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RESEARCH ARTICLE

# Figures and figure supplements

Independent evolution of functionally exchangeable mitochondrial outer membrane import complexes

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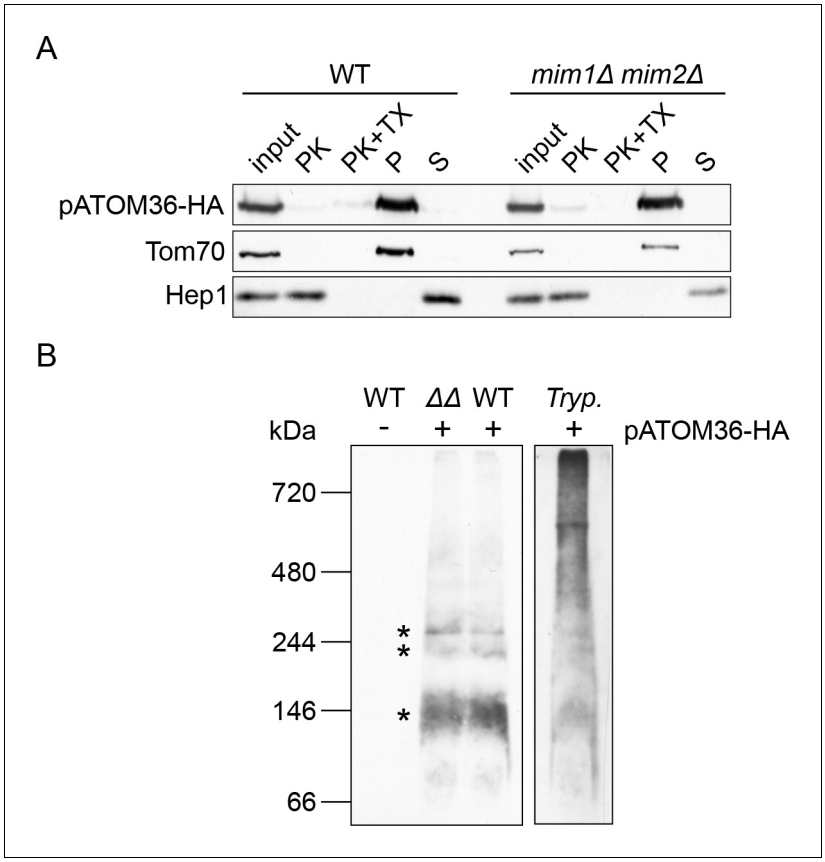


**Figure 1.** pATOM36 forms native-like complexes in the yeast mitochondrial OM. (**A**) Mitochondria isolated from WT or *mim1Δ/mim2Δ* cells expressing pATOM36-HA were left intact or lysed with Triton X-100 (TX) before they were subjected to treatment with proteinase K (PK). Alternatively, other samples were subjected to alkaline extraction followed by separation by centrifugation to pellet (P) and supernatant (S) fractions. All samples were analysed by SDS-PAGE followed by immunodecoration with antibodies against the HA-epitope, the OM receptor protein Tom70, or the matrix soluble protein Hep1. (**B**) Mitochondria were isolated from yeast WT cells transformed with an empty plasmid (-) or from WT and *mim1Δ/mim2Δ* (*ΔΔ*) cells expressing pATOM36-HA (+). Isolated yeast organelles and mitochondria-enriched fraction from *T. brucei* (Tryp.) cells expressing pATOM36-HA were lysed with 1% digitonin. All samples were then subjected to BN-PAGE followed by immunodecoration with an antibody against the HA-tag. pATOM36-containing complexes are indicated with an asterisk.

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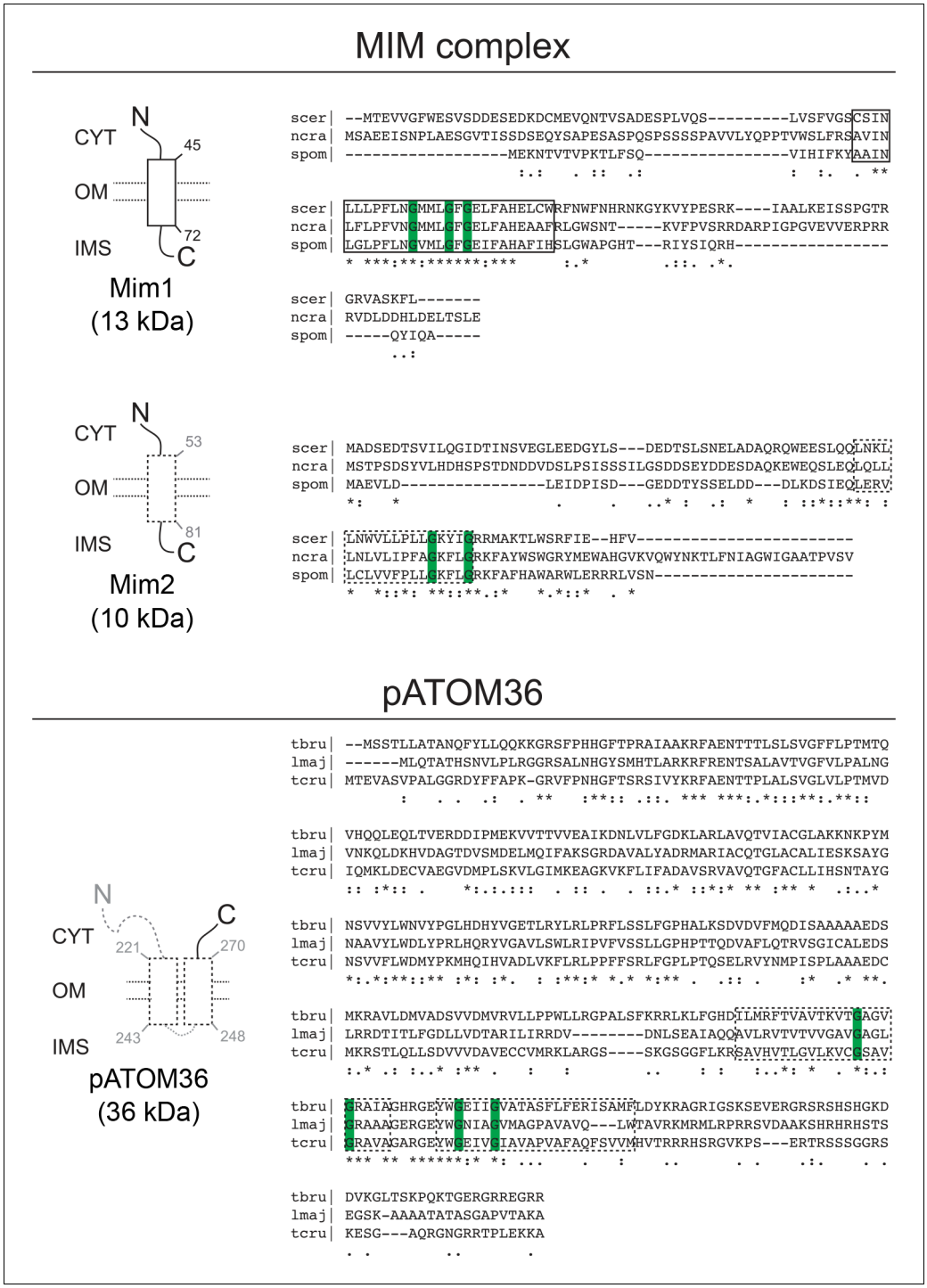


**Figure 1—figure supplement 1.** Topologies and protein sequence alignments of Mim1, Mim2 and pATOM36. Left panels: Schematic representation of the experimentally determined and predicted topologies of Mim1, Mim2 and pATOM36. The transmembrane segment (TMS) of Mim1 has been experimentally characterised (solid line), whereas those of Mim2 and pATOM36 are predicted by PolyPhobius with TOPCONS (dashed lines). The amino acid positions of the TMSs are illustrated and the protein sizes are in brackets. CYT, cytosol; OM, outer mitochondrial membrane; IMS, intermembrane space. Right panels: Multiple protein sequence alignments of Mim1, Mim2 and pATOM36. Verified and predicted TMSs are highlighted by solid and dashed lines, respectively. Conserved glycine residues are marked in green. scer, *Saccharomyces cerevisiae*; ncra, *Neurospora crassa*; spom, *Schizosaccharomyces pombe*; tbru, *Trypanosoma brucei*; lmaj, Leishmania major; tcru, *Trypanosoma cruzi*.
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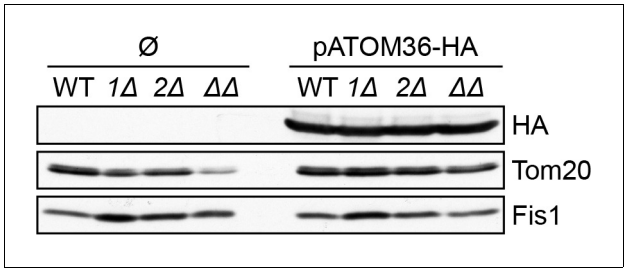


**Figure 1—figure supplement 2.** pATOM36-HA is expressed in the transformed cells. Whole cell lysate of wild type (WT), mim1Δ (1Δ), mim2Δ (2Δ) and mim1Δmim2Δ (ΔΔ) cells transformed with either an empty plasmid (Ø) or a plasmid encoding for pATOM36-HA were obtained. The samples were analysed by SDS-PAGE and immunodecoration with the indicated antibodies.

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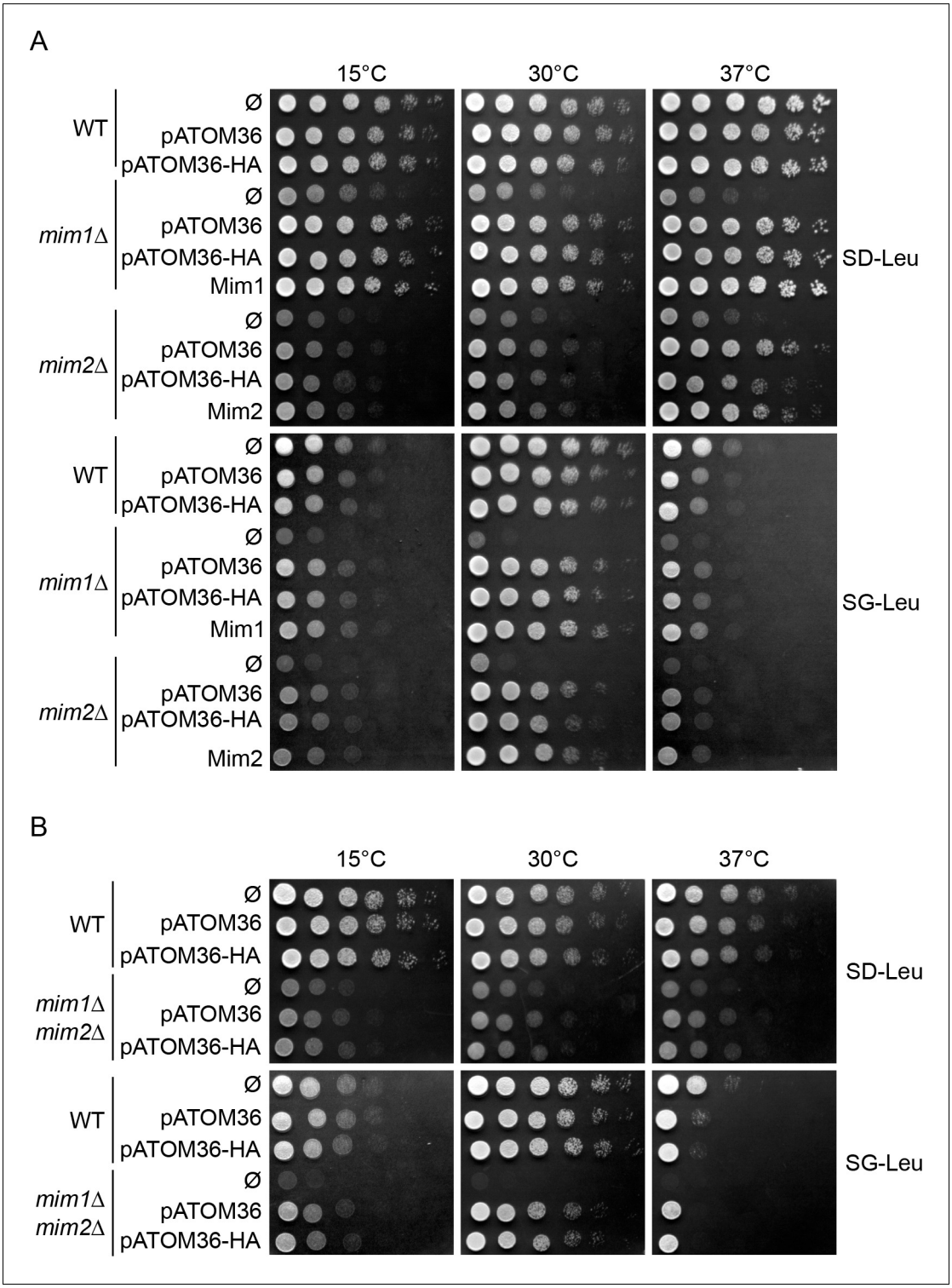


**Figure 2.** pATOM36 rescues the growth defects of cells lacking Mim1, Mim2 or both. (**A**) The indicated strains transformed with an empty plasmid (Ø) or with a plasmid expressing pATOM36 or its HA-tagged variant were tested at three different temperatures by drop-dilution assay for growth on synthetic medium containing either glucose (SD-Leu) or glycerol (SG-Leu). For comparison, plasmid-encoded Mim1 or Mim2 were transformed into *mim1Δ* or *mim2Δ* cells, respectively. All dilutions are in fivefold increment. (**B**) Cells deleted for both *MIM1* and *MIM2* (*mim1Δ/mim2Δ*) were transformed with the empty plasmid (Ø) or a plasmid encoding either native pATOM36 or pATOM36-HA. Transformed cells were analysed by drop-dilution assay at the indicated temperatures on synthetic medium containing either glucose (SD-Leu) or glycerol (SG-Leu). All dilutions are in fivefold increment.

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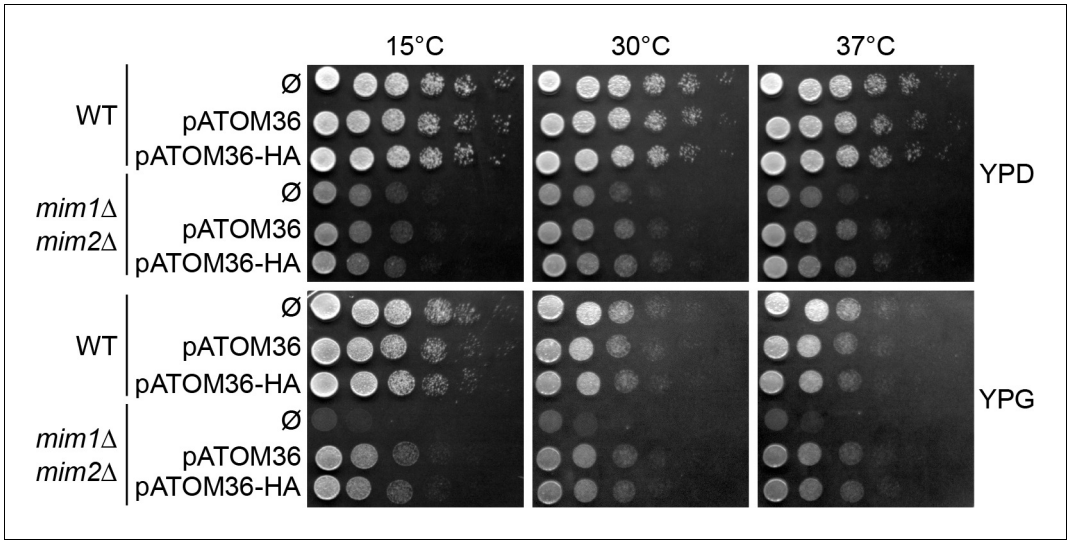


**Figure 2—figure supplement 1.** pATOM36 rescues the growth defect of mim1Δmim2Δ cells. The indicated strains transformed with an empty plasmid (Ø), a plasmid expressing pATOM36, or its HA-tagged variant were tested at three different temperatures by drop-dilution assay for growth on rich media containing either glucose (YPD) or glycerol (YPG). All dilutions are in fivefold increment.

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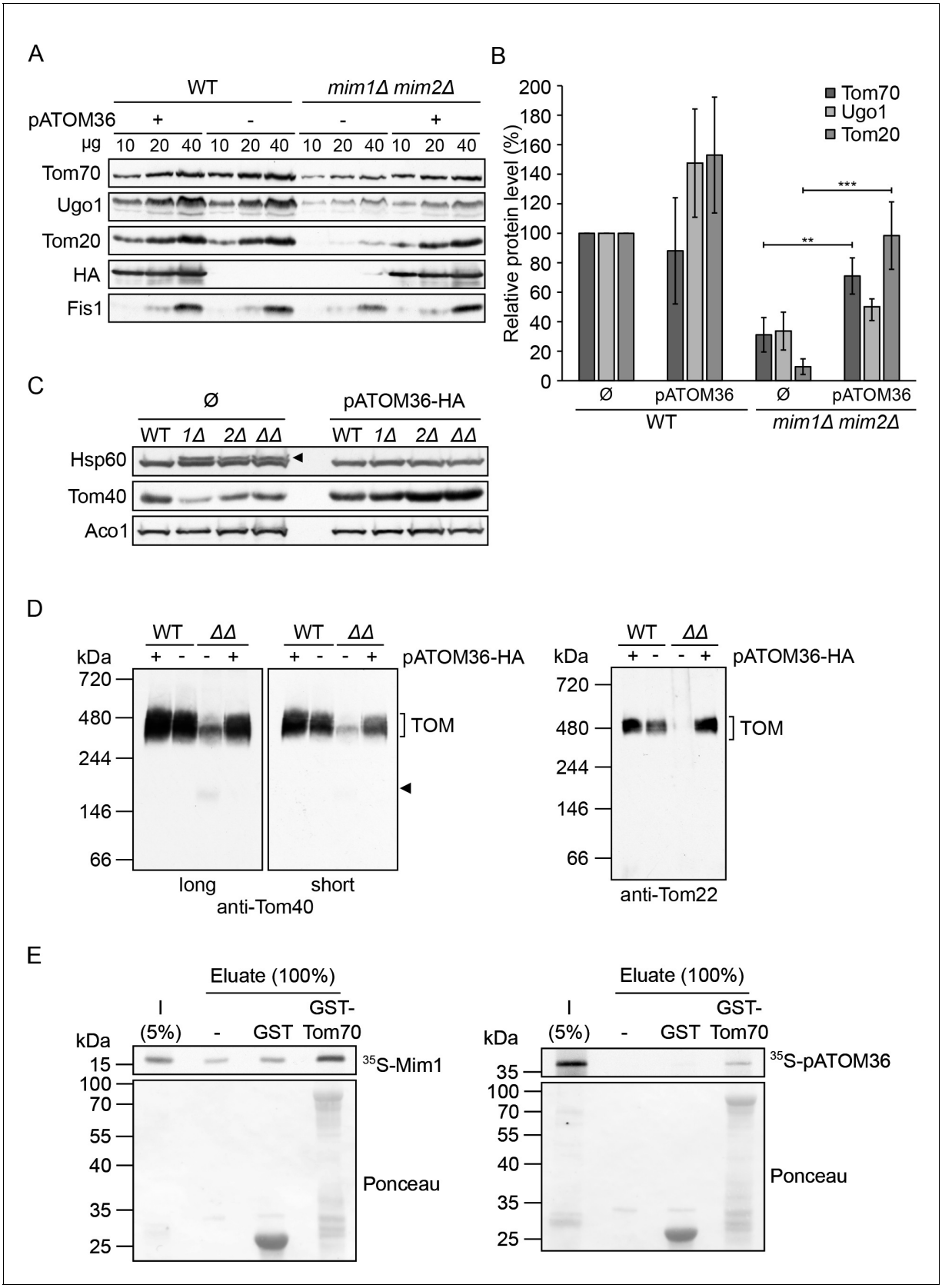


**Figure 3.** pATOM36 can compensate for the reduced steady state levels and assembly defects in cells lacking both Mim1 and Mim2. (**A**) Mitochondria were isolated from WT or *mim1Δ/mim2Δ* cells transformed with either an empty plasmid (-) or with a plasmid encoding pATOM36-HA (+). The

*Figure 3 continued on next page*

*Figure 3 continued*

specified amounts were analysed by SDS-PAGE and immunodecoration with antibodies against either the indicated mitochondrial proteins or the HA-tag. (**B**) The intensity of the bands from three independent experiments such as those presented in (**A**) was monitored. The amounts of Tom70, Ugo1 and Tom20 in the various mitochondria samples are presented as mean percentage of their levels in control organelles (WT+ Ø). The levels of Fis1 were taken as loading control. Error bars represent ± SD. **p≤0.005, ***p≤0.0005. (**C**) Whole cell lysates were obtained from WT, *mim1Δ (1Δ), mim2Δ (2Δ),* or the double deletion *mim1Δ/mim2Δ (ΔΔ)* cells transformed with either an empty plasmid (Ø) or with a plasmid encoding pATOM36-HA. Samples were analysed by SDS-PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. The precursor form of mitochondrial Hsp60 is indicated with an arrowhead. (**D**) The mitochondria described in (**A**) were solubilised in a buffer containing 1% digitonin and then analysed by BN-PAGE followed by western blotting. The membranes were immunodecorated with antibodies against the TOM subunits, Tom40 (long and short exposures) and Tom22. The TOM complex is signposted. A Tom40-containing low molecular weight complex is indicated with an arrowhead. (**E**) Mim1 and pATOM36 interact directly with Tom70. Radiolabelled Mim1 or pATOM36 (input, **I**) were incubated with glutathione beads (-) or with beads that were pre-bound to recombinant GST alone or to GST fused to the cytosolic domain of Tom70 (GST-Tom70). After washing, bound material was eluated and proteins were analysed by SDS–PAGE followed by blotting onto a membrane, and detection with either autoradiography (upper panel) or Ponceau staining (lower panel).

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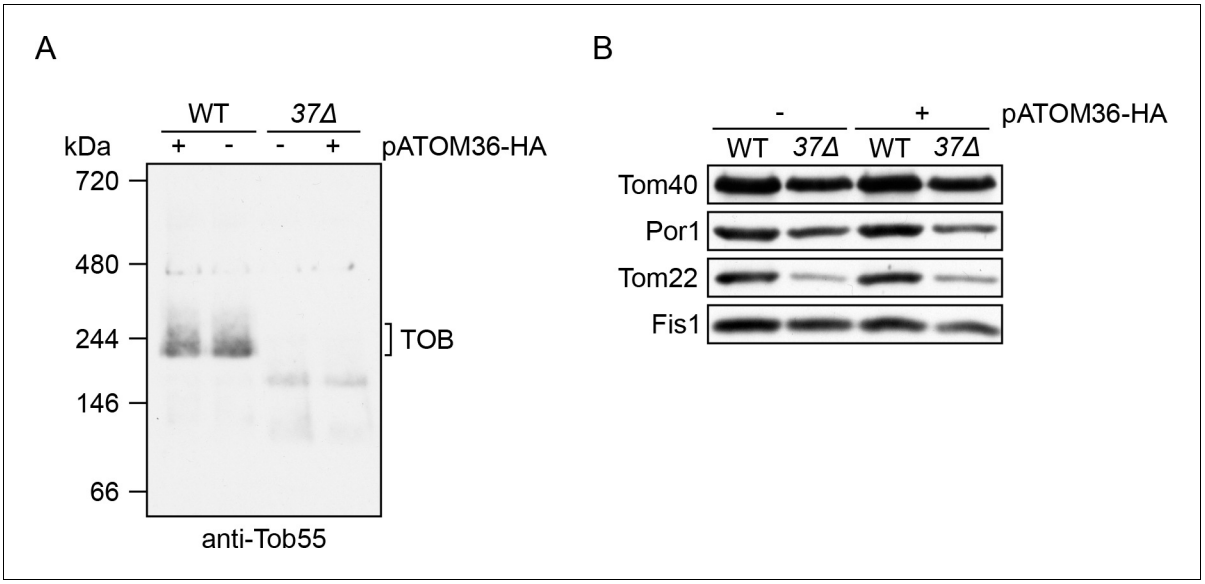


**Figure 3—figure supplement 1.** pATOM36-HA does not rescue biogenesis defects in mas37Δ cells. (**A**) Mitochondria isolated from wild type (WT) and mas37Δ (37Δ) cells transformed with either an empty plasmid (-) or a plasmid encoding for pATOM36-HA (+) were solubilised in 0.2% Triton X-100. Samples were analysed by BN-PAGE and immunodecoration with an antibody against Tob55. (**B**) Isolated mitochondria as in (**A**) were subjected to SDS-PAGE and immunodecoration with the indicated antibodies.

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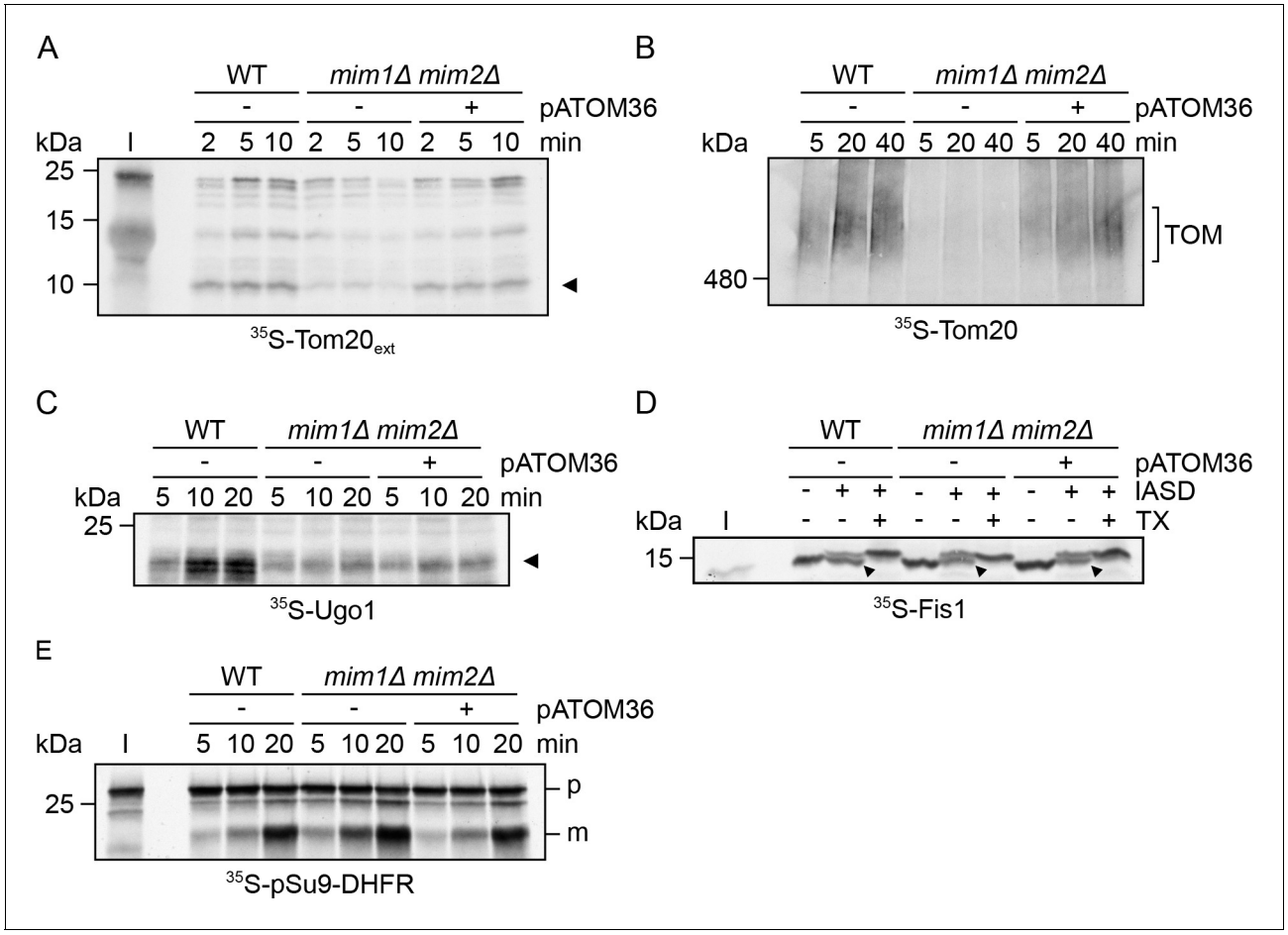


**Figure 4.** pATOM36 can rescue some of the import defects of cells lacking the MIM complex. (**A**) Mitochondria were isolated from WT cells transformed with an empty plasmid (WT-) or from *mim1Δ/mim2Δ* cells transformed with either an empty plasmid (-) or with a plasmid encoding pATOM36-HA (+). Radiolabelled $Tom20_{ext}$ molecules (5% input, I) were incubated with the indicated isolated organelles for the specified time periods. Then, mitochondria were treated with PK and analysed by SDS-PAGE and autoradiography. A proteolytic fragment of $Tom20_{ext}$, which reflects correct membrane integration, is indicated by an arrowhead. (**B**) Radiolabelled Tom20 was incubated with isolated mitochondria as in (**A**). At the end of the import reactions, mitochondria were solubilised with 0.2% digitonin and samples were analysed by BN-PAGE followed by autoradiography. The migration of Tom20 molecules assembled into the TOM complex is indicated. (**C**) Radiolabelled Ugo1 was incubated with isolated mitochondria as in (**A**). Then, mitochondria were treated with trypsin and analysed by SDS-PAGE and autoradiography. A proteolytic fragment of Ugo1, which reflects correct membrane integration, is indicated by an arrowhead. (**D**) Radiolabelled Fis1-TMC (5% input, I) was incubated with isolated mitochondria as in (**A**). Then, mitochondria were subjected to an IASD assay, re-isolated and analysed by SDS-PAGE and autoradiography. Bands representing correctly integrated Fis1-TMC are marked by an arrowhead. (**E**) Radiolabelled pSu9-DHFR (5% input, I) was incubated with isolated mitochondria as in (**A**). Then, mitochondria were re-isolated and analysed by SDS-PAGE and autoradiography. The precursor and mature forms are indicated by p and m, respectively.

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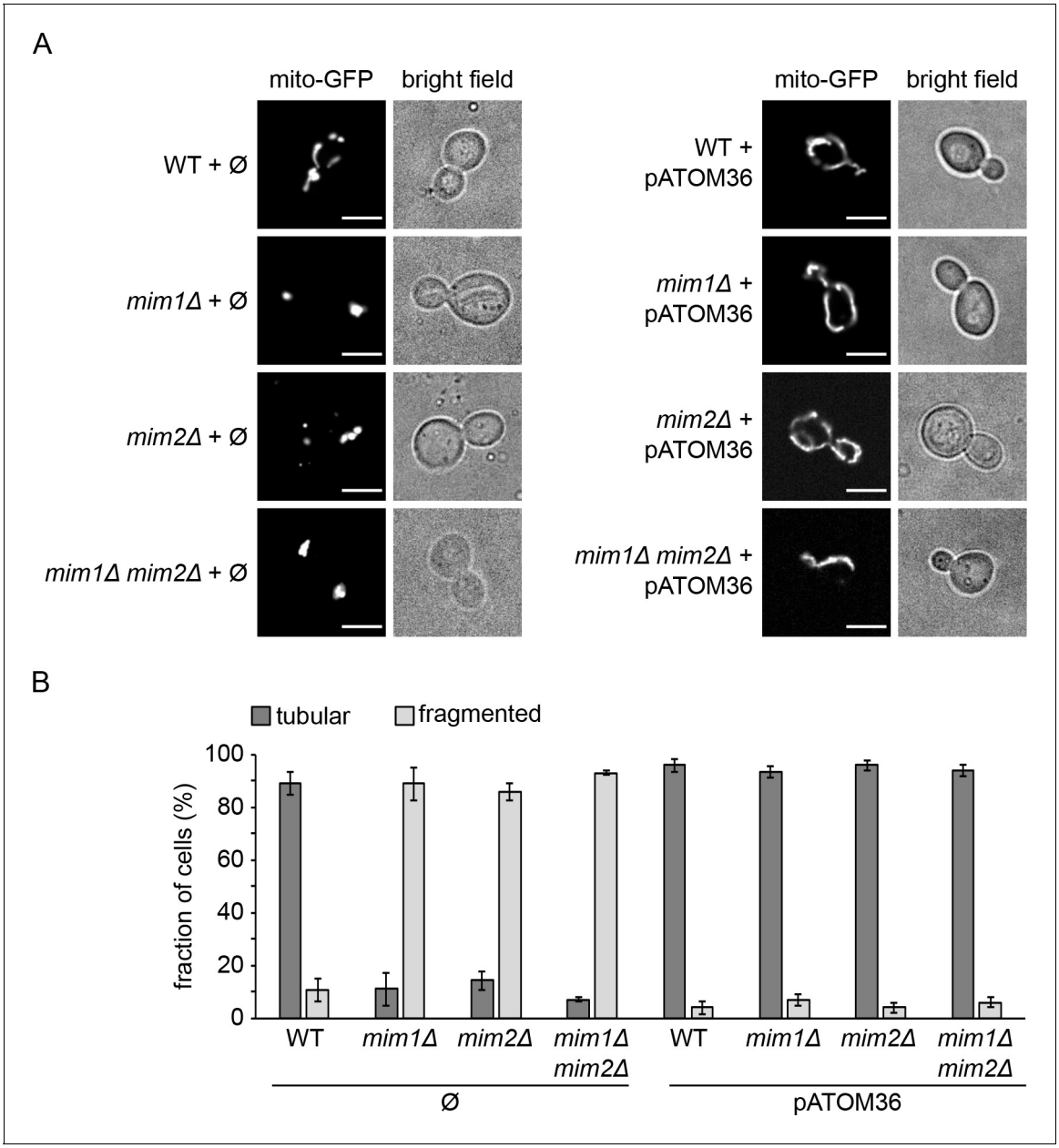


**Figure 5.** *mim1Δ* and *mim2Δ* cells expressing pATOM36 do not show altered mitochondrial morphology. (**A**) WT, *mim1Δ*, *mim2Δ*, and *mim1Δ/mim2Δ* cells harbouring mitochondria-targeted GFP (mito-GFP) were transformed with either an empty plasmid (Ø) as a control (left panels) or a plasmid encoding pATOM36 (right panels). Cells were analysed by fluorescence microscopy and representative images of the predominant morphology for each strain are shown. Scale bar, 5 µm. (**B**) Statistical analysis of the cells described in (**A**). Average values with standard deviation bars of three independent experiments with at least n = 100 cells in each experiment are shown.

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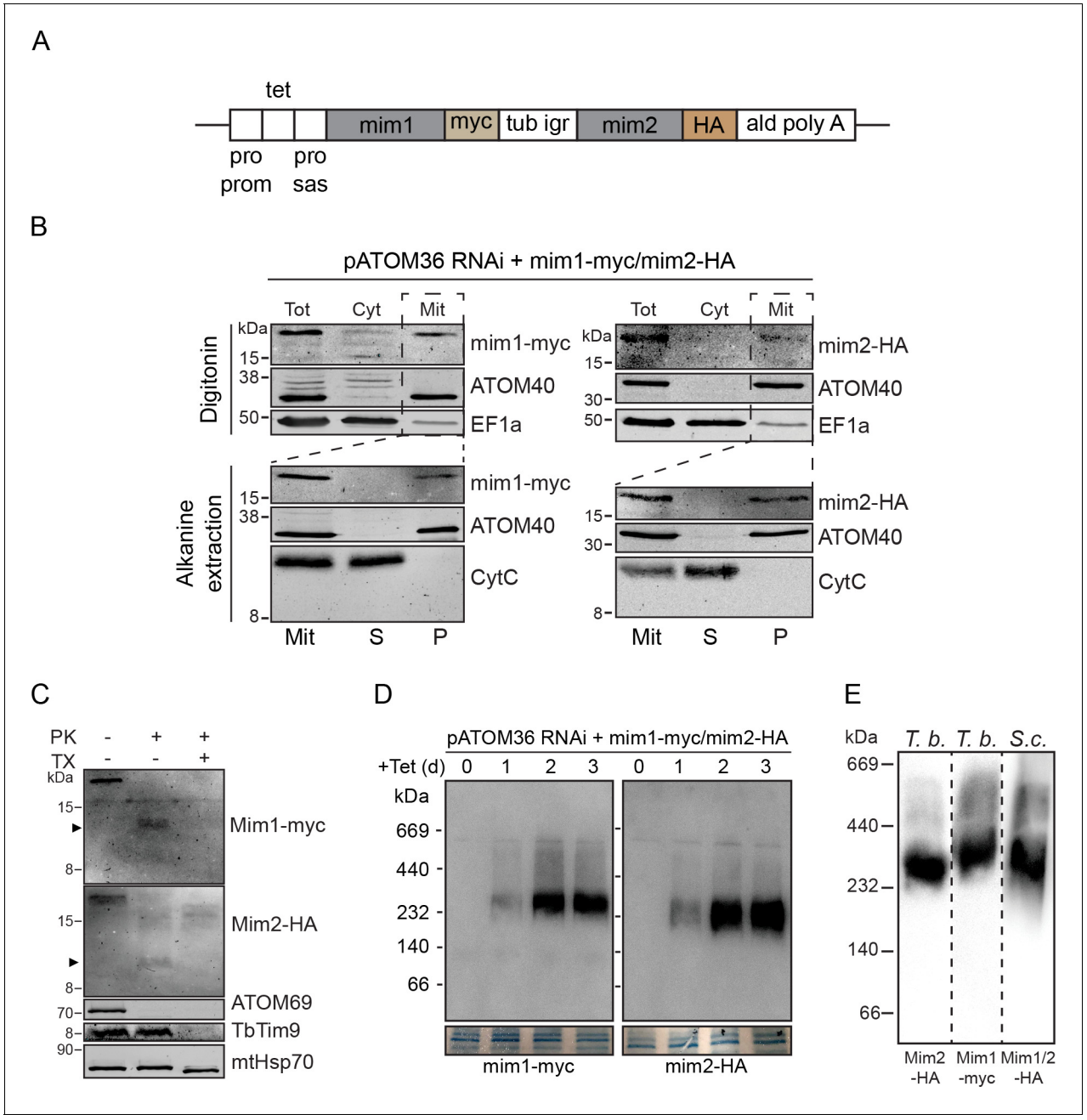


**Figure 6.** Yeast Mim1 and Mim2 form a high-molecular-weight complex in mitochondria of *T. brucei*. (**A**) Schematic representation of the insert of the pLew100-based vector that allows tetracycline-inducible expression of C-terminally myc-tagged Mim1 and HA-tagged Mim2 in *T. brucei*. Pro prom, procyclin promotor; tet, tetracycline operator; pro sas, procycline splice acceptor site; tub igr, $\alpha$- and $\beta$-tubulin intergenic region; ald polyA, 3'-UTR of the aldolase gene. (**B**) Top panels: immunoblot analysis of whole cells (Tot), soluble (Cyt) and digitonin-extracted mitochondria-enriched pellet (Mit) fractions of a tetracycline-inducible pATOM36-RNAi cell line expressing Mim1-myc and Mim2-HA. Duplicate blots were analysed for the expression of Mim1-myc (left panels) and Mim2-HA (right panels). ATOM40 and EF1a serve as mitochondrial and cytosolic markers, respectively. Bottom panels: Alkaline extraction of the mitochondria-enriched fraction (Mit) shown in the top panels. The pellet (**P**) and the supernatant (**S**) fractions corresponding to integral membrane and soluble proteins, respectively, were analysed by SDS-PAGE and immunodecoration. ATOM40 and CytC serve as markers for integral and peripheral membrane proteins, respectively. (**C**) Mitochondria-enriched fractions of the same cell line describe in (**B**) were left intact or lysed with Triton X-100 (TX) before they were subjected to treatment with proteinase K (PK). All samples were analysed by SDS-PAGE followed by immunodecoration with antibodies against myc and HA tags, the OM protein ATOM69, the IMS protein TbTim9, or the matrix protein mtHsp70. Note that mtHsp70 contains a folded core, which is protease resistant. A proteolytic fragment of Mim1 and Mim2 is indicated with an arrowhead. (**D**) Duplicate immunoblots from BN-PAGE analysis of mitochondria-enriched fractions of the same cell line describe in (**B**) were probed for Mim1-myc (left panels) and Mim2-HA (right panels). Sections of the coomassie-stained gels serve as loading control. (**E**) Immunoblots of a BN-PAGE analysis of

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mitochondria-enriched fractions of the *T. brucei* (*T.b.*) cell line simultaneously expressing myc-tagged Mim1 (Mim1-myc) and HA-tagged Mim2 (Mim2-HA) and isolated yeast (*S.c.*) mitochondria simultaneously expressing HA-tagged versions of Mim1 and Mim2. The immunoblots are probed with antibodies against HA- or myc-tag.

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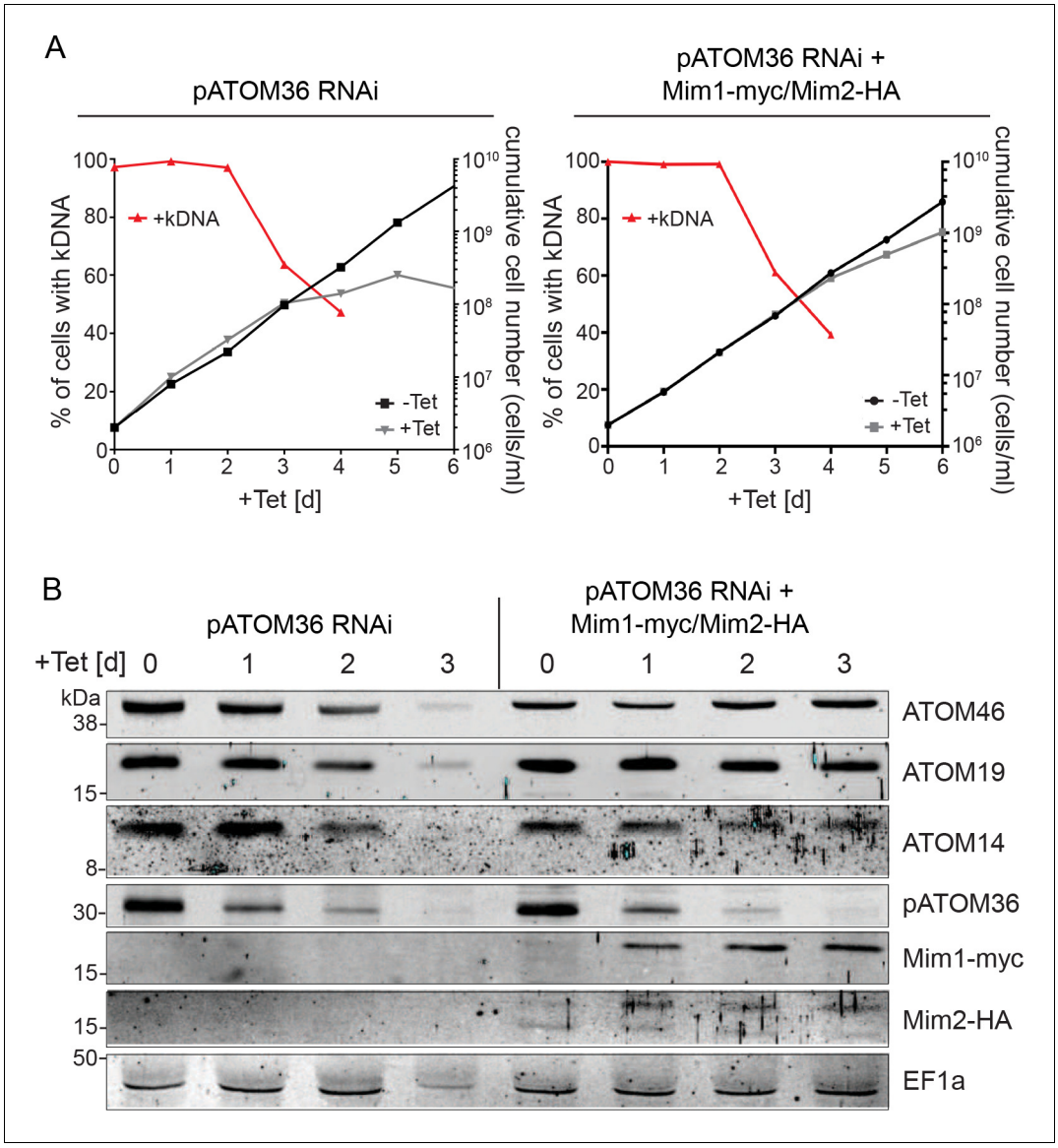


**Figure 7.** Yeast Mim1 and Mim2 complement the mitochondrial OM biogenesis phenotype of *T. brucei* cells ablated for pATOM36. (**A**) Left panel: growth in the presence and absence of tetracycline (black and grey lines, respectively) and loss of kDNA (red line) in the presence of tetracycline of the pATOM36-RNAi parent cell line. Right panel: as in the left but the analysis was done for the pATOM36-RNAi cell line that co-expresses Mim1-myc and Mim2-HA. (**B**) Whole cell lysates from the cell lines as in (**A**) were obtained after the indicated time of induction. Proteins of these samples were analysed by SDS-PAGE and immunodecoration with the indicated antibodies. ATOM46, ATOM19 and ATOM14 are subunits of the ATOM complex. Cytosolic EF1a serves as a loading control.

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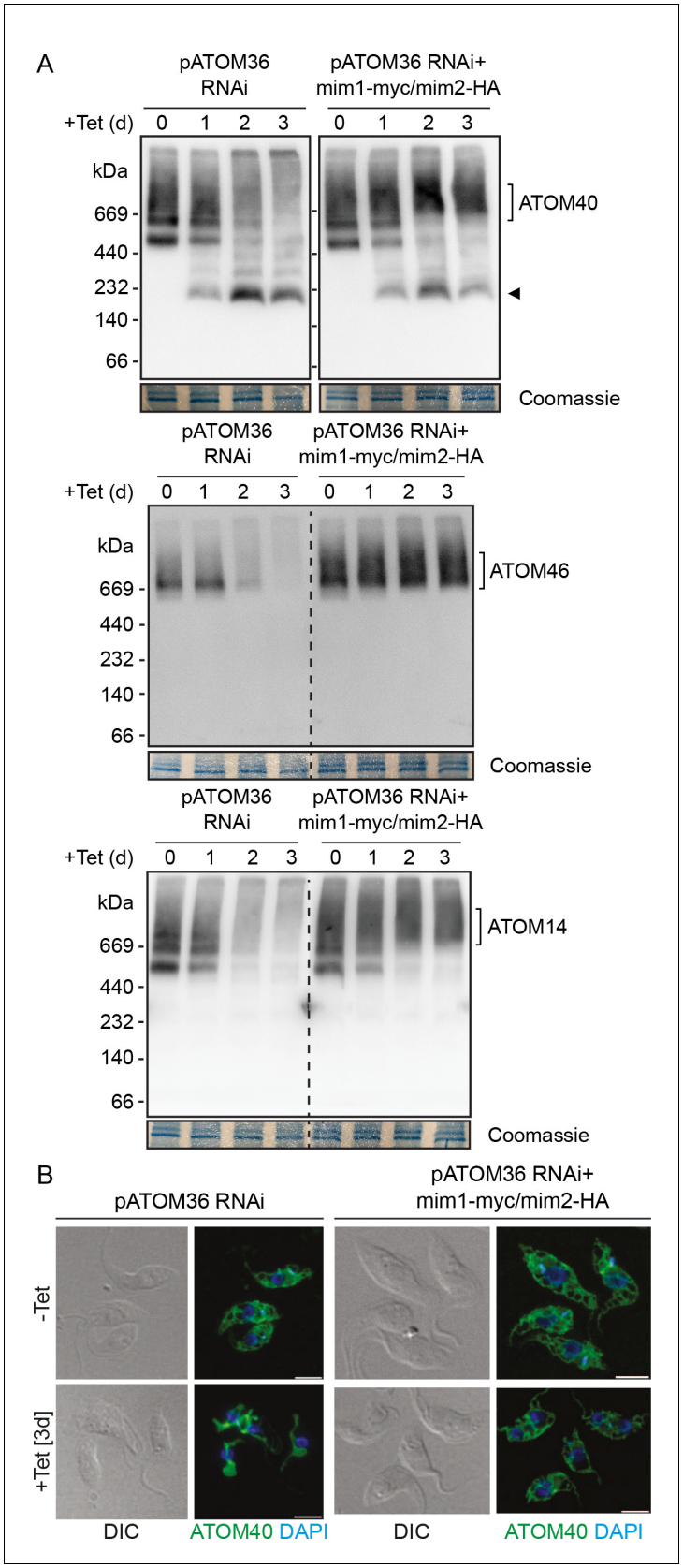


**Figure 8.** Mim1 and Mim2 rescue the assembly defect of the ATOM complex and the altered mitochondrial morphology in cells lacking pATOM36. (**A**) Mitochondria-enriched fractions from the cell lines as in ***Figure 7A***

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were obtained after the indicated time of induction. Samples were analysed by BN-PAGE followed by immunodecoration with antibodies against the indicated subunits of the ATOM complex. The migration of the ATOM complex is signposted. Sections of the coomassie-stained gels serve as loading controls. Arrowhead indicates an ATOM40-containing lower molecular weight complex. (**B**) Left images: Immunofluorescence analyses of mitochondrial morphology in the pATOM36 RNAi cell line after 0 or 3 days of induction. Right images: as in the left panels but the analysis was performed with the RNAi cell line co-expressing Mim1-myc and Mim2-HA. ATOM40 is shown in green and DAPI-stained DNA is shown in blue. DIC, differential interference contrast. Scale bar, 5 µm.

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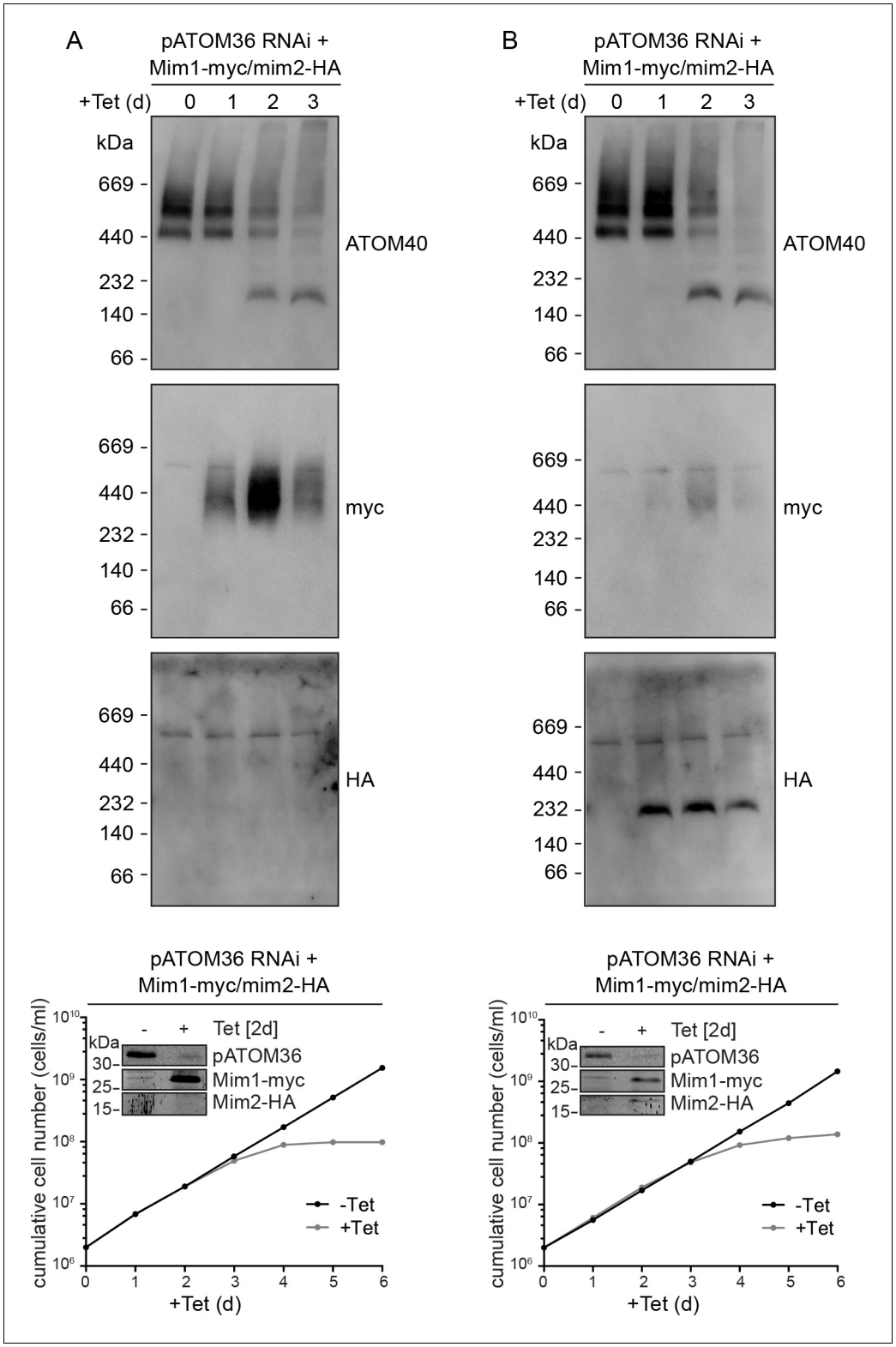


**Figure 8—figure supplement 1.** Complementing the biogenesis function of pATOM36 requires both Mim1 and Mim2. Individual clones of a pATOM36-RNAi cell line transfected with plasmids encoding myc-tagged Mim1 and HA-tagged Mim2 were analysed by BN-PAGE and subsequent immunodecoration. Clones that primarily express either myc-tagged Mim1 (**A**) or HA-tagged Mim2 (**B**) were analysed. The BN-PAGE blots were probed with anti-ATOM40 (upper panel), anti-myc (middle panel), and anti-HA (bottom panel) antibodies. Days of tetracycline

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induction (+Tet (**d**)) are indicated. Bottom graphs: growth curve for the same clone as above analysed in the presence and absence of tetracycline. Days of induction with tetracycline (+Tet [d]) are indicated. Inset: whole cell lysates of the clones were analysed by SDS-PAGE and immunodecoration with the indicated antibodies.
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