proteins that use the TIM23 translocase, including Cyb2p and Hsp60p, was similar in wild-type and *ups1Δ* mitochondria. These findings are consistent with previous experiments (Sesaki et al., 2006) and show that not all matrix-targeted proteins exhibit defective import. It is possible that the tightly folded DHFR moiety in Cyb2p fusions require higher import activities. Finally, we analyzed import of polytopic IM proteins such as an AAC and a phosphate carrier (PiC), which lack a presequence and are inserted into the IM via the TIM22 translocase. These proteins showed similar import rates into wild-type and *ups1Δ* mitochondria (Fig. 1 E). These results indicate that Ups1p is critical for the import of a subset of presequence-containing proteins targeted to the matrix and the IM via the TIM23 translocase.

Loss of Ups2p rescues defects in mitochondrial protein import and cell growth in *ups1Δ* cells

In the yeast genome, Ups1p is homologous to two other proteins with unknown function, Ylr168cp (30% identical) and Ydr185cp (25% identical; Fig. 1 F; Sesaki et al., 2006), and we named these proteins Ups2p (27 kD and 230 aa) and Ups3p (21 kD and 179 aa), respectively. Similar to Ups1p, both Ups2p and Ups3p contain the MSF1 domain (Sesaki et al., 2006). To determine whether Ups2p and Ups3p also regulate mitochondrial protein import, we examined levels of mitochondrial proteins in *ups2Δ* and *ups3Δ* cells grown in YPD (Fig. 1 A, YPD). Mgm1p, Hsp60p, and Mdj1p import and processing were not affected in *ups2Δ* and *ups3Δ* single deletion cells (Fig. 1 A, lanes 3 and 4). Surprisingly, *ups1Δups2Δ* double deletion cells were capable of producing s-Mgm1p. In addition, Hsp60p and Mdj1p were converted to their mature forms in *ups1Δups2Δ* cells (Fig. 1 A, lane 5). However, *ups1Δups3Δ* cells, like *ups1Δ* cells, showed reduced levels of s-Mgm1p and accumulation of Hsp60p and Mdj1p precursor forms (Fig. 1 A, lane 6). We also found that levels of Hsp60p and Mdj1p precursor proteins were slightly higher in *ups2Δups3Δ* and *ups1Δups2Δups3Δ* cells than in wild-type cells (Fig. 1 A, lanes 7 and 8). Thus, deletion of *UPS2* but not *UPS3* rescues defects in s-Mgm1p production as well as import of Hsp60p and Mdj1p in *ups1Δ* cells in YPD. In contrast, when *ups* mutants were grown in nonfermentable carbon sources, we found that s-Mgm1p and mature forms of Hsp60p and Mdj1p were produced normally regardless of the deletions made (Fig. 1 A, lanes 9–16).

We also found that loss of Ups2p rescues import defects in *ups1Δ* mitochondria in in vitro import assays. As shown in Fig. 1 E, *ups1Δups2Δ* mitochondria imported pb_2(167)Δ19-DHFR, pb_2(80)-DHFR, and pb_2(35)-DHFR at the wild-type rate, whereas *ups1Δ* mitochondria imported the protein more slowly. Similarly, pb_2(167)-DHFR import was partially restored in *ups1Δups2Δ* mitochondria, whereas import of AAC and PiC were not affected in *ups2Δ* or *ups1Δups2Δ* mitochondria (Fig. 1 E). Loss of Ups2p did not affect levels of import components (Fig. 1 C). These results demonstrate that import defects in *ups1Δ* mitochondria depend on Ups2p.

Loss of Ups2p also rescued growth defects in *ups1Δ* cells. *ups1Δ* cells showed growth defects in fermentable carbon sources but not in nonfermentable carbon sources as reported previously (Fig. 1 B, lane 2; Sesaki et al., 2006). *ups1Δups2Δ* cells grew normally on YPD plates, whereas *ups1Δups3Δ* cells grew similarly to *ups1Δ* cells (Fig. 1 B, lanes 5 and 6). Unlike *ups1Δ* cells, *ups2Δ* and *ups3Δ* single deletion cells as well as *ups2Δups3Δ* cells double deletion cells exhibited normal growth under YPD and YPGE conditions (Fig. 1 B, lanes 3, 4, and 7). Growth of wild-type and the triple deletion cells was indistinguishable (Fig. 1 B, lane 8). These results indicate that growth defects in *ups1Δ* depend on Ups2p.

Ups2p and Ups3p are required for mitochondrial morphology

Ups1p is required for maintaining mitochondrial morphology in fermentable carbon sources (Sesaki et al., 2006). As such, we investigated whether Ups2p and Ups3p are also involved in mitochondrial morphogenesis. To visualize mitochondria, we expressed a mitochondria-targeted GFP marker in wild-type and *ups*-deleted cells and then grew them in YPD and YPGE carbon sources. As previously reported (Sesaki et al., 2006), in *ups1Δ* cells, the majority of mitochondria (~70%) exhibit an altered morphology, exhibiting short tubules, small fragments, and aggregates when grown in YPD (Fig. 2). In contrast, wild-type mitochondria remain tubular. In *ups2Δ* cells, although the morphology of most mitochondria was similar to wild-type, a small fraction of cells (~30%) possessed mitochondria with a

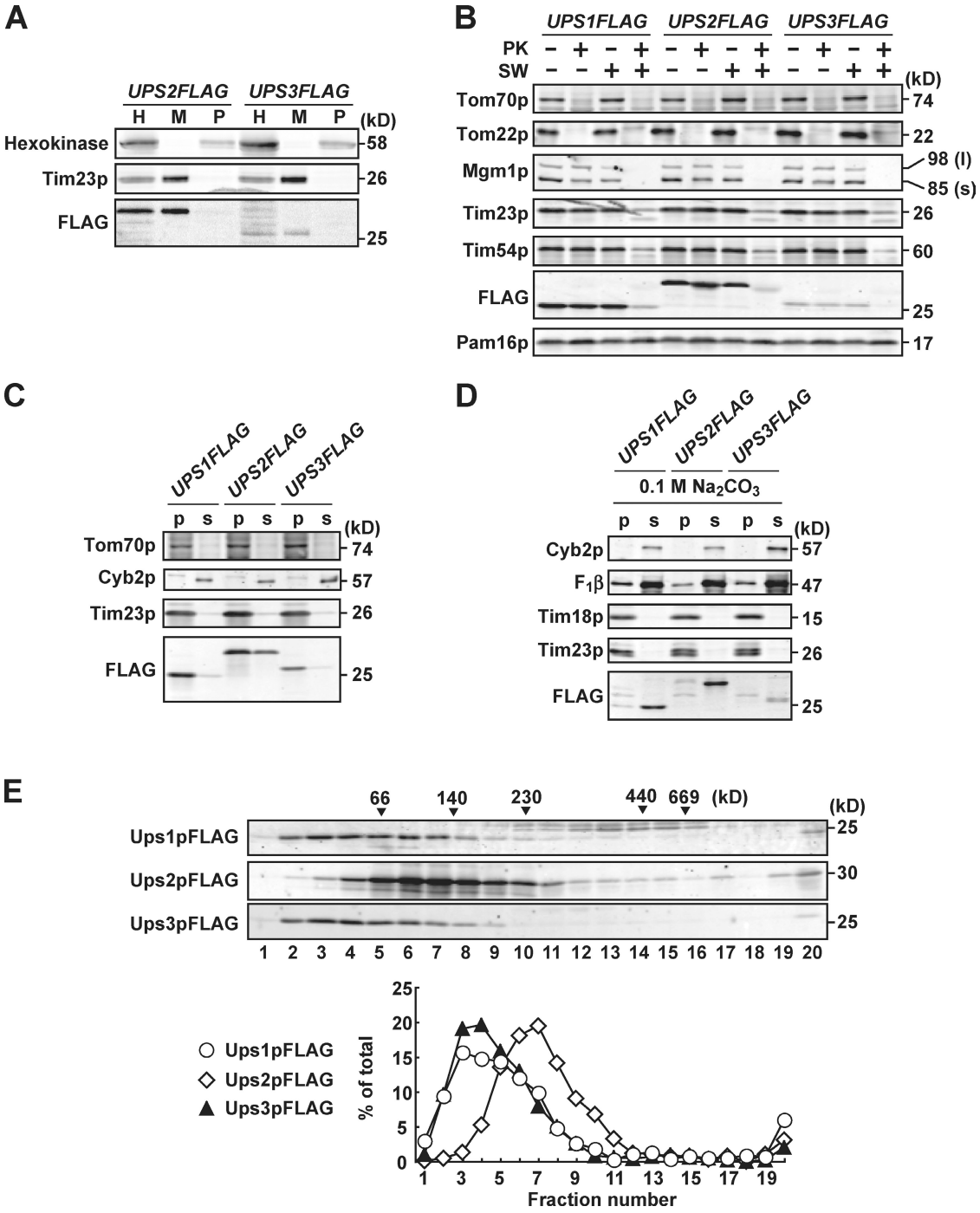

Figure 3. **Ups proteins are located in the IMS.** (A) Cells grown in YPD were homogenized (H) and separated into a mitochondrial pellet (M) and post-mitochondrial supernatant (P) by centrifugation. Proteins were analyzed by immunoblotting with antibodies against hexokinase (a cytosolic protein), Tim23p (a mitochondrial protein), and Flag. (B) Mitoplasts were generated by osmotic swelling (SW) and treated with 200 µg/ml proteinase K for 30 min on ice (PK). l, long form; s, short form. (C) After osmotic swelling, mitochondria were separated into pellets (p) and supernatants (s) by centrifugation. (D) Mitochondria were treated with 0.1 M Na_2CO_3 for 30 min on ice and separated into pellets and supernatants by centrifugation. (E) Mitochondria were solubilized with digitonin and subjected to glycerol density gradient centrifugation. Fractions were collected from the top and analyzed by immunoblotting. Quantitation of band intensity is also shown.

disorganized shape, which included relatively round structures, small nets, and aggregates (Fig. 2). However, mitochondrial shape was indistinguishable between *ups3Δ* and wild-type cells. Similar to those seen in *ups2Δ* cells, ~50% of *ups1Δups2Δ* cells exhibited mitochondria with altered morphology. Interestingly, ~90% of *ups1Δups2Δups3Δ* cells showed an altered mitochondrial shape that was similar to *ups2Δ*, *ups1Δups2Δ*, and *ups2Δups3Δ* cells. In contrast, when wild-type cells and the *ups* deletion mutants were grown in YPGE, all of them displayed a tubular mitochondrial morphology (Sesaki et al., 2006; unpublished data). Thus, Ups2p and Ups3p are important for normal mitochondrial morphology in fermentable carbon sources. Because the triple

mutants demonstrated the most severe defect, Ups proteins likely have partially overlapping but distinct roles in maintaining mitochondrial morphology.

Ups2p and Ups3p are located in the IMS

Next, we determined the mitochondrial localization of Ups2p and Ups3p in yeast cells. A previous genome-wide localization study using GFP fusion proteins showed that Ups2p and Ups3p are mitochondrial proteins (Huh et al., 2003). Consistent with this study, mitochondria isolated from cells expressing Ups2p-Flag or Ups3p-Flag demonstrated that these proteins associate with the organelle (Fig. 3 A). In fact, like the IMS-localized Ups1p (Sesaki et al., 2006), these proteins were resistant to proteolytic digestion by proteinase K (Fig. 3 B). In contrast, the OM proteins Tom70p and Tom22p were completely digested. When the OM was disrupted by osmotic swelling and mitoplasts were generated, Ups1p-Flag, Ups2p-Flag, and Ups3p-Flag were digested by proteinase K. Similarly, IM proteins possessing an IMS-exposed domain, namely Tim23p, Tim54p, and l-Mgm1p as well as the IMS protein s-Mgm1p, were accessible to proteinase K digestion only in mitoplasts (Fig. 3 B). However, the matrix protein Pam16p was not degraded by proteinase K even after the OM was opened by osmotic swelling. To determine the extent to which Ups2p and Ups3p associate with mitochondrial membranes, we disrupted the OM and pelleted the mitochondrial membranes by centrifugation (Fig. 3 C). Although approximately one third of Ups2p-Flag was released into the supernatant fractions, nearly all Ups1p-Flag and Ups3p-Flag remained associated with mitoplasts. When mitochondria were treated with sodium carbonate (Na_2CO_3), Ups1p-Flag, Ups2p-Flag, and Ups3p-Flag were extracted into the supernatant, similar to $F_1\beta$, a peripheral IM protein, and Cyb2p, a soluble IMS protein (Fig. 3 D). However, the integral membrane proteins Tim18p and Tim23p were found associated with mitochondria. Thus, Ups proteins were peripherally associated with mitochondrial membranes.

Furthermore, glycerol density gradient centrifugation of digitonin-solubilized mitochondria showed that Ups1p-Flag and Ups3p-Flag form ~60-kD protein complexes, whereas Ups2p-Flag forms ~100-kD complexes (Fig. 3 E). However, coimmunoprecipitation experiments demonstrated that Ups1p, Ups2p, and Ups3p do not interact and are likely to be in different protein complexes (unpublished data). Collectively, these results indicate that all three Ups proteins form distinct protein complexes and are peripherally associated with mitochondrial membranes in the IMS.

Roles of Ups1p and Ups2p in TIM23–PAM interactions

To understand how Ups1p and Ups2p affect protein import mediated by the TIM23 translocase, we examined the interactions between the TIM23 translocase and PAM by coimmunoprecipitation. Mitochondria expressing Tim23p-Flag were isolated and solubilized with 1% digitonin and then subjected to immunoprecipitation using anti-Flag antibodies. As shown in Fig. 4 (A and B), the levels of PAM proteins Tim44p, Pam18p, and Pam16p that coprecipitated with Tim23p-Flag were reduced 60–70% in *ups1Δ* mitochondria. In contrast, loss of Ups2p did not affect TIM23–PAM interactions. Strikingly, loss of Ups2p restored coimmunoprecipitation of PAM proteins with Tim23p in *ups1Δ* mitochondria. When subunits of the TIM23 translocase were examined, associations of Tim23p with Tim17p, Tim21p, and Tim50p were not altered in *ups1Δ*, *ups2Δ*, and *ups1Δups2Δ* mitochondria (Fig. 4, A and B). As a control, we performed coimmunoprecipitation using Tim22p-Flag and found that interactions of Tim22p with other components of the TIM22 translocase, Tim18p and Tim54p, were not affected in *ups1Δ* and *ups2Δ* mitochondria (Fig. 4, C and D). In addition, our coimmunoprecipitation experiments showed that Ups1p or Ups2p is not stably associated with components of the TIM23 translocase and PAM (Fig. S3). These results demonstrate that Ups1p is critical for maintaining stable interactions between the TIM23 translocase and PAM and that Ups2p antagonizes this Ups1p-dependent TIM23–PAM interaction.

Reciprocal coimmunoprecipitation using mitochondria expressing Pam16p-Flag was performed to confirm these results. Data in Fig. 4 (E and F) show that less Tim23p and Tim17p coprecipitated with Pam16p-Flag in *ups1Δ* mitochondria. In addition, dissociation of Tim23p and Tim17p from Pam16p was rescued in *ups1Δups2Δ* mitochondria. In contrast, loss of Ups1p did not affect interactions between Pam16p and Pam18p. Comparable amounts of Pam18p coprecipitated with Pam16p-Flag in wild-type, *ups1Δ*, *ups2Δ*, and *ups1Δups2Δ* mitochondria (Fig. 4, E and F). In addition, in *ups1Δ* mitochondria, Tim44p dissociated from Pam16p in *ups1Δ* mitochondria in an Ups2p-dependent manner.

To further analyze the assembly states of the TIM23–PAM complex in more detail, we performed glycerol density gradient centrifugation. Isolated mitochondria were solubilized in 1% digitonin and subjected to 20–40% glycerol density gradient centrifugation. In wild-type mitochondria, Tim23p and Tim17p, the channel components of the TIM23 translocase, as well as the PAM subunits Pam16p, Pam18p, and Tim44p were recovered in fractions corresponding to complexes with a molecular mass of ~300 kD (Fig. 5, A and B; fractions 11–13). To confirm that the ~300-kD complex consists of the TIM23 translocase associated with PAM proteins, we performed glycerol density gradient using Tim23p-Flag–expressing mitochondria in the presence of excess anti-Flag antibodies. Most of the Tim23p-Flag, Tim50p, Pam16p, Pam18p, and Tim44p protein within this fraction was shifted to higher molecular mass fractions (unpublished data). Fig. 5 (A and B) shows that *ups1Δ* mitochondria contained reduced amounts of Pam16p, Pam18p, and Tim44p in the ~300-kD fraction. In addition, levels of Pam16p and Pam18p in the bottom fraction increased, and the peak of Tim44p shifted to fractions corresponding to ~140-kD complexes. These results are consistent with the coimmunoprecipitation experiments shown in Fig. 4 and provide further support for the importance of Ups1p in TIM23–PAM interactions. When wild-type and *ups2Δ* mitochondria are compared, all of the proteins migrated similarly within the gradients (Fig. 5, A and C). However, loss of Ups2p rescued disassembly of the TIM23–PAM complex in *ups1Δ* mitochondria (Fig. 5, A and C). These results suggest that Ups1p is required for the assembly state of the TIM23–PAM complex and that Ups2p antagonizes this activity.

To confirm specificity of Ups1p involvement, we examined other protein complexes, including the TOM translocase

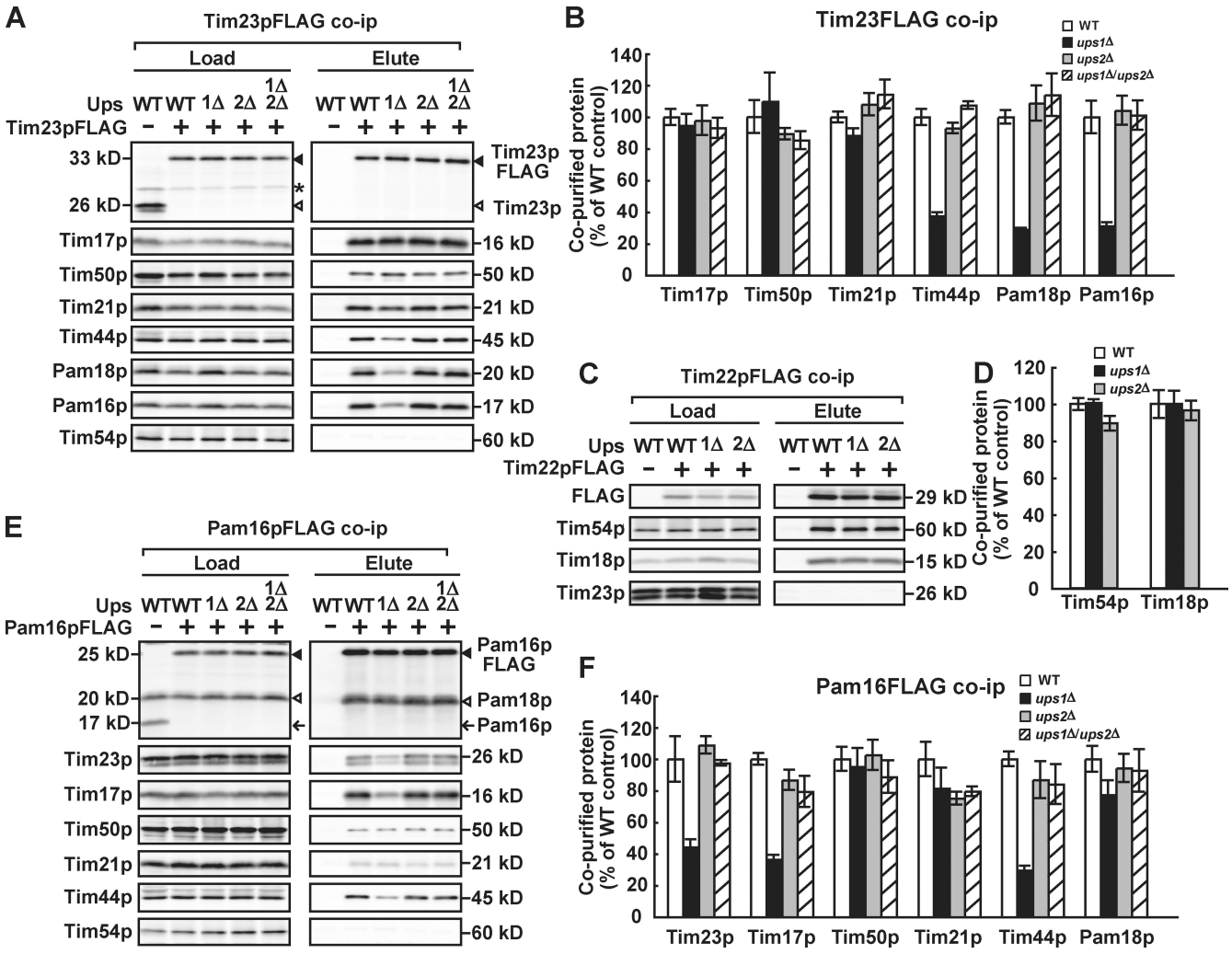

Figure 4. **Ups1p and Ups2p affect interactions between the TIM23 translocase and PAM proteins.** (A and B) Mitochondria were solubilized with digitonin and subjected to immunoprecipitation with anti-Flag M2-agarose. The mitochondrial lysate (Load; 15%) and bound proteins (Elute; 100%) are shown. Proteins were analyzed by immunoblotting using the indicated antibodies. The asterisk indicates a nonspecific band. Band intensity was normalized relative to that detected in wild-type (WT) mitochondria in B. Values are mean ± SEM (n = 4). (C and D) Immunoprecipitation was performed as described in A using mitochondria expressing Pam16p-Flag. Quantitation of band intensity is shown in D. (E and F) Immunoprecipitation was performed as described in A using mitochondria expressing Tim22p-Flag. (D and F) Values are mean ± SEM (n = 3). co-ip, coimmunoprecipitation.

with Tom40p, the TIM22 translocase containing Tim18p and Tim22p, Tim54p, and a complex containing PiC (Fig. 5, D and E). These complexes were unaffected by Ups1p deletion, indicating that this protein is specifically required for regulating the assembly state of the TIM23–PAM complex.

Ups1p and Ups2p remodel the TIM23 translocase

Although protein–protein interactions in the TIM23 translocase appeared to be intact in our coimmunoprecipitation and glycerol density gradient experiments, these interactions were unstable in *ups1Δ* mitochondria (Fig. 6). To assess this, we examined the effect of increasing amounts of Triton X-100 on interactions within the TIM23 translocase. In these experiments, 1% digitonin and up to 0.1% Triton X-100 were added to isolated mitochondria expressing Tim23p-Flag, which was immunoprecipitated. In the absence of Triton X-100, similar amounts of Tim17p, Tim21p, and Tim50p coimmunoprecipitated with Tim23p-Flag in wild-type and *ups1Δ* mitochondria (Fig. 6 A), which is consistent with our earlier results (Fig. 4). Remarkably, increasing Triton X-100 resulted in increased dissociation of Tim17p and Tim21p from Tim23p-Flag in *ups1Δ* mitochondria relative to wild-type mitochondria (Fig. 6 A). However, interaction between Tim23p-Flag and Tim50p was similar in wild-type and *ups1Δ* mitochondria regardless of the Triton X-100 concentration used. When 0.1% Triton X-100 was added, Tim23p-Flag interaction with Tim17p, Tim21p, and Tim50p was almost completely abolished (Fig. 6 A). These results indicate that Ups1p specifically stabilizes Tim23p interactions with Tim17p and Tim21p.

To determine whether Ups2p is involved in regulating this Ups1p-dependent stabilization of the TIM23 translocase, coimmunoprecipitation using wild-type, *ups1Δ*, *ups2Δ*, and *ups1Δups2Δ* mitochondria was performed in the presence of 1% digitonin and 0.025% Triton X-100 (Fig. 6 B). The stability of Tim23p–Tim17p and Tim23p–Tim21p interactions was not affected in *ups2Δ* mitochondria. However, loss of Ups2p restored

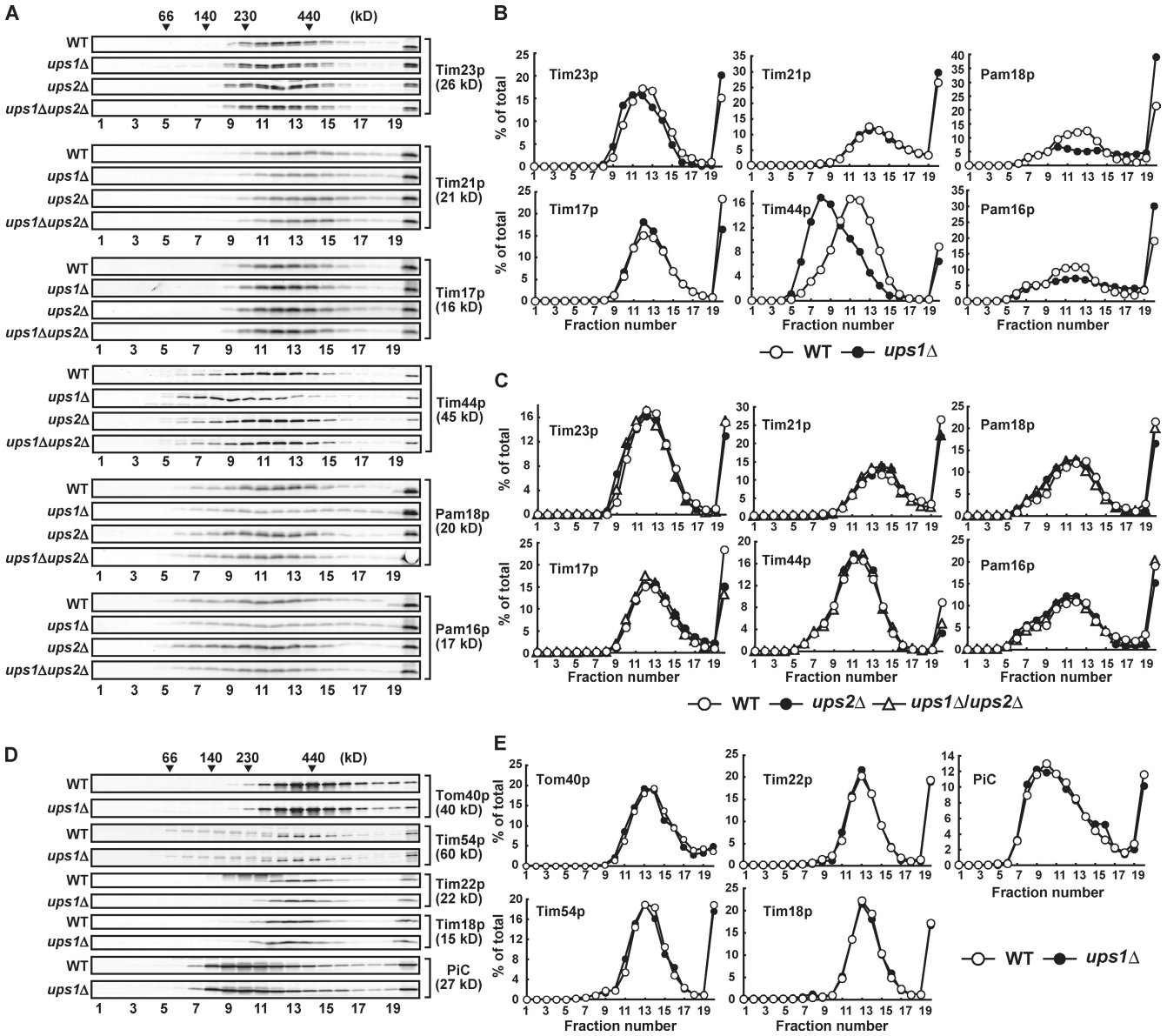

Figure 5. **Analysis of the TIM23–PAM complex in *ups* mutants.** (A–C) Mitochondria isolated from cells grown in YPLac were solubilized with digitonin and analyzed by glycerol density gradient centrifugation. Migration patterns of components of the TIM23 translocase and PAM in wild-type (WT) and *ups1Δ* mitochondria (B) and in wild-type, *ups2Δ*, and *ups1Δups2Δ* mitochondria (C) are shown. (D and E) Migration patterns of Tom40p, Tim22p, Tim18p, Tim54p, and PiC in wild-type and *ups1Δ* mitochondria. Molecular masses are shown in kilodaltons.

resistance to Triton X-100 in *ups1Δ* mitochondria, as similar amounts of Tim17p, Tim21p, and Tim50p coprecipitated with Tim23p-Flag in wild-type, *ups2Δ*, and *ups1Δups2Δ* mitochondria (Fig. 6 B). Collectively, these results demonstrate that Ups2p controls the Ups1p-dependent interactions of Tim23p with Tim17p and Tim21p but not with Tim50p within the TIM23 translocase and suggest that Ups1p and Ups2p affect the assembly state of the TIM23 translocase.

PAM proteins dissociate from the TIM23 translocase in *ups1Δ* mitochondria (Figs. 4 and 5). To test whether this dissociation causes Tim23p–Tim17p and Tim23p–Tim21p interactions to be sensitive to Triton X-100 and unstable, we examined mitochondria lacking Pam17p, which is required for TIM23–PAM interactions (van der Laan et al., 2005). Consistent with a previous study (van der Laan et al., 2005), Tim23p-Flag interactions with Pam16p, Pam18p, and Tim44p were decreased in *pam17Δ* mitochondria (unpublished data). Coimmunoprecipitation in the presence of increasing Triton X-100 resulted in similar amounts of Tim17p, Tim21p, and Tim50p being coprecipitated with Tim23p-Flag in wild-type and *pam17Δ* mitochondria (Fig. 6 C). Therefore, these data clearly demonstrate that the integrity of the TIM23 translocase is independent of PAM proteins.

The Ups1p and Ups2p pathway is distinct from Pam17p

To determine the functional relationship of Ups proteins with Pam17p and Tim21p, which also control TIM23–PAM interactions (Chacinska et al., 2005; van der Laan et al., 2005; Popov-Čeleketić et al., 2008), we generated double mutants of the *PAM17* or *TIM21* gene in combination with *ups1Δ* and *ups2Δ* and then spotted cells

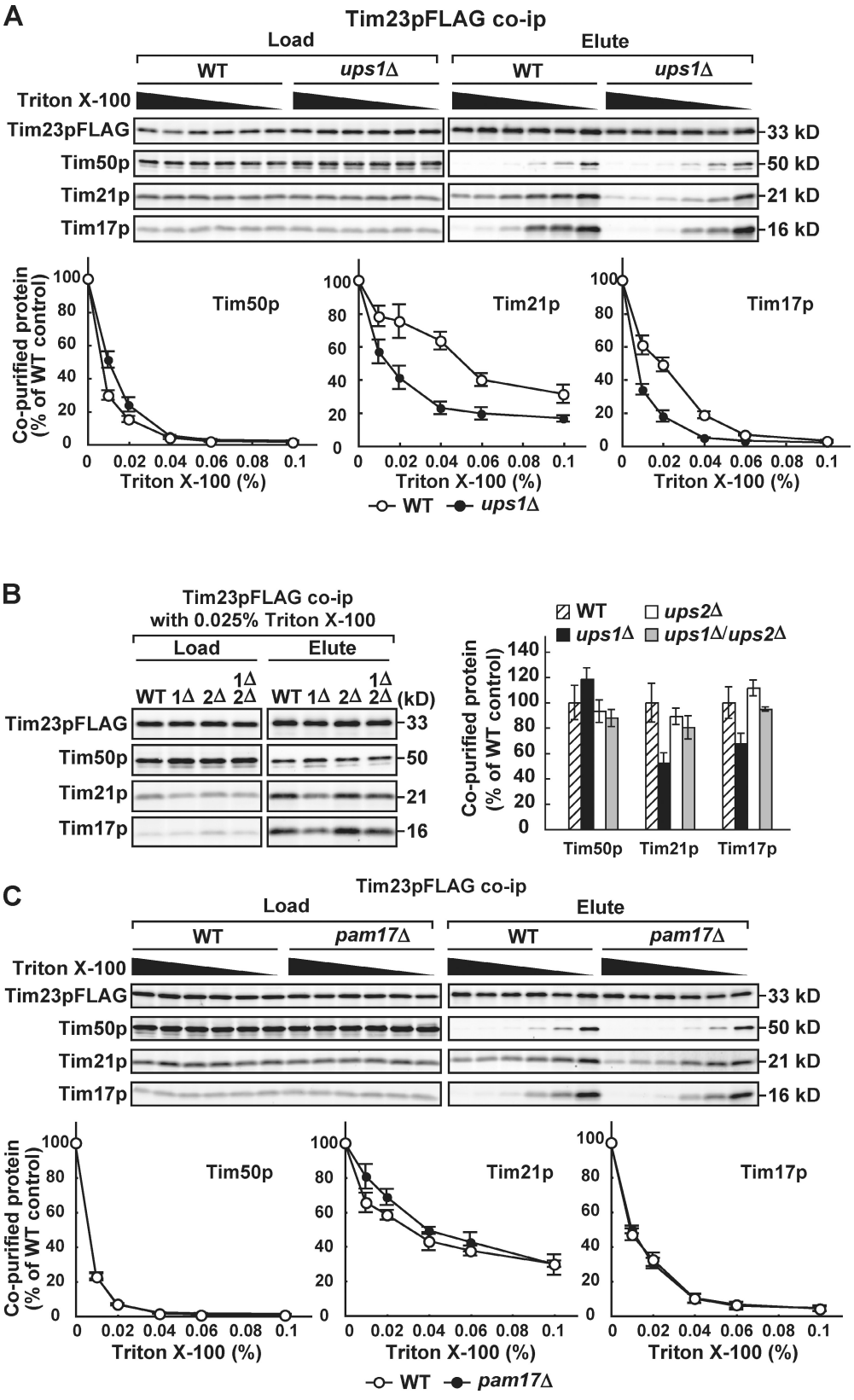

Figure 6. **Analysis of protein–protein interactions in the TIM23 translocase in *ups* mutants.** (A) Wild-type (WT) and *ups1Δ* mitochondria expressing Tim23p-Flag were solubilized in digitonin buffer containing the indicated concentrations of Triton X-100 and then incubated with anti-Flag agarose. 10% of lysate (Load) and 100% of bound proteins (Elute) were analyzed by immunoblotting. Band intensity was quantitated and normalized to samples not treated with Triton X-100. (B) Wild-type and mutant mitochondria expressing Tim23p-Flag were solubilized in digitonin buffer containing 0.025% Triton X-100 and subjected to coimmunoprecipitation (co-ip) with anti-Flag agarose. Band intensity was quantitated and normalized to that of wild-type mitochondria.

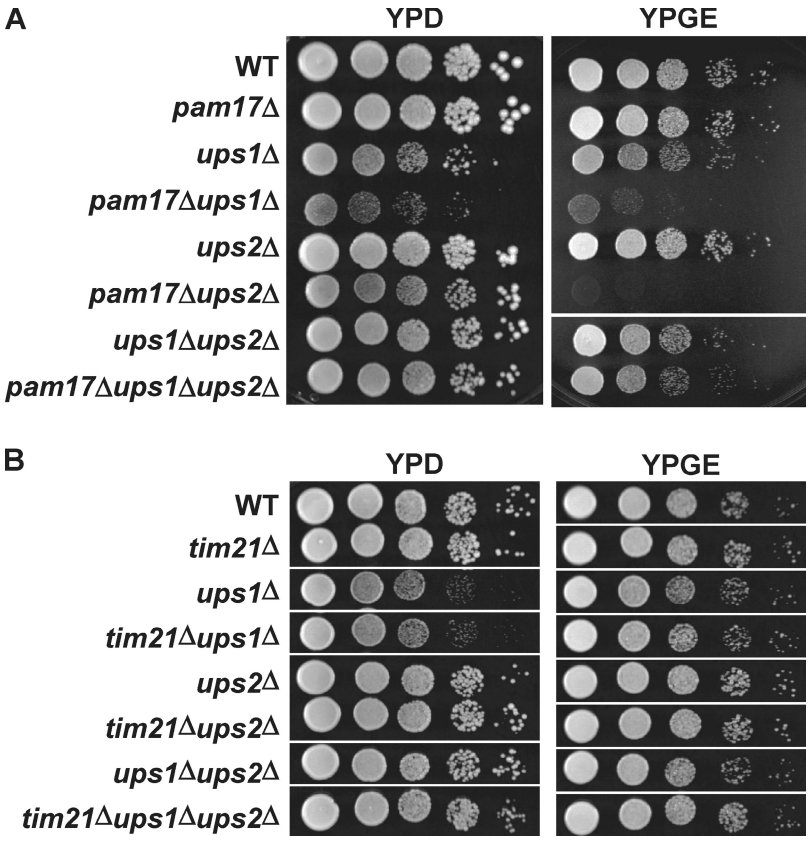

Figure 7. **Deletion of *PAM17* results in synthetic growth defects in *ups* mutants.** (A) Serial dilutions of yeast cells lacking *UPS1*, *UPS2*, and/or *PAM17* were spotted onto YPD and YPGE plates and incubated at 30°C for 2 d and 4 d, respectively. (B) Serial dilutions of yeast cells lacking *UPS1*, *UPS2*, and/or *TIM21* were spotted onto YPD and YPGE plates and grown at 30°C for 2 d and 4 d, respectively. WT, wild type.

onto fermentable (YPD) and nonfermentable (YPGE) carbon sources. Wild-type and *pam17Δ* cells grew similarly under both conditions (Fig. 7 A). However, *pam17Δups1Δ* cells showed synthetic growth defects on YPD, which were further enhanced on YPGE even though *ups1Δ* and *pam17Δ* single deletion cells grew normally on YPGE. Surprisingly, although *ups2Δ* cells did not display any growth defects on YPD and YPGE, *pam17Δups2Δ* cells showed a strong growth defect on YPGE. DAPI staining demonstrated that *pam17Δups2Δ* cells contained normal amounts of mitochondrial DNA, indicating that these growth defects are not caused by its loss, which is essential for growth in nonfermentable carbon sources (unpublished data). Interestingly, *ups1Δups2Δ* and *pam17Δups1Δups2Δ* cells grew similarly on both YPD and YPGE. These results suggest that Ups1p and Ups2p act in a pathway that is distinct yet functionally overlapping with the Pam17p pathway (van der Laan et al., 2005). In contrast, no synthetic growth defects were observed when *TIM21* deletion was combined with *ups1Δ* and *ups2Δ* (Fig. 7 B). When we examined the steady-state levels of mitochondrial proteins (Fig. S2), a precursor form of Hsp60p had accumulated in *ups2Δpam17Δ* cells in YPD (lane 9) but not in *ups2Δ* (lane 7) and *pam17Δ* (lane 3) single deletion mutants. Additional deletion of *PAM17* in *ups1Δ* cells did not increase the level of Hsp60p precursors in *ups1Δpam17Δ* cells (Fig. S2, compare lane 4 with lane 6). Interestingly, loss of Tim21p, which negatively regulates TIM23–PAM association (Chacinska et al., 2005), reduced the accumulation of Hsp60p precursors in *ups1Δups2Δtim21Δ* cells (Fig. S2, compare lane 10 with lane 11).

Ups1p and Ups2p control the level of cardiolipin in mitochondria

A recent study has shown that cardiolipin is required for the association of TIM23 translocase with PAM (Kutik et al., 2008). To determine whether Ups1p and Ups2p modulate TIM23 assembly through cardiolipin, we measured the level of cardiolipin in mitochondria isolated from wild-type, *ups1Δ*, *ups2Δ*, *ups3Δ*, *ups1Δups2Δ*, *ups1Δups3Δ*, *ups2Δups3Δ*, and *ups1Δups2Δups3Δ* cells. As controls, we also used *crd1Δ* and *taz1Δ* cells, which possess reduced cardiolipin levels (Chang et al., 1998; Gu et al., 2004). Cells were grown in the presence of ^{32}Pi in YPGE media before collection of crude mitochondrial factions from these cells (Claypool et al., 2006). Total phospholipids were extracted in chloroform/methanol and separated by thin-layer chromatography (Vaden et al., 2005; Claypool et al., 2006). As shown in Fig. 8, the level of cardiolipin was considerably reduced in *ups1Δ* mitochondria. This decreased cardiolipin level was rescued by additional loss of Ups2p in *ups1Δups2Δ* mitochondria. In contrast, loss of Ups3p did not restore cardiolipin levels in *ups1Δups3Δ* mitochondria. Similar to *crd1Δ* and *taz1Δ* cells, phosphatidylethanolamine (PE) levels were higher in *ups1Δ* mitochondria, perhaps caused by compensatory mechanisms in

(C) Coimmunoprecipitation was performed using mitochondria isolated from wild-type and *pam17Δ* cells expressing Tim23p-Flag and quantitated as described in A. (A–C) Values are mean ± SEM (n = 3).

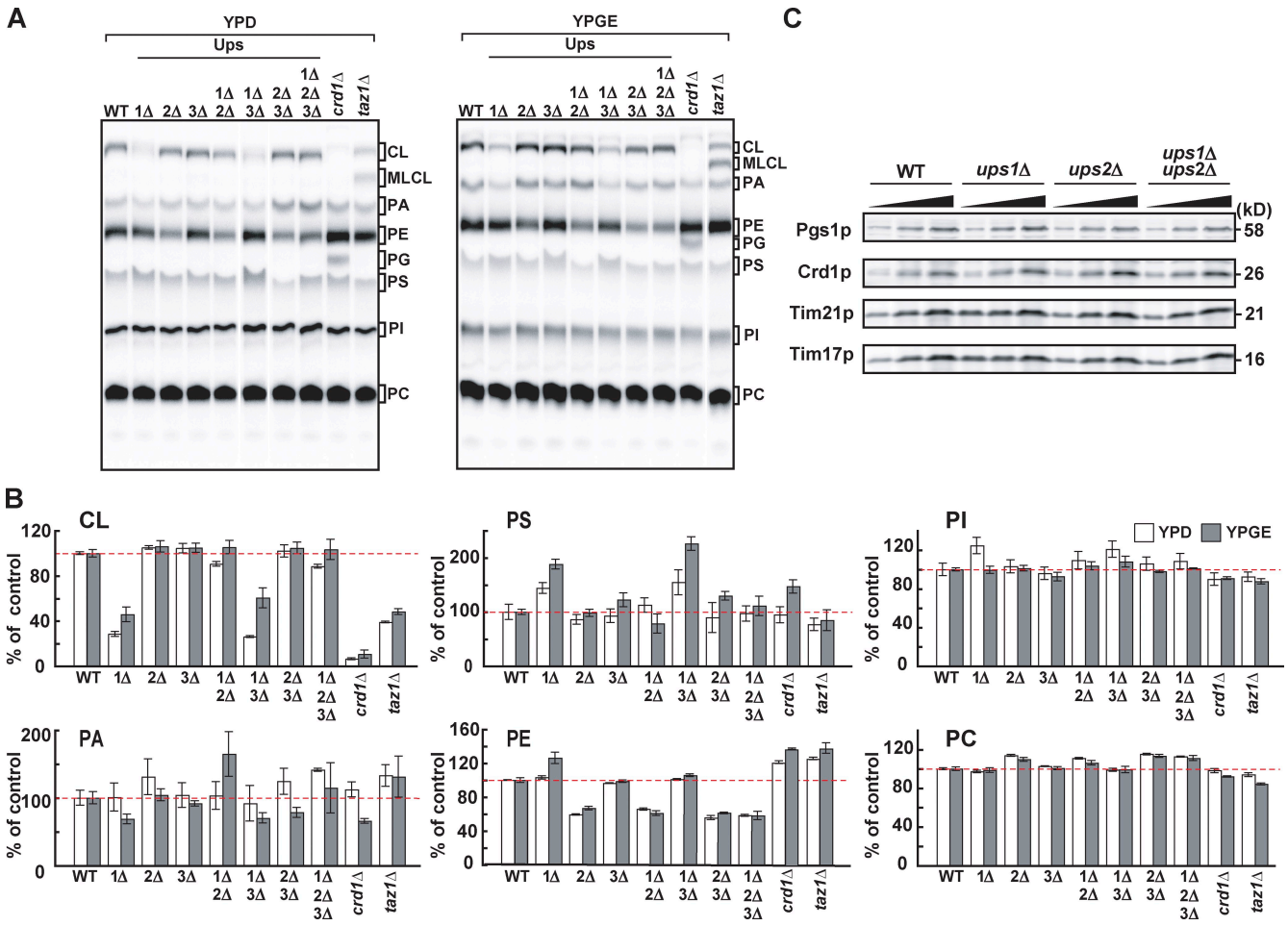

Figure 8. **Analysis of mitochondrial phospholipids in *ups* mutants.** (A) Cells lacking UPS1 (1Δ), UPS2 (2Δ), and/or UPS3 (3Δ) were grown in YPD or YPGE in the presence of ^{32}Pi. Phospholipids were extracted from crude mitochondrial fractions and separated on thin-layer chromatography. CL, cardiolipin; MLCL, monolysocardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine. (B) The relative amounts of phospholipids. Amounts of each lipid relative to total phospholipids were determined, and those detected in wild-type (WT) mitochondria were set to 100% (red dashed lines). Values are mean ± SEM (n = 3 in YPD and 4 in YPGE). (C) Mitochondria isolated from cells grown in YPLac were analyzed by immunoblotting.

response to reduced cardiolipin levels (Gu et al., 2004; Zhong et al., 2004). In contrast, decreased amounts of PE were observed in *ups2Δ*, *ups1Δups2Δ*, *ups2Δups3Δ*, and *ups1Δups2Δups3Δ* mitochondria, suggesting that Ups2p is required for the maintenance of normal levels of PE independent of Ups1p and Ups3p. In addition, we found that phosphatidylserine (PS) levels were increased in *ups1Δ* and *ups1Δups3Δ* mitochondria. In contrast, other phospholipids such as phosphatidylcholine, phosphatidylinositol, and phosphatidic acid were not affected considerably in *ups* mutant cells. Similar phospholipid compositions were observed in mitochondria isolated from YPD-grown cells (Fig. 8). As a control, the level of rate-limiting enzymes in cardiolipin biosynthesis was not affected in *ups1Δ* mitochondria, as confirmed by immunoblotting using antibodies against Crd1p and Pgs1p (Fig. 8 C). Thus, our data demonstrate that Ups1p and Ups2p control phospholipid levels, including cardiolipin, PE, and PS, in mitochondria and suggest that Ups proteins affect the assembly state of TIM23 translocase through altered levels of cardiolipin.

Because the assembly of AAC also depends on cardiolipin (Jiang et al., 2000; Claypool et al., 2008; Kutik et al., 2008), we examined AAC assembly in *ups* mutants. We imported ^{35}S-labeled AAC into isolated mitochondria and analyzed its assembly using blue native (BN) PAGE. As shown in Fig. 9 A, we found that the assembly of AAC was defective in *ups1Δ* mitochondria and that assembly was restored in *ups1Δups2Δ* mitochondria. In addition, we examined Tim23p and showed that its assembly was also defective (Fig. 9 B).

In Fig. 6, we demonstrate that Ups1p is required for protein–protein interactions within the TIM23 translocase. To determine whether this role of Ups1p is also mediated by cardiolipin, we performed similar coimmunoprecipitation experiments using *crd1Δ* cells expressing Tim23p-Flag in the presence of increasing amounts of Triton X-100 (Fig. 10). Similar to *ups1Δ* mitochondria, *crd1Δ* mitochondria showed higher sensitivity to increasing amounts of Triton X-100. In the absence of Triton X-100, similar amounts of Tim17p, Tim21p, and Tim50p coimmunoprecipitated with Tim23p-Flag in wild-type and *crd1Δ* mitochondria. Increasing Triton X-100 caused increased dissociation of Tim17p and Tim21p from Tim23p-Flag in *crd1Δ* mitochondria relative to wild-type mitochondria (Fig. 10). However, interaction between Tim23p-Flag and Tim50p was indistinguishable in wild-type and *crd1Δ* mitochondria regardless of the

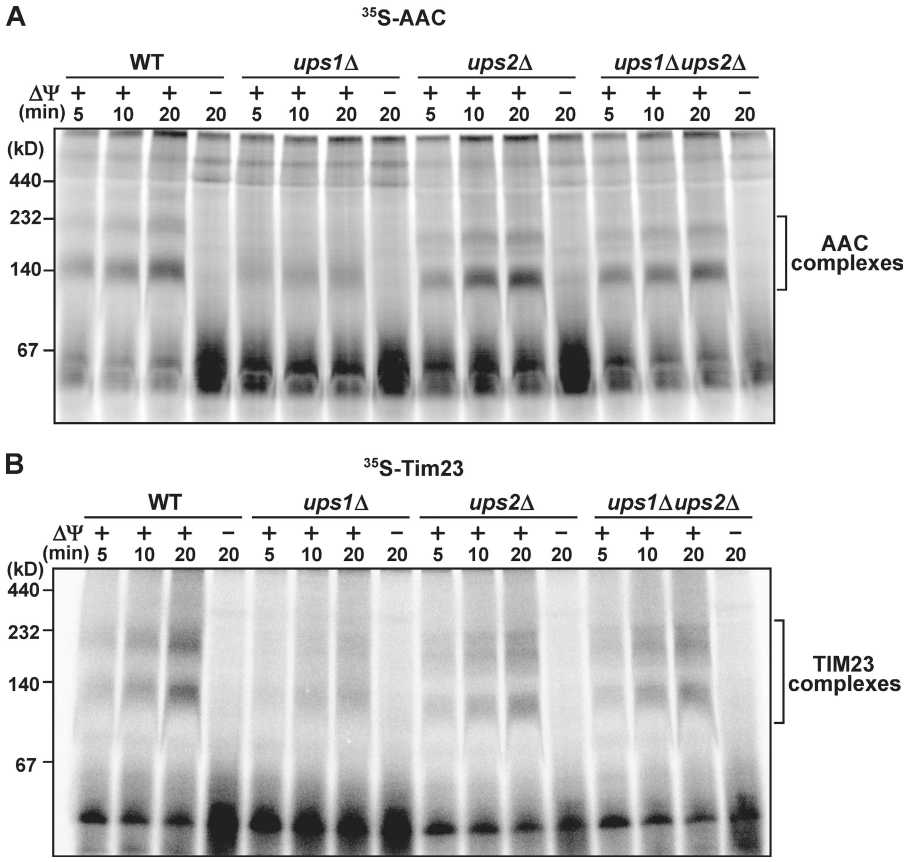

Figure 9. **Assembly of AAC and Tim23p in *ups* mutants.** (A and B) Mitochondria isolated from cells grown in YPLac were incubated with ^{35}S-labeled AAC (A) or Tim23 (B) proteins. The mitochondria were lysed with digitonin at the indicated time points and analyzed by BN-PAGE. As a negative control, 10 µg/ml valinomycin was added to the reactions (−ΔΨ). WT, wild type.

Triton X-100 concentration used. These results indicate that cardiolipin specifically stabilizes Tim23p interactions with Tim17p and Tim21p.

Discussion

In this study, we showed that Ups1p and Ups2p control the level of cardiolipin in mitochondria. Cardiolipin is a unique phospholipid with four fatty acid chains and is present mainly in the mitochondrial IM (Li et al., 2007). Within the IM, cardiolipin stabilizes the electron transport chain supercomplex between complexes III and IV through direct interaction of their subunits (Pfeiffer et al., 2003; Zhang et al., 2002, 2005). It has been proposed that cardiolipin can function as a flexible interface between these complexes (Schägger, 2002). In addition to the electron transport chain supercomplex, recent studies demonstrated that cardiolipin also functions to maintain other IM complexes, the TIM23 translocase (Kutik et al., 2008), and the AAC complex (Jiang et al., 2000; Claypool et al., 2008). In particular, Tam41p/Mmp37p, which is required for the TIM23–PAM association, mediates biosynthesis of cardiolipin (Tamura et al., 2006; Kutik et al., 2008). Similarly, mutants with decreased levels of cardiolipin exhibit destabilized TIM23–PAM complexes and compromised protein import (Jiang et al., 2000; Kutik et al., 2008). Our current data extend these findings and further substantiate the physiological importance of cardiolipin in TIM23 assembly and therefore protein import. Specifically, we found that cardiolipin mediates not only TIM23–PAM association but also protein–protein interactions within TIM23 translocase (Figs. 6 and 10). Our findings account for the requirement of cardiolipin in functional reconstitution of motor-free TIM23 translocase (van der Laan et al., 2007). Consistent with our study, a recent study has also shown that Ups1p and Ups2p/Gep1p regulate cardiolipin levels in mitochondria (Osman et al., 2009).

The mechanism by which Ups1p and Ups2p control cardiolipin levels remains to be determined. One possible role for Ups proteins is the regulation of enzyme activity within the cardiolipin biosynthetic pathway. For example, the cardiolipin synthase Crd1p is predicted to contain three transmembrane domains with an N-terminal presequence, thereby rendering it likely to possess a short loop in the IMS. The catalytic site of Crd1p is located on the matrix side of the IM (Schlame and Haldar, 1993). It is possible that Ups proteins regulate the catalytic activity of Crd1p through its IMS domain. Alternatively, Ups1p and Ups2p may antagonistically regulate the conversion of phosphatidylglycerol-phosphate to phosphatidylglycerol, which is a substrate of Crd1p. Another possible role for Ups proteins is to regulate lipid import into mitochondria. For example, PS is imported into mitochondria from the ER through physical connections between these two organelles followed by conversion to PE within mitochondria (Achleitner et al., 1999). Defects in phospholipid import may affect the levels of mitochondrial phospholipids. Because the level of PE is lower in *ups2Δ* mitochondria, Ups2p may facilitate import of PS into mitochondria from the ER. A third possibility is that these proteins sort

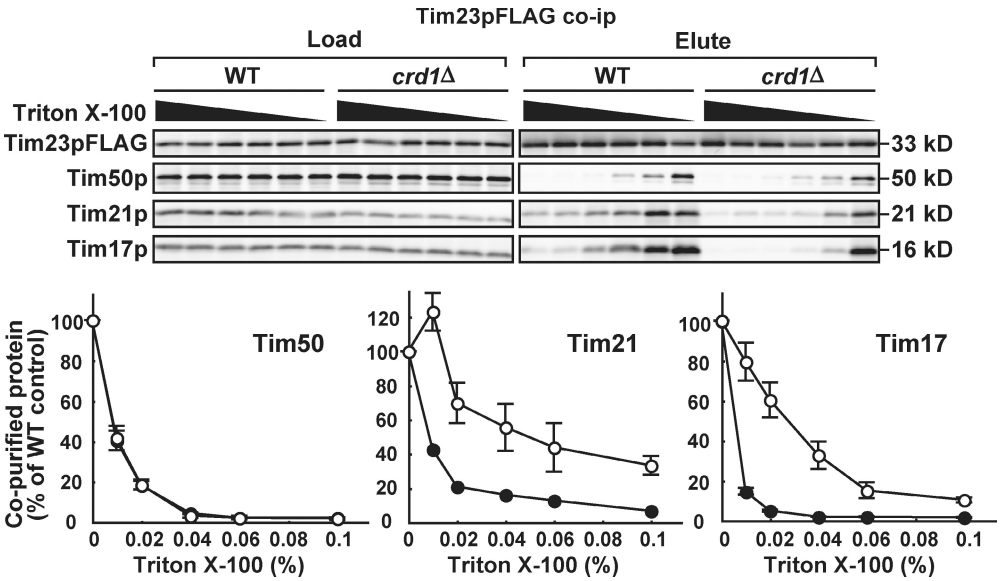

Figure 10. **Cardiolipin is required for interactions in the TIM23 translocase.** Wild-type (WT) and *crd1Δ* mitochondria expressing Tim23p-Flag were solubilized in digitonin buffer containing the indicated concentrations of Triton X-100 and then incubated with anti-Flag agarose. 10% of lysate (Load) and 100% of bound proteins (Elute) were analyzed by immunoblotting. Relative amounts of protein were quantitated and normalized to samples that were not treated with Triton X-100. Values are mean ± SEM (n = 3). co-ip, coimmunoprecipitation.

phospholipids within mitochondria. For example, to be delivered to the site of biosynthesis and function, phospholipids may be transferred from one leaflet to another as well as from the OM to the IM at contact sites where both membranes are positioned closely (Ardail et al., 1991; Epand et al., 2007). Ups proteins may mediate these processes in the IMS. Finally, Ups proteins may regulate the degradation of cardiolipin and other phospholipids. It is possible that synthesis of cardiolipin and PE is not affected in *ups* mutants, but that these lipids are instead degraded rapidly. A better understanding of Ups function in the maintenance of phospholipids requires further study.

A previous study showed that the level of *CRD1* mRNA was increased in nonfermentable carbon sources (Su and Dowhan, 2006). Consistent with this, we found that the level of cardiolipin was higher when *ups1Δ* cells were grown in YPGE than in YPD (Fig. 8). Similarly, cell growth, mitochondrial membrane potential, and protein import also improved in YPGE-grown *ups1Δ* cells compared with YPD-grown *ups1Δ* cells. Increased levels of cardiolipin could partially stabilize protein complexes in the IM and improve cell physiology even in the absence of Ups1p. To further support this idea, decreased levels of TIM22 translocase subunits (e.g., Tim22p, Tim18p, and Tim54p) were observed in YPD but not in YPGE possibly because of compromised integrity of TIM22 translocase. Interestingly, Tam41p, which is required for cardiolipin biosynthesis (Kutik et al., 2008), was up-regulated in *ups1Δ* cells in YPD, suggesting a physiological compensation pathway when cardiolipin levels are decreased.

We have shown that the Ups1p and Ups2p play important roles in mitochondrial morphogenesis in addition to mitochondrial import and the control of phospholipid levels. Although mitochondria appeared to be fragmented in *ups1Δ* cells, mitochondrial fusion was not grossly defective as mitochondrial fusion occurs during yeast mating (Sesaki et al., 2006). We also found that the levels of two fusion components, Ugo1p and Fzo1p, are not reduced and that exogenous expression of s-Mgm1p does not restore tubular mitochondria (Sesaki et al., 2006). In contrast to mitochondria in *ups1Δ* cells, mitochondria in double and triple *ups* mutants formed small net- and ringlike structures. In some cells, short tubules were extended from these mitochondria. These morphologies are similar to those seen in *mdm31Δ* and *mdm32Δ* cells (Dimmer et al., 2005). Mdm31p and Mdm32p are IM proteins that have been suggested to play roles in mitochondrial morphology, mitochondrial DNA inheritance, and maintenance of cardiolipin levels (Dimmer et al., 2005; Osman et al., 2009). Interestingly, Mdm31p and Mdm32p genetically interact with Mmm1p and Mdm10p, which are OM proteins involved in mitochondrial morphogenesis, protein import, and phospholipid maintenance (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997; Hobbs et al., 2001, Meisinger et al., 2004, 2006, 2007; Osman et al., 2009). Thus, it is possible that Ups1p and Ups2p, in collaboration with these proteins, may link phospholipid regulation and morphogenesis.

The function of Ups1p is evolutionarily conserved among eukaryotes (Sesaki et al., 2006). In the human genome, there are four proteins related to yeast Ups proteins. Of these, PRELI (protein of relevant evolutionary lymphoid interest) shows the highest homology to Ups1p and can functionally replace Ups1p in yeast cells. Exogenous expression of PRELI has been shown to rescue the levels of s-Mgm1p, the mitochondrial morphology defect, and the growth defect in yeast *ups1Δ* cells (Sesaki et al., 2006). In addition, we found that PRELI can restore cardiolipin levels in *ups1Δ* cells (unpublished data). These observations suggest that PRELI regulates cardiolipin levels in human mitochondria. PRELI is a mitochondrial protein (Fox et al., 2004; Sesaki et al., 2006) that is highly expressed in the liver, lymph node, and leukocytes (Guzman-Rojas et al., 2000). A recent study showed that PRELI

also regulates apoptosis (Tahvanainen et al., 2009). It would be of interest to directly determine whether PRELI is required for the maintenance of cardiolipin levels and whether the functions of Ups2p and Ups3p are also conserved in humans.

Materials and methods

Strains, media, and genetic methods

Yeast strains used in this study are listed in Table S1. Complete disruption of *UPS1*, *UPS2*, *UPS3*, *TIM21*, *PAM17*, and *CRD1* was accomplished by PCR-mediated gene replacement with a pair of primers, 1729/1730, 1744/1745, 1742/1743, PTM64/PTM65, PTM87/PTM88, and PTM185/PTM186, respectively (Brachmann et al., 1998). The *HIS3*, *URA3*, and *kanMX4* genes from the pRS303, pRS306, and pRS400 plasmids were used as disruption markers (Brachmann et al., 1998). The Flag-tagged Ups strains and Tim22 strain were constructed by homologous recombination in FY833 using the Flag-kanMX4 cassette from p3Flag-kanMX (Gelbart et al., 2001) and pTYE247 (Tamura et al., 2006), respectively, with a pair of primers, HS23/HS24 (Ups1p-Flag), HS25/HS26 (Ups2p-Flag), HS27/HS28 (Ups3p-Flag), and PTM176/PTM177 (Tim22p-Flag). The sequences of the primers are listed in Table S2. Cells were grown in YPD (1% yeast extract, 2% polypeptone, and 2% glucose), YPGE (1% yeast extract, 2% polypeptone, 2% glycerol, and 3.2% ethanol), or YPLac (1% yeast extract, 2% polypeptone, and 3% lactic acid, pH 5.6). Cells that have the *kanMX4* gene were selected on YPD containing 500 µg/ml G418. Standard genetic techniques were used (Adams et al., 1997).

In vitro protein import

Mitochondria were isolated from wild-type (FY833), *ups1Δ* (YRJ2011), *ups2Δ* (YHS0001), and *ups1Δups2Δ* (YHS0005) cells grown in YPLac at 30°C as described previously (Tamura et al., 2006). Radiolabeled precursor proteins were synthesized using rabbit reticulocyte lysate by coupled transcription/translation in the presence of [^{35}S]methionine (Promega; Sesaki et al., 2006). Isolated mitochondria were incubated with radiolabeled precursor proteins in 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 $MgCl_2$, 2 mM ATP, 2 mM NADH, 1% BSA, 0.5 mM creatine phosphate, and 0.1 mg/ml creatine kinase) at 25°C. The import reaction was stopped by addition of 10 µg/ml valinomycin. To remove nonimported proteins, the reaction mixture was treated with 50 µg/ml proteinase K for 20 min on ice. Proteinase K was then inactivated by addition of 1.5 mM PMSF. Mitochondria were isolated by centrifugation, and the imported proteins were resolved by SDS-PAGE and radio imaging using a molecular imager (PharosFX Plus; Bio-Rad Laboratories) and Quantity One (Bio-Rad Laboratories) and Photoshop (Adobe) softwares.

Microscopy

Cells were observed using a microscope (Axioskop; Carl Zeiss, Inc.) with a 100× NA 1.3 Plan-Neofluar objective. Fluorescence and differential interference contrast images were captured with a digital camera (Orca ER; Hamamatsu Photonics) using Open Laboratory software version 3.0.8 (PerkinElmer) and processed with Photoshop software.

Coimmunoprecipitation

500 µg of mitochondria was solubilized at 2 mg protein/ml in digitonin buffer (1% digitonin, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% [vol/vol] glycerol, 0.1 mM EDTA, and 1 mM PMSF). After centrifugation at 16,100 *g* for 15 min, 200 µl of the supernatant was diluted with 800 µl of coimmunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% [vol/vol] glycerol, 0.1 mM EDTA, and 1 mM PMSF) containing 10 µl of anti-Flag M2-agarose (Sigma-Aldrich). 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, 1 mM PMSF, and protease inhibitor cocktail [Sigma-Aldrich]), and then the bound proteins were eluted by boiling in SDS-PAGE sample buffer. The eluted proteins were resolved by SDS-PAGE followed by immunoblotting. For immunoblotting, proteins were visualized by fluorophores conjugated with secondary antibodies (ZyMax goat anti–rabbit IgG [H + L] Cy5 conjugate and/or Alexa Fluor 488 goat anti–mouse IgG [H + L]; Invitrogen) and analyzed using a PharosFX Plus molecular imager and Quantity One and Photoshop softwares. For immunoprecipitation in the presence of Triton X-100, up to 0.1% Triton X-100 was included in both the digitonin buffer and wash buffer.

Density gradient centrifugation

Mitochondria were solubilized at 2 mg protein/ml in digitonin buffer for 20 min on ice and then centrifuged at 16,100 *g* for 15 min. The 200-µl supernatant was placed onto a 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 and then centrifuged at 45,000 rpm for 15 h in a rotor (SW55Ti; Beckman Coulter) at 4°C. After centrifugation, 270-µl fractions were collected from the top. Proteins were precipitated with 10% TCA and then resolved by SDS-PAGE followed by immunoblotting.

Phospholipid analysis

Yeast cells were diluted to an OD_{600} = 0.02 in 2 ml YPD in the presence of 10 µCi/ml ^{32}Pi and cultivated for 20 h. When YPGE was used, cells were diluted to an OD_{600} = 0.1 and grown for 36 h. Phospholipids were extracted from crude mitochondrial fractions and separated by thin-layer chromatography as previously described (Vaden et al., 2005; Claypool et al., 2006). To examine the level of Crd1p and Pgs1p, we performed immunoblotting of isolated mitochondria using antibodies to these proteins (gifts from N. Pfanner, N. Gebert, and N. Wiedemann, University of Freiburg, Freiburg, Germany).

BN-PAGE

150 µg of mitochondria was solubilized in 40 µl 1.0% digitonin buffer (1.0% digitonin, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10% glycerol) for 20 min on ice. After removal of insoluble material by centrifugation at 15,700 *g* for 20 min, the supernatant was mixed with 2 µl of 20× BN buffer (4% Coomassie brilliant blue G250 and 100 mM 6-aminohexanoic acid) and subjected to BN-PAGE (5–13% gradient gel with 4% stacking gel) for 200 min at either 250 V or 7 mA. Anode and cathode buffer for BN-PAGE was prepared according to Wittig et al. (2006).

Online supplemental material

Fig. S1 shows that the precursor of Hsp60p and Mdj1p is accumulated in the cytosol and mitochondrial surface in *ups1Δ* cells. Fig. S2 shows the effects of Tim21p and Pam17p loss on steady-state levels of Hsp60p in *ups* mutants. Fig. S3 shows that Ups1p and Ups2p do not interact with components of the TIM23 translocase and PAM. Tables S1 and S2 show the yeast strains and PCR primers used in this study, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200812018/DC1.

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