The core MICOS complex subunit Mic60 has been substituted by two cryptic mitofilin-containing proteins in Euglenozoa

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ABSTRACT

Cristae enclose respiratory chain protein complexes, making them the bioenergetic subcompartments of mitochondria. The MICOS complex is among the inducers of sharp membrane curvature needed for crista formation. Resembling the respiratory chain complexes, MICOS is organized around a core protein, the mitofilin-domain bearing Mic60, that was inherited from the alphaproteobacterial progenitor of mitochondria. Extant alphaproteobacteria express Mic60 to form their own bioenergetic subcompartments, demonstrating the permeance of Mic60's form and function during the long span of prokaryotic and eukaryotic evolution. Yet, unlike virtually all aerobic eukaryotes, Mic60 is not encoded in any genome of the multifarious protists that comprise the phylum Euglenozoa, including trypanosomes. Here, we show that Mic60 has been replaced in euglenozoans by two cryptic mitofilin domain-containing MICOS subunits, Mic34 and Mic40. Sharply contrasting Mic60 from two domains of life, these are not integral membrane proteins. Reverse genetics revealed they are intertwined with the oxidative protein folding pathway required for mitochondrial–and crista–biogenesis, veiling a potential membrane remodeling role. Nevertheless, Mic34 binds to phospholipid bilayers *in vitro*. Mic34 and Mic40 heterologous expression remodels gammaproteobacterial cytoplasmic membranes, like prokaryotic and eukaryotic Mic60. Astoundingly, Mic34 overexpression elaborates the simplified tubular mitochondrion of a *Trypanosoma brucei* life cycle stage with repressed oxidative phosphorylation. The structural correspondence of Mic34's mitofilin domain to Mic60's predicted mutations to conserved motifs that ablated Mic34's mitochondria-remodeling activity. Thus, the mitofilin protein family is more diverse than originally supposed, with two of its structurally most divergent members altering the core of euglenozoan MICOS.

SIGNIFICANCE STATEMENT

Cristae are mitochondrial subcompartments that give the organelle its distinctive ruffled appearance. They house multiprotein-complex machinery needed for cellular respiration. These crista-embedded complexes are built around an immutable protein core inherited from the bacterial symbiont that engendered mitochondria. Mic60 represents one such core protein. Because of its fundamental role in crista formation, Mic60 is present in virtually all organisms with cristae, and even in the closest known bacterial relatives of mitochondria, reflecting Mic60's symbiotic origin. Euglenozoan protists are a strange exception, lacking a recognizable Mic60 despite having cristae. Instead, they have two cryptic Mic60-like proteins that evaded recognition due to their radically altered structures. This demonstrates that even the normally conserved cores of ancient protein complexes can change during evolution.

INTRODUCTION

Cristae are the bioenergetic subcompartments of mitochondria that house the molecular machinery for cellular respiration (1). Their biogenesis involves the extreme bending of the mitochondrial inner membrane (IM) by dedicated–and usually multiprotein–molecular machineries (2). Rows of ATP synthase dimers are responsible for positive curvature at cristae rims (3) whereas the Mitochondrial contact site and Cristae Organizing System (MICOS) complex forms both crista junctions, constricted zones of negative curvature (4). Additionally, MICOS forms contact sites between the IM and outer membrane.

While ATP synthase dimerization appears to be a eukaryotic invention (3), the keystone MICOS subunit Mic60 is of more ancient origin, being directly inherited from the alphaproteobacterial symbiont that gave rise to mitochondria (5, 6). Mic60's function in the formation of bioenergetic subcompartments and double membrane contact sites is conserved in extant alphaproteobacteria (7), supporting the hypothesis that the cenancestor of mitochondria and alphaproteobacteria already possessed the requisite machinery to form cristae (8). Indeed, the shared properties of eukaryotic and prokaryotic Mic60 most parsimoniously imply that their common ancestor had the capacity to remodel their inner membranes into crista-like subcompartments (9). Also, like the respiratory chain complexes that are embedded in cristae, MICOS is also assembled around a proteinaceous core inherited from the alphaproteobacterial symbiont (10). Core architectures are necessarily more conserved than the surrounding shell of often lineage-specific subunits to stabilize the functionality of these protein complexes (11).

The canonical domain architecture of Mic60 (5, 6) forms a tertiary structure that allows the protein's homotetramerization, which has been proposed to tubulate membranes (12). A transmembrane domain (TMD) embeds its N-proximal segment into the IM. A central coiled coil (CC) domain provides an interaction interface bringing together two antiparallelly oriented Mic60 homodimers into a tetramer. The C-terminal mitofilin domain, made up of eight alpha helices, directly interacts with phospholipids on the opposite side of a tubulated membrane. Via this mechanism, Mic60 has been proposed to be the primary factor in crista junction constriction (13).

Given its central role in crista biogenesis, Mic60 is ubiquitously encoded in the genomes of aerobic eukaryotes, with only a few exceptions (5, 6). Flagellated protists belonging to the phylum Euglenozoa represent one such anomaly. Euglenozoans are a sub-clade within the eukaryotic supergroup Discoba (14). This phylum is named after Euglenida, a subdivision containing free-living, mostly freshwater protists (Fig. 1A). Two sister clades branch next to Euglenida: Diplonemea, a group of predominantly marine flagellates, and Kinetoplastea, named after the kinetoplast, a massive network of mitochondrial DNA that comprises the organelle's single genome (15). A species-poor group Symbiontida forms a fourth euglenozoan lineage (16), but it is not considered further in this study due to limited sequence resources.

Until it was isolated from the kinetoplastid *Trypanosoma brucei* (17), the MICOS complex was only known from animals and yeast, grouped together in the diverse Opisthokonta clade (14). *T. brucei* MICOS has some commonalities with opisthokont MICOS, such as having the other central subunit, Mic10. While *T. brucei* MICOS differs in having two Mic10 paralogs, one paralog associates with ATP synthase dimers (18), like in yeast (19). However, as predicted by the aforementioned bioinformatic screen, *T. brucei* MICOS lacked a canonical Mic60. At the time of discovery, a subunit bearing a superficially similar domain architecture,

but lacking the characteristic mitofilin domain, was annotated as a putative Mic60 (17). However, here we present *in silico* and experimental evidence that two other MICOS subunits named Mic34 (Uniprot ID: Q57ZZ1) and Mic40 (Q57YH2) truly represent cryptic mitofilin family proteins. Thus, we henceforth will refer to the originally misannotated Mic60 (Q38DZ0) as Mic24. This discovery has expanded the mitofilin proteins to include members that implement the eponymous domain unconventionally.

RESULTS

The soluble MICOS subunits Mic34 and Mic40 are paralogs that each contain a mitofilin domain

A *bona fide* ortholog of Mic60 was not identified in the MICOS complex isolated from *T. brucei* (17). However, when the largest *T. brucei* Mic34 and Mic40 MICOS subunits were searched against the Pfam-A (v.37) database using HHpred (20), which implements sensitive protein homology detection based on pairwise comparison of profile hidden Markov models (HMMs), both retrieved the family PF09731.14, *i.e.* mitofilin as the best hit with convincing statistical significance (98.0% and 96.77% probabilities for Mic34 and Mic40, respectively). We then built a more representative profile HHM based on Mic34 and Mic40 multiple sequence alignments (MSAs) of a phylogenetically comprehensive set of homologs retrieved from all major Euglenozoa groups: Kinetoplastea, Diplonemea, and Euglenida (Dataset S1). The improved taxonomic coverage of the query MSA increased the sensitivity of the homology detection by HHpred and reinforced the credibility of the matches to mitofilin for Mic34 and Mic40 by increasing the probabilities to 99.16% and 98.03%, respectively.

Inspection of the HHpred Mic34 and Mic40 alignments to the mitofilin protein family entry showed that the Mic60 region detected to be homologous to one or the other euglenozoan protein differs (Fig. 1A). For the Mic34-Mic60 comparison the match was restricted to a C-terminal part of the former and a region of the latter exactly corresponding to the ‘mitofilin domain’ as delineated by Benning et al. (21). The match between Mic40 and Mic60 extended further upstream to include a larger part of the Mic60’s central coiled-coil (CC) region. This alignment covered almost the full Mic40 protein, except for a short N-terminal region, which corresponds to a part of Mic40 not detected by mass spectroscopy in a previous study, implying it potentially is not part of the mature protein (17).

To further illuminate the relationship between Mic60 and euglenozoan Mic34 and Mic40, we carried out a phylogenetic analysis that was restricted to the mitofilin domain apparently shared by all three proteins. As anticipated, the resulting tree (Fig. S1) groups sequences representing the three different proteins. The most parsimonious scenario explaining the origin of Mic34 and Mic40 starts with a canonical *mic60* gene in the euglenozoan ancestor followed by its duplication into the eventual Mic34 and Mic40 paralogs. Subsequent independent sequence divergence affecting most pronouncedly the N-terminal region of the encoded protein. Owing to the presence of both Mic34 and Mic40 orthologs in all three major euglenozoan lineages, all these events must have happened before their divergence.

The mitofilin domain is the most conserved primary structure of prokaryotic and eukaryotic Mic60 proteins (5, 6). To bolster confidence that Mic34 and Mic40 are cryptic mitofilin proteins, we employed AlphaFold2 predictions of the mitofilin domains starting from its second alpha helix (α2) (21) found in the three representatives from each euglenozoan group, namely *T. brucei* (Kinetoplastea), *Hemistasia phaeocysticola* (Diplonemea), and *Euglena gracilis* (Euglenida) (Fig. 1B). These tertiary structures were aligned to the mitofilin domain of discoban *Naegleria gruberi* Mic60, which overlaps the theoretical *Saccharomyces*

cerevisiae and the experimentally solved *Chaetomium thermophilum* (12) mitofilin domains with a root-mean-square distance (RMSD) of <1 Å; *N. gruberi*'s mitofilin aligned to each fungal one equivalently as the two fungal structures did to each other (Fig. S2A). Expectedly, the Mic34 three representative euglenozoan mitofilin domains are well aligned (Fig. S2B).

A linear map of Mic34 and Mic40's mitofilin domain alpha helices α2-α8 (21) was derived from the high confidence predicted structures (Fig. 1C) and multiple sequence alignments used to make the aforementioned profile HMM (Fig. 1A) and phylogenetic tree (Fig. S1). The extended loop between α2 and α3 in Mic60 *S. cerevisiae* (and to a lesser extent *N. gruberi*) is considerably reduced in Mic34 and Mic40. Lipid binding site (LBS) 1, which is necessary for optimal lipid binding and yeast cristae junction formation (22), was present in all examined Mic34 and Mic40. In almost all structural alignments, a highly conserved and functionally important aromatic amino acid (7) within LBS1 perfectly overlapped (Fig. 1B, S1C). This phenylalanine is staggered in *T. brucei* compared to all the other LBS1 structures examined (Fig. 1B, S2B) because it is shifted by one amino acid in the kinetoplastid primary structures (Fig. S5F). Discoban LBS1 appears to be an amphipathic helix with the hydrophobic face oriented like fungal LBS1; a notable exception is *T. brucei* Mic34, whose hydrophobic face is shifted ~90º counterclockwise (Fig. S2D).

The adjacent LBS2 (α5) was much more variable, consistently being the least reliably predicted motif that aligned poorly to the reference *N. gruberi* mitofilin domain (Figs. S2C). Furthermore, it is missing in *H. phaeocysticola* Mic34 and Mic40. The orientation of the last three alpha helices α6-8 is well conserved in all three representative Mic34 proteins. In Mic40, this is a more malleable feature, with *T. brucei* and *E. gracilis* having a shorter alpha helix downstream of α8, and α6 fused to LBS2 or missing, respectively (Fig. 1C). In summary, the mitofilin domain structure of Mic34 appears to be more similar to canonical Mic60 than Mic40.

Mic34 and Mic40 are involved in the mitochondrial intermembrane space assembly pathway that affects mitochondrial morphology

To verify *in silico* predictions that Mic34 and Mic40 are cryptic mitofilins, we examined how their depletion in procyclic stage *T. brucei* (PCF), which bears mitochondria with mature cristae that house an active electron chain (23), affects the organelle's morphology. As expected (17, 24), induced RNAi-silencing of Mic34 and Mic40 resulted in growth inhibition (Fig. S3A). Qualitative (Fig. 2A, S3B) and quantitative (Fig. 2B) assessment of mitochondrial complexity showed that branching was reduced at 3- and 5-day time points (TP3 and TP5) after RNAi induction, with the organelle exhibiting a tubular appearance in the latter. *S. cerevisiae mic60* null mutants also exhibit alternations to the mitochondrial network (25).

Interpretation of the observed morphological defects may be confounded by downregulation of proteins imported into the intermembrane space (IMS) by the Mitochondrial Intermembrane Space Assembly (MIA) pathway (26), which has been observed upon Mic34 silencing (24). To see if Mic40 depletion has the same effect on IMS proteins as Mic34, we first verified that the MIA substrate Tim9 (27) was downregulated during the time course of RNAi induction (Fig. S3C). To gain a comprehensive view on the cohort of proteins that may rely on Mic40 for their import, we performed a mitoproteome-wide analysis (*i.e.* depletome) of Mic40 TP3 and TP5 relative to wild type *T. brucei* (Fig. 2C-D). Like Mic34 (24), the MICOS subcomplex completely residing in the IMS peripherally to the IM (24), was first downregulated followed by downregulation of MIA substrates (Fig. 2C-D). Indeed, the Mic40 depletome showed significant overlap with both Mic34 (Fig. 2D, left) and Mic20 (Fig. 2D, middle), a thioredoxin-like subunit of the peripheral MICOS subcomplex (17, 28). Thus, Mic40 also plays an integral part in IMS protein import by MIA.

Returning to the question of whether IMS import defects may underlie the reduction in mitochondrial complexity seen in Mic34 and Mic40 knockdown cells, we measured the degree of branching in TP3 and TP5 Erv1 RNAi PCF (Fig. 2B, S3B). Erv1 is a sulfhydryl oxidase that plays an essential role in the MIA pathway (26, 27), and is not a component of *T. brucei* MICOS (17, 29). Erv1 depletion phenocopies Mic34 and Mic40's effect on mitochondrial complexity to the same degree, which was previously reported based on qualitative observations (30, 31). The Mic34 and Mic40 depletomes overlap with Erv1's (27), albeit to a lesser degree than Mic20's. It should be noted that peripheral MICOS subunits were also downregulated upon Erv1 silencing. (27), possibly leading to the observed morphological alteration. Consistent with this hypothesis, these subunits were by far the most affected at TP3 (Fig. 2D), when simplification of the mitochondrion was first observed during Mic40 downregulation (Fig. 2A-B). However, while these results do not rule out that Mic34 or Mic40 are directly involved in membrane remodeling, we still cannot decouple this function from its established but still unexplained role in the MIA pathway.

Mic34 binds to phospholipids *in vitro*

First, we decided to test if Mic34 and Mic40 can deform liposomes *in vitro*, as has been demonstrated for Mic60 (9, 22). For this experiment, recombinant *T. brucei* Mic34 and Mic40 bearing N-terminal His-tags (rMic34 and rMic40, respectively) were expressed in *Escherichia coli*, where they sequestered into inclusion bodies (Fig. S4A-B). After their purification by sequential affinity and size exclusion chromatography, rMic34 and rMic40 were solubilized with varying concentrations of the mild detergent n-dodecyl β-d-maltoside (DDM). While the majority of rMic34 proved to be soluble in 0.1% DDM, rMic40 was poorly solubilized in any of the tested DDM concentrations (Fig. S4C-D). Thus, we excluded rMic40 from further analysis.

Next, 0.1% DDM solubilized rMic34 was loaded onto large unilamellar vesicles (LUVs) comprised of IM phospholipids (9, 32), followed by DDM removal. A fraction of rMic34 was carbonate extracted from the LUVs (Fig. 3A), which is the expected behavior of the IMS-periphery localized Mic34 ((17) and also see below). Surprisingly, most rMic34 acted as an integral protein in this biochemical assay. To eliminate the possibility of DDM-mediated rMic34 insertion into the membrane, we performed a Proteinase K protection assay to see if Mic34 is binding the exterior of the LUV (Fig. 2B). The protease completely degraded rMic34 in the liposome sample even in the absence of the membrane-disrupting detergent sodium dodecyl sulfate (SDS), mimicking the behavior of the *S. cerevisiae* rMic19, which does not directly interact with phospholipids (12).

Next, we assayed whether rMic34 deformed the LUVs by flotation on Histodenz density gradients (Fig. 3C). As previously reported (9, 32) , LUVs alone float to the lightest fractions of the gradient. After incubation with rMic34, LUVs with or without cardiolipin formed multiple bands in denser fractions of the gradient, reminiscent of canonical Mic60 (9). The presence of rMic34 in these fractions was verified by immunoblotting (Fig. 3D), indicating that rMic34 binding is weighing down the LUVs. The signal in the heaviest fraction corresponds to unbound rMic34, as seen in the rMic19-incubated LUV negative control.

To see if the striated banding pattern we observed often corresponds to LUV deformation (9, 32), LUVs that flotated into lower density fractions were isolated and observed by negative-stain transmission electron microscopy (TEM). However, we could not reliably observe any obvious differences between the naked LUVs and those incubated with rMic34, independently of the presence of cardiolipin in the LUV membrane (Fig. 3E).

This lack of LUV deformation may be because rMic34 does not have *in vitro* membrane deformation activity or this activity is inhibited by the 0.1% DDM treatment. To test the latter hypothesis, we compared the liposome deformation activity of Epsin N-terminal Homology Domain (ENTH) that underwent 0.1% DDM solubilization prior to incubation with Folch liposomes spiked with 5% PI(4,5)P2 (DDM+) to that of untreated ENTH (33) (DDM-). DDM+ ENTH bound to the outside of liposomes (Fig. 3B-C). However, liposome tubulation was only observed in the absence of the detergent treatment (Fig. 3F). Thus, we are unable to address Mic34's putative membrane remodeling activity *in vitro* with our current experimental set up. Nevertheless, we conclude that Mic34 binds phospholipids *in vitro* in a cardiolipin-autonomous way like yeast Mic60 (22).

Mic34 and Mic40 are capable of remodeling the cytoplasmic membrane of a gammaproteobacterium upon export to the periplasm

After establishing that Mic34 is able to bind phospholipid membranes, we next investigated whether Mic34 and Mic40 have membrane-bending activity *in vivo*. We employed an assay that has been used to demonstrate that Mic60 has this capacity (9). Mic34 and Mic40 from *T. brucei* were heterologously expressed in the gammaproteobacterium *E. coli*. To facilitate their export into the periplasm, which represents the proteobacterial analog of the mitochondrial IMS, each MICOS subunit was fused to maltose binding protein (MBP).

First, *E. coli* transformed with various MBP expression constructs were separated into periplasm and spheroplast fractions (Fig. 4A). As expected, MBP alone was completely exported into the periplasm. This and the retention of T7 polymerase within the spheroplasts (Fig. S5A) verified successful fractionation. While a minor portion of Mic34-MBP and Mic40-MBP was observed in the periplasm, they were enriched in the spheroplast fraction (Fig. 4A). Carbonate extraction of the spheroplasts was done to see whether Mic34-MBP and Mic40-MBP were embedded in the membrane (Fig. 4A), like the integral subunit Mic24 fused to MBP (Fig. S5B). While some Mic34-MBP and Mic40-MBP were observed in the integral fraction–reminiscent of Mic34's behavior in LUVs (Fig. 3A)–most accumulated in the soluble fraction (Fig. 4A). Our interpretation is that Mic34-MBP and Mic40-MBP tightly associate with periplasm-facing part of the cytoplasmic membrane because (1) the protein is partially observed in the periplasm fraction and (2) Mic34 and Mic40 are sorted into inclusion bodies when expressed in the cytoplasm (Fig. S4A-B).

Next, we looked if Mic34 and Mic40 remodeled *E. coli* membranes similarly to heterologously expressed full-length *S. cerevisiae* Mic60 or just its IMS part (9). After establishing by TEM that we can replicate this experiment (Fig. S5C), including reproducing that MBP alone does not affect *E. coli* ultrastructure (Fig. 4B), we observed Mic34-MBP and Mic40-MBP expressing *E. coli* by electron tomography (Fig. 4C) and TEM (Fig. S5D). Mic34-MBP and Mic40-MBP had respectively 5 and 3 times more vesicles observed by TEM in the periphery of the cell as compared to the parental strain (Fig. 4D). Immunogold labelling of the MBP moiety showed that both chimeras were also observed in the periphery, associated with the majority of vesicles. Electron tomography allowed us to better resolve these vesicles, which accumulated in the periplasm (Fig. 4C-D, Movies S1 and S2). Furthermore, we used this method to determine that the bulk of MBP-visualizing gold nanoparticles were associated with the vesicles (Fig. S5E). Together these results indicate that Mic34-MBP and Mic40-MBP are directly responsible for cytoplasmic membrane remodeling when heterologously expressed in *E. coli*, phenocopying *S. cerevisiae* Mic60 in this assay.

To test whether the predicted LBS helices of the more conventional Mic34 (Fig. 1, S5F) are important for membrane remodeling, as has been demonstrated for *S. cerevisiae* Mic60 (22), we expressed a periplasm-targeted LBS deletion mutant (Mic34ΔLBS1+2). We first tested

whether the ~6 kDa smaller mutant was expressed (Fig. 4E) and targeted to the periplasmic face of the *E. coli* cytoplasmic membrane (Fig. S5A). As expected, this mutant failed to remodel the cytoplasmic membrane, having about the same number of vesicles as the parental strain (Fig. 4D-E).

In conclusion, with an assay based on heterologous expression in *E. coli*, we were able to show that (1) Mic34 and Mic40 have the capacity to remodel membranes and (2) Mic34's LBS is directly involved in this activity. Thus, we provide empirical evidence that Mic34 and Mic40 are extremely diverged mitofilin family proteins.

Overexpression of Mic34 causes reticulation of a morphologically simplified trypanosomal mitochondrion

The long slender bloodstream form (BSF) *T. brucei* has a morphologically simplified mitochondrion that runs as a tube along the cell body opposite the flagellum, reflecting the cessation of cellular respiration in this life cycle stage (23, 34, 35). All MICOS complex subunits are downregulated in this stage compared to PCF (17). Thus, expression of electron transport chain subunits is necessarily triggered during BSF differentiation into PCF (36), corresponding with mitochondrial reticulation.

Ectopic expression of V5-epitope tagged Mic34 (Mic34-V5$^+$) with 1000 ng/ml doxycycline in wild type BSF was considerably higher than that of endogenous Mic34 (Fig. 5A). Surprisingly, this overexpression reproducibly induced cytostasis after 24 hours, with induction by even 10 ng/ml doxycycline effectively inhibiting growth (Fig. 5A, S6A-B). Mic40-V5$^+$ ectopic expression only mildly inhibited growth (Fig. S6B), perhaps due to its lower expression (S6A). After verifying that Mic34-V5$^+$is targeted to the mitochondrion (Fig. S6C), we assayed how mitochondrial morphology was affected after 36 hours of Mic34-V5$^+$ due to its cytostatic effect. Remarkably, many Mic34-V5$^+$ cells exhibited profound reticulation of their mitochondrion (Fig. 5C), with ~2.3 times more branching than in the wild type BSF organelle (Fig. 5D). Aparant fragmentation of the organelle was also observed (Fig. S6D). However, we did not notice any obvious changes to the cristae that were sporadically observed by TEM (Fig. S6E). This is consistent with the uninterrupted absence of electron transport chain subunits and unaffected F_OF_1- ATP synthase expression in Mic34-V5$^+$ cells (Fig. S6F). Furthermore, IMS protein levels were not altered, unlike during Mic34 depletion in PCF (24). Thus, the observed morphological changes are likely due to Mic34-V5$^+$ acting on the mitochondrion directly.

We were concerned that Mic34-V5$^+$ branching phenotype was an artifact of cell cycle arrest. The mitochondrion becomes more branched during cell cycle progression to ensure inheritance of the organelle after cytokinesis (34, 35). Thus, a defect to this process could secondarily cause a reticulated mitochondrion (Fig. 5E). To address this issue, we counted the number of kinetoplasts and nuclei in Mic34-V5$^+$ cells bearing a reticulated mitochondrion; branching starts during kinetoplast division, perceptively increasing as the cell goes from having a single nucleus and kinetoplast (1N1K) to two separate kinetoplasts (1N2K) and eventually two separate nuclei (2N2K) (Fig. 5E). The vast majority of unsynchronized Mic34-V5$^+$ cells with a reticulated mitochondrion were 1N1K (Fig. 5C, F), at early cell cycle stages that normally have a tube-like mitochondrion. As expected, wild type BSF exhibited branched mitochondria mostly in the later 2N2K cell cycle stage. Thus, we conclude that Mic34-V5$^+$ overexpression directly affects mitochondrial morphology.

We hypothesized that the Mic34 LBS alpha helices may be important for the observed cell cycle arrest due to mitochondrial remodeling. We overexpressed mutants in which both (Mic34ΔLBS1+2$^+$) or just LBS1 (Mic34ΔLBS1$^+$) were deleted (Fig. S5F). Like WT Mic34,

these mutants were also targeted to the mitochondrion (Fig. S6C). After 24 hours of overexpression growth inhibition severity remarkably correlated with the extent of LBS deletion (Fig. 5A). The mild growth inhibition in Mic34ΔLBS1+2^{+} could be explained by its relatively lower expression compared to others (Fig. 5B, *c.f.* lane 4 with 2 and 4). However, Mic34ΔLBS1+2^{+} and Mic34-V5^{+} induced with 10 ng/ml doxycycline (Fig. 5A, lane 4) are equivalent, with the latter exhibiting more dramatic growth inhibition than the deletion mutant. Together with our previous *E. coli* data, we conclude that the LBS of the mitofilin-like Mic34 plays an important functional role as it does in canonical Mic60.

Tertiary (Fig. 1B-C) and primary structural alignments predicted that the conserved LBS1 phenylalanine of *T. brucei* LBS1 (F175) would be a critical residue for the observed Mic34 mitochondria remodeling given it and an upstream arginine (R) residue were shown to be essential for yeast Mic60 remodeling activity (22). We expressed a double point mutant affecting these amino Mic34^{R170D_F174D}, which was equivalently expressed compared to Mic34-V5+)(Fig 5G, S6C) and targeted to the mitochondrion (Fig. S6C). Mic34^{R170D_F174D} growth was almost restored to levels in the uninduced counterpart (Fig 5G), which may be explained by a mitigation of apparent fragmentation seen in Mic34^{R170D_F174D} compared to Mic34-V5^{+} (Fig. 5H, S6D). Thus, we conclude that Mic34 was directly responsible for the mitochondrial remodelling observed upon its overexpression Furthermore, the cytostatic effect of Mic34-V5^{+} may be linked with the apparent fragmentation that also occurs.

DISCUSSION

Here, we provide *in silico* and experimental evidence supporting the conclusion that euglenozoan MICOS contains two soluble subunits that represent cryptic mitofilin family proteins. In contrast to Mic60 from two domains of life, Mic34 and Mic40 do not contain TMD that embeds them into a phospholipid bilayer, as supported by its repeated association with the periphery of membranes in this and another (17) study. It has been proposed before in passing (11, 37) that the ancestral Mic60 got split into two parts, one represented by the integral membrane Mic24 and the other by the soluble, Mic34 in the forebear of Euglenozoa. Our results corroborate that Mic34 indeed represents a cryptic mitofilin domain-containing protein. Additionally, we demonstrate that the still hypothetical split of integral and soluble components was followed by duplication of the latter, mitofilin domain-containing part, resulting in the paralogous Mic34 and Mic40 pair. Admittedly, the phylogenetic tree inferred from mitofilin domain sequences is not well resolved and does not support this scenario, as it would need Mic34 and Mic40 to branch most closely to Mic60 from Heterolobosea (the sister group to Euglenozoa). However, this is not unexpected, given the sequence divergence of the sampled mitofilin family proteins and the resulting challenges for phylogenetic inference; note also that the branching of Mic60 itself conforms less to organismal phylogeny than that of Mic34 and Mic40.

The presence of cryptic mitofilin domains in Mic34 and Mic40 predicts that these are membrane remodeling proteins like conventional Mic60 from prokaryotes and eukaryotes. However, we were unable to verify Mic34 and Mic40's capacity to remodel phospholipid bilayers *in vitro* here. Nevertheless, this assay did reveal Mic34 can bind liposomes. Thus, we had to resort to assaying their putative role in membrane remodeling by other means. Heterologous expression of Mic34 and Mic40 deformed *E. coli* cytosolic membranes, the prokaryotic analog to the mitochondrial IM (7, 8), as yeast and alphaproteobacterial Mic60 did (9). However, a vital difference should be noted here: Mic60 caused the cytoplasmic membrane to invaginate inward into the cytoplasm whereas Mic34 and Mic40 promoted the accumulation of vesicles within the IMS. This may be due to the cryptic mitofilins acting in the IM differently than Mic60 or that heterodimerization between Mic34 and Mic40 is a

prerequisite for recapitulating the mechanism of conventional Mic60, which acts on membranes as a homooligomer (12). Nevertheless, Mic34 requires the LBS motifs it shares with Mic60 (22) for cytoplasmic membrane vesicularization, consistent with the notion it is a cryptic member of the mitofilin protein family.

Mic34 ectopic expression in a *T. brucei* BSF with a reduced, tubular mitochondrion causes elaboration into a branched organelle. This parallels the enhanced interconnectivity of mitochondria upon Mic60 overexpression in primary and immortalized neuronal cell lines (38). These assayed Mic34 membrane-deformation activities *in vivo* rely on its predicted LBS motifs, further supporting Mic34 and its paralog Mic40 as diverged mitofilin protein family members. LBS1 contains a conserved aromatic amino acid (7) that is essential for Mic60's *in vitro* and *in vivo* activities (22). This residue along with an upstream arginine residue is also essential for Mic34-mediated BSF-mitochondria remodeling, as predicted. Indeed, the aromatic residue in *C. thermophila* Mic60 is buried inside the hydrophobic interaction interface of the Mic60 homodimer (12). Whether this residue plays a similar role in Mic34 homooligomerization and/or heterooligomerization remains an open question.

Mic34 overexpression did not alter cristae dramatically in BSF *T. brucei*, contradicting Mic60 overexpression phenotypes in yeast (25) and alphaproteobacteria (7), manifested in the branching of their respective bioenergetic subcompartments (*e.g.* cristae). However, this inconsistency can be explained by the lack of an active electron transport chain in BSF (23, 36), which were present when Mic60 was overexpressed in yeast and bacteria. Thus, an electron transport chain may be necessary for populating and stabilizing elaborated crista membranes. Alternatively, but not in a mutually exclusive way, Mic34 may act stoichiometrically with Mic40 and/or (an)other MICOS subunit(s) to shape crista.

One clear divergence from canonical Mic60 is that Mic34 (24) and Mic40 (this study) appear to be directly integrated into the MIA pathway for IMS protein import in PCF, like the thioredoxin-like Mic20 subunit (17, 28). In contrast, while yeast Mic60 augments the Mia40 sulfhydryl oxidase's mediation of MIA (39), Mic60 deletion does not lead to depletion of MIA substrates (40) like Mic34 and Mic40 silencing. The MIA defects seen upon depletion of Mic34 and Mic40 complicate the interpretation of crista defects given that putative respiratory chain subunits and assembly factors, plus the small Tim proteins, are imported via MIA. Moreover, the latter are needed for import of IM proteins (41, 42), many of which are assembled into respiratory chain complexes (43). Based on our results, the MIA pathway also is important for maintaining reticulated mitochondria in PCF, as shown by Erv1 simplifying mitochondrial complexity to the same degree as Mic34 and Mic40. However, it is possible that concomitant downregulation of the soluble MICOS subunits Mic20 and Mic32–and by extension, perhaps the rest of this subcomplex–may be responsible for the morphological phenotype of Erv1 RNAi after all (27). Thus, at this time we cannot verify nor refute the hypothesis that MICOS plays a role in maintaining a reticulated mitochondrial network *via* its demonstrated membrane remodeling activity.

It is worth noting that this work has implications about the subcomplex architecture of MICOS throughout eukaryotes. In opisthokonts, two adjacent integral membrane subcomplexes are each assembled around their respective core subunit, Mic10 and Mic60 (4, 37). It is reasonable to expect this 'horizontal' (relative to the membrane) configuration in the majority of aerobic eukaryotes because they have both Mic10 and Mic60 (5, 6). Uniquely, trypanosomal–and by extension likely also euglenazoan–MICOS has a vertical architecture: a membrane-embedded subcomplex containing two Mic10 paralogs and a peripheral subcomplex comprised of soluble subunits located within the IMS (24). This divergent subcomplex architecture likely corresponds to the soluble nature of IMS localized Mic34 and

Mic40 (17). Significantly, the existence of the Mic34/Mic40 subcomplex implies that a mitofilin domain represents the core of a MICOS module regardless of whether not it is directly IM anchored. Perhaps the Mic60 subcomplex is more predisposed to IM detachment than the Mic10 subcomplex. In opisthokonts, the only soluble MICOS subunit Mic19 (plus its paralog Mic25 in vertebrates) is part of the Mic60 complex (5, 6, 12, 22). In contrast, the persistence of the trypanosomal Mic10 subcomplex within the IM suggests it is perpetually integral IM MICOS module.

In the diverged euglenozoans and opisthokonts, a mitofilin protein represents the largest subunit in the MICOS complex. This may reflect the mitofilin domain's role in bridging juxtaposed membranes at contact sites and crista junctions as well as being an interaction hub to facilitate coordination of MICOS other fundamental pathways (44, 45). Such a central role in MICOS is likely a product of the mitofilin domain's ancient, pre-mitochondrial origin (7). Unlike the rather widespread loss of Mic60 in anaerobes without cristae, the transfiguration of Mic60 into Mic34/Mic40 appears to be a singular event that occurred in exclusively Euglenozoa, joining other unique mitochondrial synapomorphies of this phylum (46).

Our work here also shows that the mitofilin protein family is more diverse than originally thought, given the conservation of Mic60 in alphaproteobacteria and mitochondria (7). It is unknown whether the duplication of the soluble mitofilin proteins is an adaptation to their loss of a TMD. However, it is clear that Mic34 is under a higher selection pressure than Mic40, hinting that it perhaps takes on at least a few of the functions of conventional Mic60 in other eukaryotes. Perhaps the apparent malleability of the Mic40 mitofilin domain during euglenozoan evolution allows the MICOS complex to be fine-tuned to the needs of the lineage's subdivisions. This reveals surprising variability of the mitofilin family of proteins found in the organismal diversity occurring in two domains of life.

MATERIAL AND METHODS

Mitofilin sequence dataset generation

Homologs of *T. brucei* Mic34 and Mic40 were identified by a combination of blast (47) and HHMER3 searches (48) in a variety of databases and sequence resources, including the NCBI non-redundant protein sequences database, TriTryp (49), EukProt v3 (50), and sequence assemblies and annotations accompanying individual publications (details provided in Dataset S1). Briefly, homologs were first collected with blast (considering hits with e-value <1e-5) and aligned with MAFFT v7 (https://mafft.cbrc.jp/alignment/server/; (51)). The HMMER3 package was then used for building a profile HMM for each MSA and searching the target protein sequence sets (using the hmmbuild and hmmsearch command, respectively). Newly identified hits missing in the original MSA and passing the program-defined inclusion threshold were collected, added to the MSA and the whole search was repeated until no new significant hits were retrieved or the search started to retrieve hits that were found not to represent euglenozoan Mic34 or Mic40 proteins. In a few cases homologs were obtained by tblastn searches against genome or transcriptome assemblies and the sequence of the respective encoded proteins were deduced by conceptual translation.

Only a subset of identified sequences was retained for further analyses, taking into account the degree of their completeness, whether both Mic34 and Mic40 homologs were identified for the given species (some of the sequences resources, especially the various euglenid single-cell transcriptomes, are very incomplete and did not yield both expected homologs), and keeping the taxon sampling balanced (i.e. avoiding the inclusion of highly similar

sequences from closely related species). As a result, we obtained a set of Mic34 and Mic40 homologs from 44 euglenozoan taxa encompassing representatives all three main euglenozoan clades as well as their various sublineages, In a few cases the sequences were attributed to a wrong organism in the given database, stemming from contamination of the source protist cultures used for transcriptome sequences by a co-cultured euglenozoan, whose identity was determined by investigating informative marker sequences present in the same assembly, such as the 18S rRNA gene or elongation factor 2 (EF2) protein. All the sequences were manually curated to achieve their accuracy (e.g. by removing artificial N-terminal extensions frequently present in protein sequences automatically inferred from transcriptome assemblies); in some cases the existing truncated sequences were extended or even completed by manually recruiting original Illumina sequencing reads identified by iterative blast searches against the respective sequencing runs in the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra). All the Mic34 and MIc40 sequences included in the final dataset and the related metadata are provided in (Dataset S1).

HHpred searches

Homology of Mic34 and Mic40 to other proteins was rigorously examined with HHpred (20) as available at the MPI Bioinformatics Toolkit server (https://toolkit.tuebingen.mpg.de/tools/hhpred). The searches with queries representing individual sequences, i.e. *T. brucei* Mic34 and Mic40, were done with the default parameters, i.e. with initial three iterative HHblits searches against the UniRef30 database, which in both cases retrieved a set of exclusively kinetoplastid homologs that were then included by the program into the query MSA, used then for building a profile HMM and searching the target database of profile HMMs based on HMM-HMMM comparisons. In the case of searches in which the query was the custom Mic34 or Mic40 MSA (in each case computed with MAFFT from the final set of 44 selected euglenozoan representatives), no initial HHblits searches were allowed and the query MSA was directly used for creating the query profile HMM. For reference we report results of HHpred searches against the Pfam-A_v37 database, which all retrieved the Pfam database entry PF09731.14 as the only significant hit. Alternatively, we also searched the COG_KOG_v.1 database and identified with both Mic34 and Mic40 (individually or as an MSA) the same entry as the best (and significant) hit, i.e. KOG1854, which represent eukaryotic Mic60 proteins, reinforcing the results of the searches against Pfam-A.

Molecular phylogenetic analysis

A set of reference eukaryotic Mic60 sequences was gathered by selecting proteins from a broader sequence set obtained by searching the EukProt v3 database with HMMER3 and a profile HMM built from the seed alignment of the Pfam family PF09731 (mitofilin). Sequences were selected to include a representative from different major eukaryote lineages, with a denser sampling for the supergroup Discoba, owing to the presumed origin of the euglenozoan Mic34 and Mic40 from an ancestral discoban Mic60. Additional discoban Mic60 sequences were identified by HMMER or blast searches in relevant sequence resources from species not included in EukProt v3 and added to the dataset (details provided in Dataset S1). The Mic60 sequences were combined with the Mic34 and Mic40 sequences used in HHpred analyses (see above) and a multiple alignment was built using MAFFT v7 and the L-INS-I iterative refinement method. The alignment was restricted to the C-terminal region corresponding to the mitofilin domain and used for tree inference performed the ETE3 3.1.3 pipeline (52) as implemented on the GenomeNet server (https://www.genome.jp/tools-bin/ete). Specifically, the alignment was trimmed with trimAl v1.4.rev6 (53), removing columns with >20% of gaps, and the tree was inferred using RAxML v8.2.11 (54), with the

default parameters, the substitution model PROTGAMMAJTT, and 100 bootstrap replicates. The resulting tree was visualized in an arbitrarily rooted form using iTOL v6 (55) and further processed using a graphical editor.

Structural predictions

The HHpred server was used to detect the mitofilin domain of Mic34 and Mic40 using hidden Markov model profiles to interrogate the following manually set structural and domain databases: PDB_mmCIF70_18_Jun; Pfam-A_v36; UniProt-SwissProt-viral70_3_Nov_2021; TIGRFAMs_v15.0. E-values reported in Fig. 1A are for the top hit 'PF09731.12; Mitofilin'. AlphaFold 2 predictions for *T. brucei* Mic34 and Mic40 and *N. gruberi* Mic60 were performed according to a previously established pipeline catered to discoban proteins (56). Mitofilin domains were aligned in ChimeraX by restricting the matchmaker to the two domains (57). The mitofilin alpha helices were mapped by manual inspection of the aforementioned structural predictions and crosschecked with multiple sequence alignments to identify deletions and fusions in the mitofilin domains.

***Trypanosoma brucei* culturing, transfection, fractionation and growth analysis**

The PCF Mic34, Mic40 and Erv1 inducible RNAi and SMOXp parental (58) cell lines were generated with the same constructs plus strategy and then grown as previously described (17, 30). The pleomorphic AnTat1.1 BSF cell line was cultivated in HMI-9 media (59) at cell densities ≤8 × 10^5 cells/ml to maintain pleomorphism. Cells were transfected using a Amaxa Nucleofector (X-001) with the pT7-3xV5 vector (60), in which wild type and mutated *mic34* and *mic40* was cloned to facilitate their inducible ectopic expression by addition of 1 µg/ml of doxycycline to the media. Cells were fractionated into cytoplasmic and organellar fractions as previously described (17). Cell densities were measured on a Beckham Coulter Z2 Cell and Particle Counter.

Mass spectroscopy analysis

For depletome data, mitochondria from 10^8 parental SMOXp, TP3 and TP5 Mic40i *T. brucei* cells were crudely isolated as before (17) and snap frozen in liquid nitrogen for storage at -80ºC prior to LC-MS/MS mass spectroscopy analysis, which was performed and the data visualized by a previously established pipeline (61) with slight modifications. Briefly, for sample prep, SP4 (Solvent Precipitation SP3) protocol without beads was used (62). Pellets of enriched mitochondria (50 µg of protein) were solubilized by SDS (final concentration 1% [w/v]), reduced with TCEP [tris(2-carboxyethyl)phosphine], alkylated with chloroacetamide and digested sequentially with Lys-C and trypsin. Samples were desalted on Empore C18 StageTips, dried in a speedvac, and dissolved in 0.1% TFA + 2% acetonitrile. About 0.5 µg of peptide digests were separated on a 50 cm C18 column using 90 min elution gradient and analyzed in DIA mode on an Orbitrap Exploris 480 (Thermo Fisher Scientific) mass spectrometer equipped with a FAIMS unit.

Recombinant Mic34, Mic19 and ENTH expression and purification

The Mic34 open reading frame, omitting the region encoding the first 14 amino acid residues, was cloned into the pSKB3 vector via the BamHI and HindIII restriction sites in frame with the N-terminal His tag sequence (28). The BL21(DE3)-derived C4 *E. coli* strain (63) was transformed with the plasmid. Upon reaching 0.6 OD_{600}, rMic34 production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37ºC. Isolated inclusion bodies containing rMic34 were dissolved in 8 M urea. This mixture was loaded into ÄKTAprime Plus liquid chromatography system connected the 1 ml HisTrap column (GE Healthcare), followed by washing with lysis buffer (8 M urea, 2 mM Tris pH 7.2, 200 mM

NaCl, 1mM EDTA) at a 1 ml/min flow rate. An increasing gradient of elution buffer (lysis buffer with 400 mM imidazole) was introduced into the column; rMic34 was eluted at 40% (v/v) elution buffer. Fractions in which rMic34 was detected by Coomassie stained SDS-PAGE were pooled and loaded onto a HiLoad 16/600 Superdex 200 pg column (Cytiva) for size exclusion in 8 M urea, 150 mM NaCl, 10 mM Tris-HCl, pH 8. Purest fractions were chosen based on SDS-PAGE analysis and the protein was refolded through step-wise dialysis against refolding buffer (0.1% DDM, 150 mM NaCl, 20 mM HEPES, pH 7.4)

The *S. cerevisiae MIC19* coding sequence was cloned into the pGEX-6P-1 vector (Cytiva) via the BamHI and SalI restriction sites with a C-terminal TEV cleavage site and His-tag, and in frame with the N-terminal GST tag. BL21(DE3) *E. coli* were transformed with the plasmid and grown to an OD600 of 0.6 before induction with 1 mM IPTG for 20 h at 18°C. Cells were lysed in lysis buffer (complete protease inhibitor cocktail without EDTA [Roche], 5% glycerol, 50 mM imidazole, 150 mM NaCl, 20 mM HEPES, pH 7.4), cleared by ultracentrifugation at 70000 × g for 25 min and loaded onto a His-Trap column (Cytiva) using an Äkta purifier chromatography system. Unbound proteins were washed out with 10 column volumes (CV) of lysis buffer and bound proteins eluted in 500 mM imidazole lysis buffer. Imidazole was dialysed out in dialysis buffer (5% glycerol, 150 mM NaCl, 20 mM HEPES, pH 7.4). The GST tag was removed via PreScission protease (Cytiva) and removal of GST and PreScission protease on glutathione Sepharose (GE Healthcare) and the His-tag via incubation with TEV protease in the presence of 14 mM β-mercaptoethanol and removal of His-tag and TEV protease on nickel-nitrilotriacetic acid (NiNTA) agarose beads (Macherey-Nagel).

The *Rattus norvegicus* ENTH domain of Epsin was purified based on a previously published protocol (64). In short, the protein was expressed with an N-terminal GST tag from the pGEX-6-P2 vector in BL21(DE3) cells. Expression was induced in LB medium with 1 mM IPTG for 3 h at 30°C. The cells were harvested and lysed in lysis buffer (complete protease inhibitor cocktail without EDTA [Roche], 150 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1 mM DTT, pH 7.4). Lysed cleared by ultracentrifugation was loaded onto a GST-Trap column (Cytiva) using an Äkta purifier chromatography system. Bound protein was eluted in lysis buffer containing 10 mM reduced glutathione and peak fractions were concentrated. The GST tag was cleaved with Precision Protease at 4°C overnight. To separate cleaved GST, the buffer was then exchanged to low salt buffer (50 mM NaCl, 50 mM HEPES, pH 8) using a Sephadex G-25 column (GE Healthcare) and the sample applied onto a HiTrap Q column (Cytiva). The bound ENTH domain was eluted by steadily increasing the NaCl concentration to 1 M. Concentrated peak fractions were further purified by size exclusion on a HiLoad 16/600 Superdex 75 pg column (Cytiva) in 150 mM NaCl, 20 mM HEPES, pH 7.4, and ENTH-containing fractions were pooled.

LUV preparation

Egg l-α-phosphatidylcholine (PC), egg l-α-phosphatidylethanolamine (PE), liver l-α-phosphatidylinositol (PI), brain l-α-phosphatidylserine (PS), Heart cardiolipin (CL), and brain L-α-phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) were purchased from Avanti Polar Lipids, Inc. Folch bovine brain extract Fraction I was purchased from Sigma Aldrich. IMM (48% PC/24%PE/16%PI/4%PS/8%CL), IMM-CL (56% PC/24%PE/16%PI/4%PS) and Folch + 5% PI(4,5)P2 lipid mixtures were dried under nitrogen flow to form thin lipid films. Then they were dried in a desiccator for at least 3 h. Lipid films were hydrated in rehydration buffer (150 mM NaCl and 20 mM HEPES buffer, pH 7.4). Resulting LUVs were subjected to repeated freeze–thaw cycles and extruded through a 100-nm diameter polycarbonate filter (Whatman). Protein was added to liposomes in 0.1% DDM rehydration buffer with a molar protein/lipid ratio of 1:500.

Flotation assay, sodium carbonate extraction, proteinase K protection

Flotation assays were performed in nonionic Histodenz (Sigma-Aldrich) density gradients. LUVs were placed on the bottom of the ultracentrifugation tubes, and a discontinuous 40/30/20/10/2% Histodenz density gradient was built up from high density to the lowest at the top of the tube. LUVs were ultracentrifuged for 1 h at 273,000 × g and 4°C. Afterwards, the gradient was dissected into nine equal fractions, precipitated with 10% TCA, and analyzed by SDS-PAGE. For sodium carbonate extraction and proteinase K protection assay, LUVs were flotated in a two-step density gradient consisting of 40% and 2% Histodenz. LUVs and co-flotating proteins were collected from the interphase. For sodium carbonate extraction, LUVs were incubated with 3 volumes of 100 mM Na_2CO_3', pH 11, for 30 min at RT before ultracentrifugation at 167,000 × g for 30 min at 4°C to separate membrane-bound from membrane-dissociated proteins. The supernatant containing lose protein was TCA-precipitated before both fractions were analysed by SDS-PAGE. For proteinase K protection assay, collected LUVs were washed in 8 volumes of rehydration buffer (150 mM NaCl, 20 mM HEPES, pH 7.4), pelleted at 167 000 × g for 30 min at 4°C and again resuspended in rehydration buffer before being incubated with 40 µg/ml proteinase K, or buffer, in the presence or absence of 1% SDS for 15 min at RT. Then, 2 mM PMSF was added and the samples incubated for 10 min at RT before being TCA-precipitated, resuspended in SDS-loading dye containing 1 mM PMSF and analysed by SDS-PAGE. As a control, proteins in absence of LUVs were treated in the same way.

***Escherichia coli* culturing, transformation and subcellular fractionation**

Coding sequences of *T. brucei* Mic34, Mic40 and Mic24 (omitting the regions encoding the first 14, 28, and 7 amino acid residues, respectively) were cloned into the BamHI and HindIII restriction sites of the pMAL-p2X vector and transformed into BL21(DE3) *E. coli*. The induction of MBP chimeras, which are targeted to the periplasm, was done as previously (9), except using LB broth. The subcellular fractionation of *E. coli* was performed as before (9), except the integral proteins remaining in the cytoplasmic membrane of the spheroplasts were separated by 0.1M Na_2CO_3 pH 11.5 treatment like previously (17).

Mic34 site directed mutagenesis and deletion of LBS motifs

A PCR reaction was performed on the pT7-3V5-Mic34 plasmid using oligos LUK0227 and LUK0280, the former incorporating the mismatches necessary for introducing the desired point mutations within the mitofilin domain of Mic34. ΔLBS1 and ΔLBS1+ 2 deletion mutants were generated using either pT7-3V5-Mic34 or pMAL-p2X-Mic34 templates for a PCR reaction with a primer downstream of LBS1 (ΔLBS1-R) and upstream of either LBS1 (ΔLBS1-F) or LBS2 (ΔLBS2-F), respectively. Primer sequences are:

LUK0280	5'-TATCGTTGAGGCGGGGAAAGCTCCCG-3'
LUK0227	5'-GCGCGTCCGAGATTATCGGCCGTTACATCCACAACAGCTGGTGA-3'
ΔLBS1-R	5'-TGAAGGGATGCTGTTTATGTACTTGAG-3'
ΔLBS1-F	5'-GCTCCCGCGGCTGAGCCCGTTC-3'
ΔLBS2-F	5'-TTCCGCACATCACTAAGCCCAACC-3'

These oligos containing 5'phosphates were ligated using T4 ligase (NEB) following purification of the PCR product and DpnI digestion (to remove the original plasmid). The resulting plasmid was verified by sequencing.

Immuno-electron tomography and transmission electron microscopy

The cell suspension was fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M HEPES buffer for 1 h at RT. The cells were washed three times in

0.1M HEPES buffer and embedded in 10% gelatine preheated at 37°C. The gelatine blocks were immersed in 2.3 M sucrose for 72 h. The sample was placed on a stub and frozen in liquid nitrogen, then cryo-sectioned into 80-100 nm sections, using a cryo-ultramicrotome EM UC6 (Leica). The sections were placed on a formar/carbon-coated nickel grid (300 Mesh) and allowed to thaw. The grids were then placed in 0.1M HEPES buffer. After treatment with blocking buffer (BB) composed of 1% fish skin gelatin dissolved in HEPES for 1h, the grids were incubated with anti-MBP antibody diluted 1:20 in BB at RT for 30 min. After washing in BB, the sections were incubated with the Protein A conjugated with 5 nm gold nanoparticles (UMC, Utrecht) diluted 1:50 in BB and incubated for 45 min at RT. Controls for nonspecific binding of the secondary antibody were performed by omitting the primary antibody. Finally, the sections were washed in HEPES, dH2O, stained with uranyl acetate, embedded in methylcellulose, and inspected using a JEM-1400Flash transmission electron microscope (JEOL).

Localization and analysis of immunogold labelling were performed utilizing electron tomography. A JEM 2100F (JEOL) transmission electron microscope equipped with a direct electron detector K2 Summit (Gatan) and controlled with the software package SerialEM was used to acquire tilt series images in the range +57° to -63° with a tilt step of 1°. Electron tomography reconstruction and filtering using nonlinear anisotropic diffusion were performed in the software package IMOD (65).

LUVs were imaged using a Talos L120C electron microscope equipped with a Ceta 16M camera.

Vesicle and nanoparticle counting

Particle detection was performed by applying a density threshold to enhance the contrast between the nanoparticles and the surrounding membrane. Particle counting was conducted using the ImageJ software, where the occurrence of nanoparticles and vesicles was analyzed with a precision limit of 5 nm. Vesicle counting was performed blind in 23 cells.

Light microscopy and image analysis

Images for 2D branching analysis were acquired on a BX63 widefield microscope (Olympus) equipped with a 100× UPLSAPO NA 1.40 objective lens (Olympus), with an X-Cite excitation light source (Excelitas), U-FUNA and U-FBWA fluorescence filter cubes (Olympus), and a DP74 camera (Olympus) set to the 8-bit grayscale mode. Raw 8-bit grayscale images were converted into the 32-bit format. Images were filtered using the difference of Gaussians s = 2 and 4. Background subtraction was done in Fiji (66) using the sliding paraboloid and the radius of 20.0 pixels. Image thresholding was done using the triangle method. Branching quantification and analysis was performed in Fiji using the 2D analysis option of the Mitochondria Analyzer plugin (67). We measured mitochondria branching and length for 100 complete cells for each condition. All statistical analyses were performed using the GraphPad Prism software, using an unpaired two-tailed Student's t-test. A *p*-value of less than 0.05 was considered statistically significant.

Images for fragmentation analysis and 3D reconstruction were acquired on a FV3000 laser scanning confocal microscope (Olympus). The microscope was equipped with a UAPON 100× TIRF NA 1.49 objective lens (Olympus), 405 nm and 488 nm lasers for excitation (Coherent), quadband dichroic beamsplitter TRF89902 ET (Chroma), and high-sensitivity GaAsP spectral detectors with detection windows set to 420-470 nm and 500-540 nm, respectively. Z-stacks were acquired using Nyquist sampling and processed using the 3D constrained iterative deconvolution with the maximum likelihood algorithm and the theoretical point spread function in cellSens Dimension (Olympus). Creation of 3D models

was done in Imaris 10.0 (Oxford Instruments) using manual thresholding for surface creation and removal of small objects. For fragmentation analysis maximum intensity projections were taken from Z-stacks and processed in Fiji using the Mitochondria Analyzer plugin. Single cells were cropped from the images and subjected to image thresholding using the default parameters in Mitochondria Analyzer with the Block size of 1.85 micron and the C-value of 3. The number of non-connected fragments for individual cells was counted from binary images. We measured 35-50 cells for each condition. All statistical analyses were performed using the GraphPad Prism software, using an unpaired two-tailed Mann-Whitney test in GraphPad Prism. A *p*-value of less than 0.05 was considered statistically significant.

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD059459. Mic34 and Mic40 MSAs are available in the Figshare data repository (XXXX).

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FIGURE LEGENDS

Fig. 1. The N-termini of Mic34 and Mic40 retain a mitofilin domain.
(A) Schematic representation of HHpred results using representative profile HHMs. The searches detected statistically significant matches (the respective values indicated in the figure) between both Mic34 and Mic40 and the Pfam family PF09731.14 (*i.e.*, Mic60), with the specific matching regions delimited by the grey zones. The three proteins are drawn to scale according to the reference proteins for each HMM (arbitrarily Mic34 and Mic40 from *T. brucei* and *Dictyostelium discoideum* Mic60). For Mic60 (PF09731.14) three different structural/functional regions are delimited, including a TMD (TM), a coiled-coil region (boundaries as reported (22) for *C. thermophila* Mic60), and the mitofilin domain as recently redefined (21).
(B) The AlphaFold2 predicted structures of the putative mitofilin domains of Mic34 and Mic40 (light purple) aligned to the predicted mitofilin domain of discoban *N. gruberi* Mic60 (alpha helices colored as in B). The root-mean-square distance (RMSD) of the alignments is given in red below the model. Scale bar, 10 Å. Dashed-boxed insets show alignment of conserved LBS1 aromatic amino acid. Scale bar, 1 Å. Major euglenozoan clades depicted as in (15): Eugl., Euglenida; Dipl., Diplonemea; Kin., Kinetoplastea.
(C) Map of the mitofilin alpha-helical motifs from yeast and *N. gruberi* (Ng) Mic60, plus representative Mic34 and Mic40 from the three clades in A. Mitofilin alpha helices are color coded and correspond to names on top. The conserved aromatic amino acid residue of LBS1 is color coded based on the key below the uppermost LBS1 (F, phenylalanine; W, tryptophan; Y, tyrosine). Abbreviations: α, alpha helix; Ct, *C. thermophilum*; Eg, *E. gracilis*; Hp, *H. phaeocysticola*; LBS, lipid binding site; Sc, *S cerevisiae*; Tb, *T. brucei*. The PDB accession number for the solved CtMic60 mitofilin domain structure (12), in which LBS2 was necessarily deleted, is given in parentheses.

Fig. 2. Mitochondrial remodeling by Mic34 and Mic40 seems to be coupled with their involvement in the mitochondrial intermembrane space assembly pathway.
(A) Representative 3D reconstruction of mitochondria in wild type (WT) and Mic34 knockdown *T. brucei* cells at time points (TPs) 3 and 5 days after Dox induction. Mitochondria labeled by an anti-HSP70 antibody are in green; the nucleus and kinetoplast DNA (arrowhead) are in blue. Scale bar, 1 µm.
(B) Scatter plot showing the number of mitochondrial branches per cell (n=100) in WT plus Mic34, Mic40, and ERV1 RNAi at TP 3 and 5 of doxycycline induction. Data are presented as individual values with their mean and standard deviation indicated by bar and whiskers. Statistical significance: **p = 0.01 and ****p < 0.0001.
(C) Schematic representation of the MICOS complex and ERV1. Peripheral MICOS subunits whose depletomes were determined are colored in yellow. Integral membrane components are shown in gray. The lipid bilayer is represented as a double gray line. The ERV1 dimer is depicted in red.
(D) Volcano plots of Mic40 depletomes measured at TP3 (top row) and TP5 (bottom) of RNAi induction. Each row shows the same Mic40 TP3 and TP5 depletome data colored to highlight overlap with the depletomes of (left to right) Mic34 (24), Mic20 (17, 28), and Erv1 (27). Points corresponding to MICOS subunits are individually labelled as in C. X-axis, Log_2-transformed fold change of Mic40 RNAi over WT; y-axis, -log of P-value of measurement from triplicates.

Fig. 3. Mic34 binds to phospholipid bilayers *in vitro*.
(A) Carbonate extraction of Mic34 loaded large unilamellar vesicles (LUVs) with 25 or 75 mM sodium carbonate. Supernatant, S; pellet, P.

984 **(B)** Proteinase K (PK) protection assay of LUVs loaded with Mic34 or Mic19 and Folch
985 liposomes loaded with ENTH visualized with Coomassie Brilliant Blue staining after SDS-
986 PAGE.
987 **(C)** Flotated LUVs with and without (-CL) cardiolipin separated on aa noncontinuous
988 Histodenz density gradient (schema on left) loaded with recombinant Mic34. Compare
989 banding pattern with LUVs flotated alone (right).
990 **(D)** Histodenz density gradient fractions resolved by SDS-PAGE and stained with
991 Coomassie Brilliant Blue or transferred to a membrane for incubation with an anti-His
992 antibody (Mic34 with and without CL).
993 **(E)** Negatively stained LUVs loaded with Mic34. Scale bar, 100 nm.
994 **(F)** Negatively stained Folch + 5% PI(4,5)P2 liposomes incubated with the membrane
995 remodeling protein ENTH that did (+DDM) or did not (DDM-) undergo prior solubilization with
996 0.1% DDM. Scale bar, 200 nm.
997
998 **Fig. 4. Heterologously expressed Mic34 and Mic40 remodels the cytoplasmic**
999 **membrane of *E. coli* in an LBS-dependent manner upon periplasm export.**
1000 **(A)** Schema (left) of periplasting by osmotic shock to separate spheroplast (*i.e. the*
1001 compartment bound by the cytoplasmic membrane) and periplasm fractions. The former was
1002 fractioned by carbonate extraction to separate integral and peripheral membrane proteins.
1003 Western blots on right show the localization of MBP-Mic34, MBP-Mic40, and MBP alone in
1004 *E. coli*, as detected with an anti-MBP antibody.
1005 **(B)** Representative transmission electron micrographs of *E. coli* when MBP expression is not
1006 (left) and is (right) induced. Scale bar, 200 µm.
1007 **(C)** Representative electron tomograms of immunogold-labeled MBP-Mic34 and MBP-Mic40
1008 using an anti-MBP antibody. Full cells are shown in the main image with dashed boxes
1009 corresponding to magnified inset images that depict either unmarked or traced images. In
1010 the latter, pink line traces the cytoplasmic membrane, red the outer membrane, and orange
1011 the vesicles; yellow circles highlight electron-dense gold nanoparticles. Scale bars: main
1012 image, indicated above bar; inset, 50 nm. Insets report the depth in the Z-dimension of the
1013 depicted plane of the tomogram when applicable.
1014 **(D)** Bar graph reporting the total number of vesicles observed in TEM images (n=22) of cells
1015 expressing MBP tagged Mic34, Mic40, Mic34ΔLBS1+2, and of WT cells. The number of
1016 vesicles arising in Mic34 and Mic40 expressing cells. Vesicles directly associated with
1017 nanoparticles are shaded in light purple with their proportion shown.
1018 **(E)** Western blot probed using an anti-MBP antibody to show MBP-Mic34 and MBP-
1019 Mic34ΔLBS1+2 expression (left) and a representative TEM image of *E. coli* expressing
1020 MBP-Mic34ΔLBS1+2. Scale bar= 200 nm).
1021
1022 **Fig. 5**. **Mic34 overexpression induces reticulation of the tubular mitochondrion of**
1023 **bloodstream form *T. brucei*.**
1024 **(A)** Growth of Mic34 overexpression (O/E) as measured by cell density plotted
1025 logarithmically under different doxycycline (Dox) concentrations (left). Solid line with circles,
1026 1000 ng/ml; triangle,100 ng/mL; square,10 ng/ml. Dashed line, 0 ng/ml Dox. Standard
1027 deviation at each time point (n = 3) is not visible at this scale. Right, western blots probed
1028 with anti-Mic34 (detecting both endogenous Mic34 and V5-tagged Mic34) and anti-V5 tag
1029 antibodies, with fluorescently detected proteins used as a loading control (load).
1030 **(B)** Left, growth of cells overexpressing Mic34 WT (black circle), ΔLBS1+2 (purple square),
1031 ΔLBS1 (red triangle) by induction with 1000 ng/ml Dox (dotted line) compared to uninduced
1032 cells (solid lines). Standard deviation at each time point (n = 3) is not visible at this scale.
1033 Right, western blot probed with anti-V5 to show expression of Mic34 WT, ΔLBS1+2, and
1034 ΔLBS1 with (+) and without (–) 100 ng/ml Dox induction.

1035 **(C)** Representative 3D reconstructions of mitochondria in parental and WT Mic34 O/E cells.
1036 Mitochondria labeled by anti-HSP70 antibody are in green; the DAPI-stained nucleus and
1037 kinetoplast DNA (arrowhead) are in blue. Scale bar, 1 μm. Note all cells are 1N1K cells.
1038 **(D)** Quantification of mitochondrial branching in 100 parental and Mic34 WT O/E (36 hours
1039 after Dox induction) cells. Statistical significance: ****, $p < 0.0001$.
1040 **(E)** Schema of the *T. brucei* bloodstream cell cycle under normal conditions (yellow
1041 background with black arrows) and upon Dox-induced Mic34 overexpression (↑Mic34 O/E)
1042 (red arrow). Mitochondria are in blue and the number of nuclei (N) and kinetoplast DNA (K) is
1043 given below each cell. Dashed arrows indicate a pathway to defective 1N2K cells under
1044 normal Mic34 expression and overexpression.
1045 **(F)** Bar graph showing the percentage of cells with reticulated mitochondria from different
1046 cell cycle stages depicted in E in parental and WT Mic34 overexpression cells.
1047 **(G)** Growth of Mic34^{R170DF174D} overexpressing (Dox+) or uninduced (Dox-) cell lines. Standard
1048 deviation whiskers at each time point (n = 3) are faintly visible at 3 days in this scale. Right,
1049 western blots probed with anti-V5 tag antibody (top) and loading control (bottom; load) as in
1050 A.
1051 **(H)** Quantification of mitochondrial fragmentation in Mic34 WT and Mic34^{R170DF174D}
1052 overexpressing (+, 36 hours after Dox induction) and non-induced (-) cells. Statistical
1053 significance: *, $p < 0.05$. Number of cells counted: WT-, 42; WT+, 37; Mic34^{R170DF174D} (both +
1054 and -), 47.
1055
1056 **Supplementary Fig. S1. Maximum likelihood tree of the mitofilin domain.**
1057 A tree inferred with RAxML v8.2.11 (substitution model PROTGAMMAJTT and default
1058 parameters) from a multiple alignment of 111 sequences (233 positions after trimming).
1059 Sequences representing the mitofilin domain in Mic60 proteins from various eukaryotes (with
1060 the sampling focused on representatives of the supergroup Discoba) and Mic34 and Mic40
1061 proteins from Euglenozoa. The root was arbitrarily placed between the Mic60 and
1062 Mic34+Mic40 subtrees. Numbers at branches correspond to bootstrap values calculated
1063 from 100 replicates (shown only when ≥25%). Tip labels corresponding to euglenozoan
1064 sequences are rendered in different colors according to the classification of the source
1065 organism into one of the three main euglenozoan subgroups (see the legend on the bottom
1066 left). Sequence accession numbers are provided in Dataset S1.
1067
1068 **Supplementary Fig. S2. Mitofilin family structures.**
1069 **(A)** Structural alignments of Mic60 mitofilin domains from yeast and the discoban *N. gruberi*
1070 (RDSM values given below in red). Red arrows point to aligned LBS1 aromatic amino acids.
1071 Alpha helix α8 not shown for the *Chermochaetoides thermophila* Mic60 (CtMic60). Scale bar,
1072 10 Å.
1073 **(B)** Structural alignment of three representative Mic34 mitofilin domains (colored as in
1074 names on the left) aligned to that of *N. gruberi* Mic60 (alpha helices colored as in Fig. 1B).
1075 RMSD scores for alignment using each representative Mic34 given on left. Red arrow points
1076 to superposition of LBS1 aromatic amino acids; light blue arrow points to askew aromatic
1077 amino acid in *T. brucei* (Tb) Mic60. Scale bar, 10 Å.
1078 **(C)** Predicted AlphaFold2 of full-length *N. gruberi* (Ng) Mic60 plus representative Mic34 and
1079 40 depicted in Fig. 1. Structures are colored according to pLDDT confidence score color
1080 coding on top left. Note that LBS2 is the least confidently predicted mitofilin alpha helix.
1081 **(D)** Helical wheel projections of the LBS1 amphipathic alpha helices of representative Mic60,
1082 Mic34 and Mic40 structures. Key to depicted features in the projections are given in the top
1083 left.
1084 All abbreviations as defined in the legend of Fig. 1B.
1085

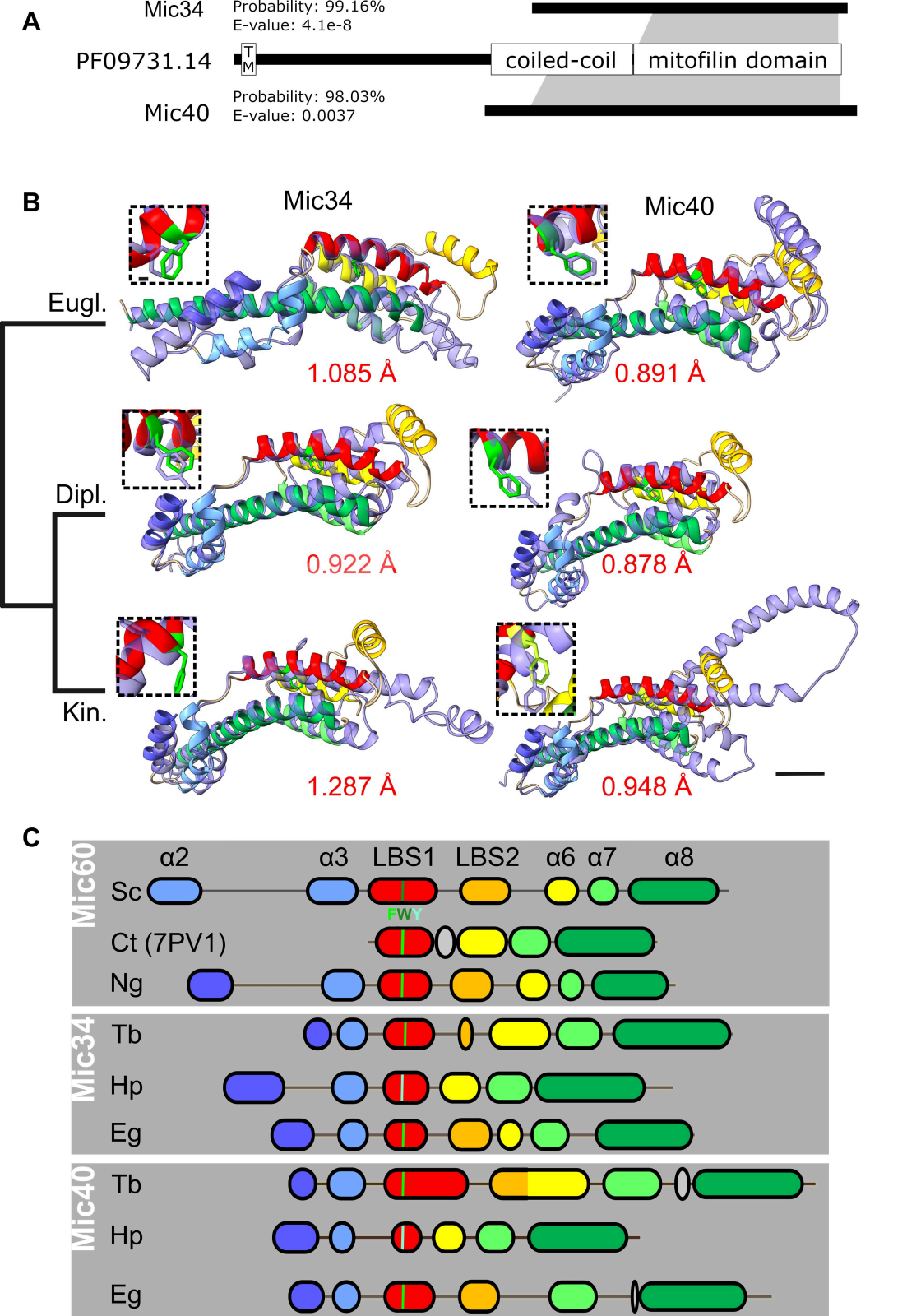
1086 **Supplementary Fig. S3. Mic34 and Mic40 depletion leads to defects in intermembrane**
1087 **space protein import that are associated with aberrant mitochondrial morphology,**
1088 **phenocopying Erv1 RNAi.**
1089 **(A)** Accumulated cell growth measurements of Mic34, Mic40, and Erv1 RNAi for 5 days of
1090 doxycycline (Dox) induction (+) as compared to non-induced cells (-). Insets show western
1091 blots probed with an antibody recognizing Mic34, Erv1, and the HA epitope attached to
1092 Mic40 expressed from one allele. The loading control for both Mic40 and Erv1 is a
1093 fluorescently detected protein on a stain-free gel, while for Mic34 an anti-Mic34 antibody
1094 nonspecific band was used as a loading control.
1095 **(B)** Gross morphology of mitochondria visualized by confocal microscopy of wild-type (WT)
1096 as well as Mic34, Mic40, and Erv1 knockdown cells, probed with mtHSP70 (green) and DAPI
1097 (blue), were taken at 3 and 5 days post-induction. Scale bar, 5 μm.
1098 **(C)** Mic34, Mic40, and Erv1 RNAi cells were collected without Dox induction (Non) and at 2
1099 (TP2), 4 (TP4), and 6 (TP6) days post-induction and proteins indicated on left were detected
1100 on western blots with specific antibodies.

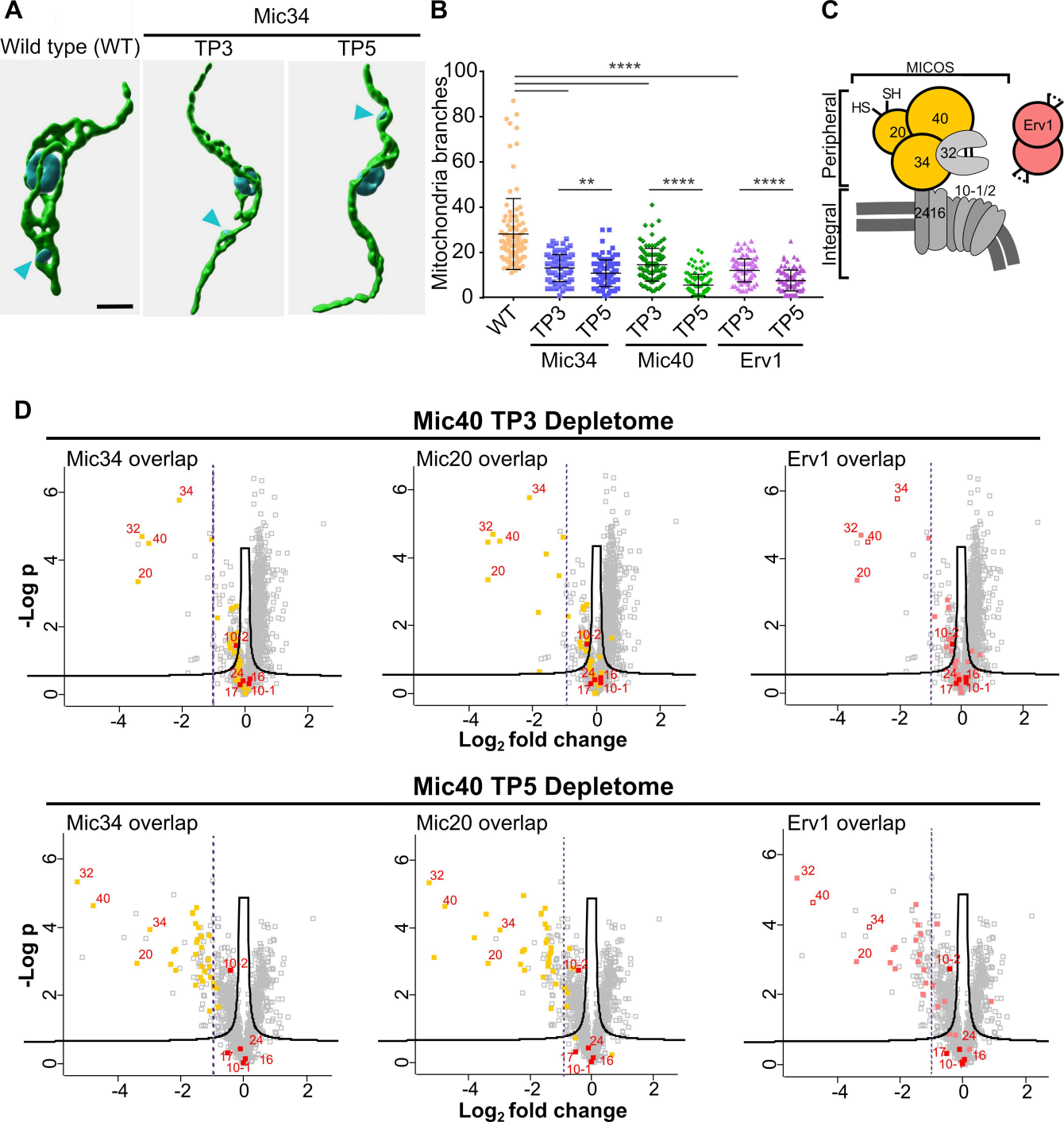
1101 **Supplementary Fig. S4. Expression and purification Mic34 and Mic40**.
1102 **(A)** Expression and purification of recombinant Mic34 resolved by SDS-PAGE with
1103 subsequent Coomassie Brilliant Blue staining from inclusion bodies (left) by His-trap affinity
1104 chromatography and size exclusion chromatography (right). Blue arrowheads to the right of
1105 gels point to recombinant protein. The affinity His-trap chromatogram with protein
1106 absorbance measured at 280 nm in milli-absorbance units (mAU) represented by blue line
1107 and conductivity in mS/cm represented by brown dashed line. The highlighted yellow region
1108 toward the end of the chromatogram shows the elution peak. The size exclusion
1109 chromatogram shows UV absorbance at 280 nm in mAU. The peak fractions collected for
1110 SDS-PAGE analysis are highlighted in red at the base of the chromatogram.
1111 **(B)** Expression and purification of recombinant Mic40. Data presented as in A.
1112 **(C)** DDM concentration titration to determine appropriate Mic34 solubilization conditions as
1113 assayed by ultracentrifugation sedimentation. S, supernatant; P, pellet.
1114 **(D)** DDM concentration titration to determine appropriate Mic40 solubilization conditions as
1115 in C.
1116
1117 **Supplementary Fig. S5**. Deformation of *E. coli* cytoplasmic membranes by periplasm-
1118 targeted mitofilin family proteins.
1119 **(A)** Osmotic shock of MBP-Mic34ΔLBS1+2 to separate spheroplasts (Sphero) and periplasm
1120 (Peri). Western blot analysis was performed using MBP antibody and T7 polymerase (T7
1121 Poly) as markers. A fluorescently detected protein visualized using a stain-free gel served as
1122 a loading control.
1123 **(B)** Fractionation and carbonate extraction of MBP-Mic24 into spheroplasts (Sphero),
1124 periplasm (Peri), integral (Integ) and peripheral (Periph) proteins.
1125 **(C)** TEM images of *E. coli* expressing full-length MBP-ScMic60-Full and soluble truncation
1126 MBP-ScMic60-IMS (9). White arrowheads point to vesicles and other deformations of the
1127 cytoplasmic membrane. Scale bar, 200 nm.
1128 **(D)** TEM images of MBP-Mic34 and MBP-Mic40, with white arrowheads pointing to
1129 nanoparticles near vesicles. Scale bar, 200 nm.
1130 **(E)** Pie chart quantifying nanoparticles associated with 100 vesicles in Mic34-MBP and
1131 Mic40-MBP in 4 cells observed by electron tomography.
1132 **(F)** Multisequence alignment (MSA) of euglenozoan Mic34 LBS1 and 2. Species are given
1133 on left and colored based on euglenozoan clade: kinetoplastid, red; diplonemid, blue;
1134 euglenid, green. Schema of *T. brucei* LBS alpha helices predicted by AlphaFold2 (Fig. 1) are
1135 immediately above MSA. Deletions made in ΔLBS mutants (left) are indicated by uppermost

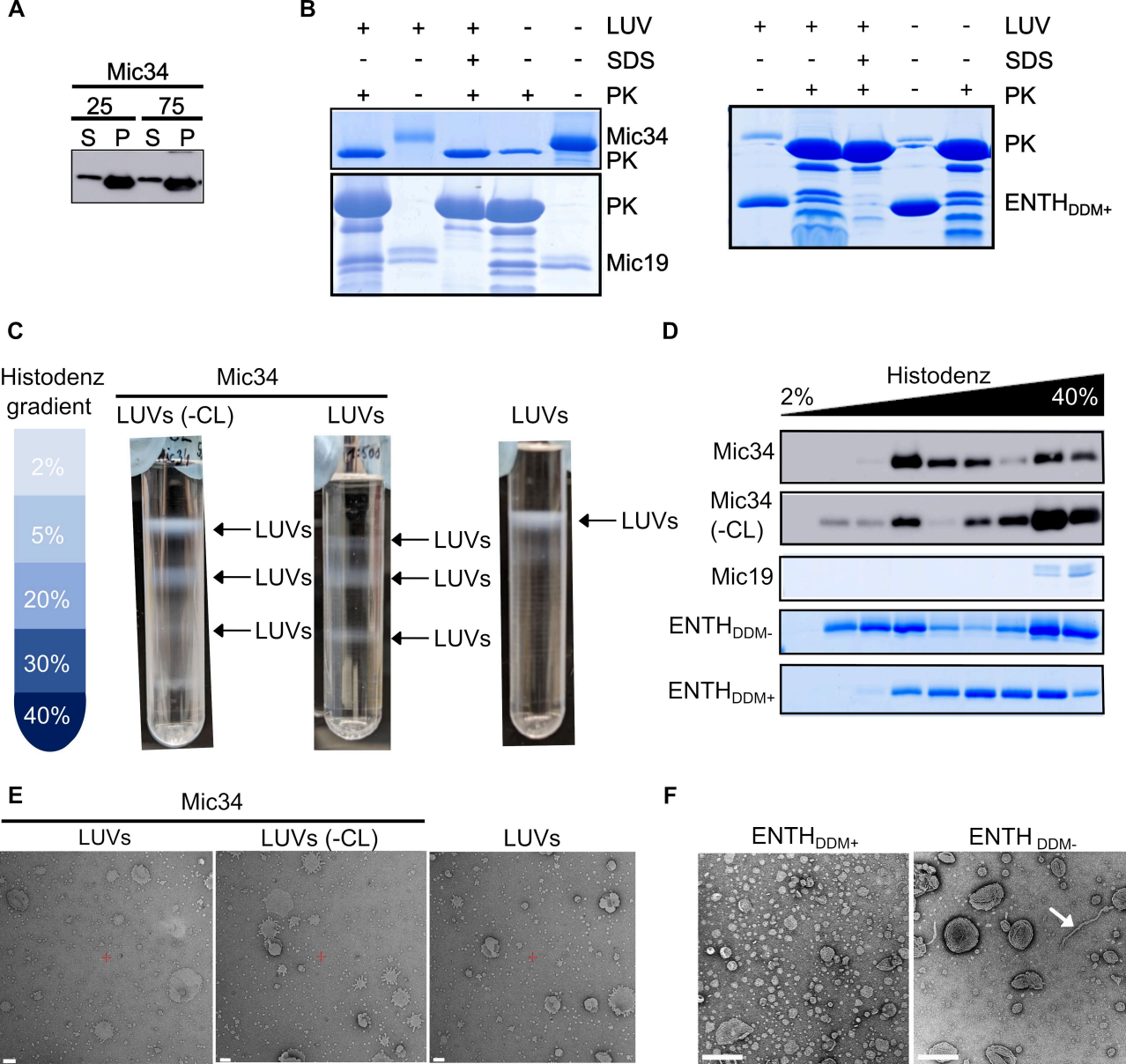
1136 black bars. Converted amino acids in TbMic34^{R170D_F174} point mutants are indicated with
1137 purple-boxed 'D's, with second one marking the conserved phenylalanine (F) of
1138 kinetoplastid, while asterisks marks conserved F position in diplonemids and euglenids.
1139
1140 **Supplementary Fig. S6. Mic34 overexpression causes growth inhibition without**
1141 **affecting protein import, OXPHOS complexes, or cristae morphology**.
1142 **(A)** Western blot analysis of Mic34 and Mic40 overexpression (O/E) in two clones and WT.
1143 Blots were probed with antibodies against V5 and mitochondrial HSP70.
1144 **(B)** Cumulative cell growth measurements of Mic34 and Mic40 O/E following three days of
1145 Dox induction. Non-induced samples are represented by a blue line, Clone 1 by an orange
1146 line, and Clone 2 by a pink line. Whiskers represent standard deviations.
1147 **(C)** Digitonin fractionation of total *T. brucei* bloodstream form cells (Tot) overexpressing
1148 examined forms of V5-tagged Mic34 (labelled on top as in Fig. 5) into cytosolic (Cyt) and
1149 mitochondrial fractions (Mit). Anti-HSP70 antibody marks fractions containing mitochondria,
1150 whereas the anti-enolase antibody marks cytosolic fractions. Note that the V5 signal
1151 fractionates with HSP70's, indicating mitochondrial targeting.
1152 **(D)** Merged Z-stack confocal slices of *T. brucei* overexpressing Mic34 (Dox+), and non-
1153 induced control (Dox-). Cell body and flagellum outlined. Green signal is from anti-HSP70
1154 antibody, which labels a protein dispersed throughout the mitochondrial matrix. Blue signal
1155 corresponds to stained DNA. Arrowheads point to mitochondrial fragments observed in the
1156 Mic34 overexpressing cells. Scale bar, 5 µm.
1157 **(E)** TEM images of Mic34 O/E after 2 days of Dox induction compared to WT. White
1158 arrowheads indicate cristae structures. Scale bar, 500 nm.
1159 **(F)** Western blot analysis of Mic34 O/E, WT BSF, and WT PCF. Antibodies used are
1160 indicated on the left of the blot.
1161
1162 **Movie S1. Electron tomogram of MBP-Mic34 deformation of the *E. coli* cytoplasmic**
1163 **membrane.** Same cell as in Fig. 4C. Red trace, outer membrane; purple trace, cytoplasmic
1164 membrane; orange trace, vesicles; green circles, gold nanoparticles that visualize the MBP
1165 moiety of the chimeric protein.

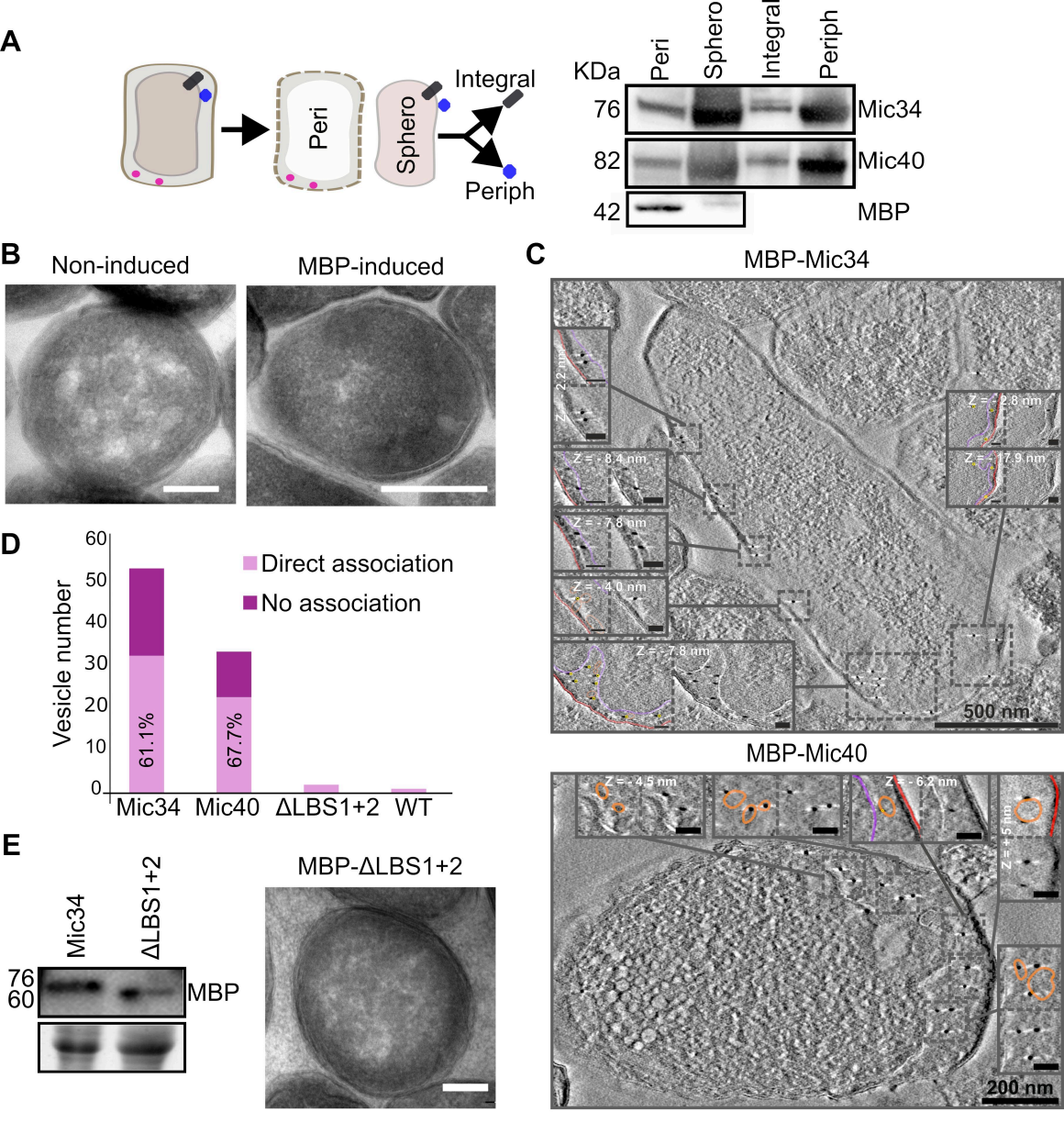
1166 **Movie S2. Electron tomogram of MBP-Mic40 deformation of the *E. coli* cytoplasmic**
1167 **membrane.** See Movie S1 legend.

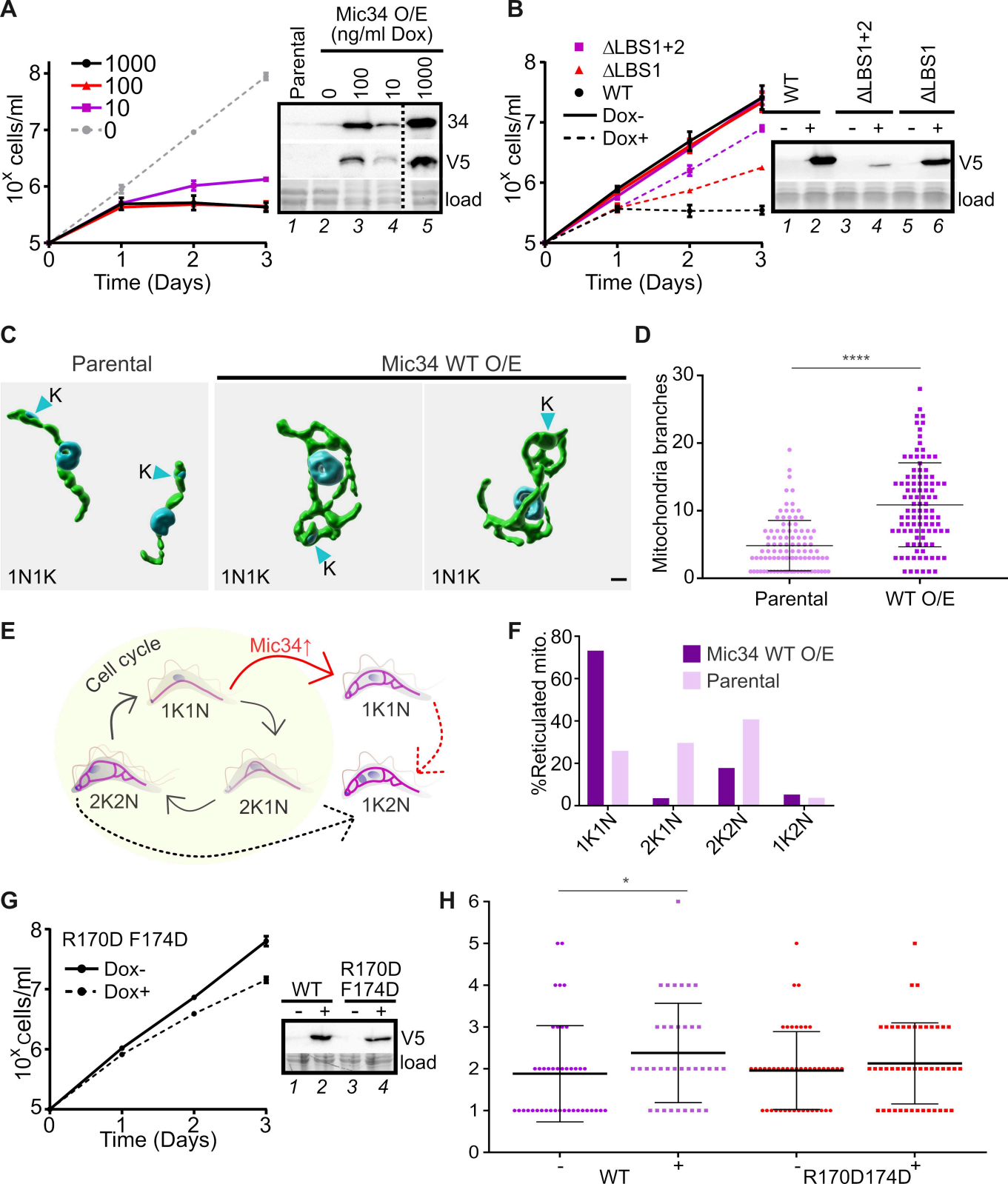
1168 **Dataset S1**. Mic34, Mic40, and Mic60 sequences analyzed in this study.


A
Mic34
Probability: 99.16%
E-value: 4.1e-8
PF09731.14
TM
coiled-coil
mitofilin domain
Mic40
Probability: 98.03%
E-value: 0.0037
B
Mic34
Mic40
Eugl.
Dipl.
Kin.
1.085 Å
0.891 Å
0.922 Å
0.878 Å
1.287 Å
0.948 Å
C
Mic60
α2
α3
LBS1
LBS2
α6
α7
α8
Sc
FWY
Ct (7PV1)
Ng
Mic34
Tb
Hp
Eg
Mic40
Tb
Hp
Eg


A
Mic34
Wild type (WT)
TP3
TP5
B
Mitochondria branches
****
**
****
****
WT
TP3
TP5
TP3
TP5
TP3
TP5
Mic34
Mic40
Erv1
C
MICOS
Peripheral
Integral
HS
SH
20
40
34
32
Erv1
10-1/2
2416
D
Mic40 TP3 Depletome
Mic34 overlap
Mic20 overlap
Erv1 overlap
-Log p
Log2 fold change
Mic40 TP5 Depletome
Mic34 overlap
Mic20 overlap
Erv1 overlap
-Log p
Log2 fold change


A
Mic34
25
75
S P S P
B
LUV
SDS
PK
Mic34
PK
PK
Mic19
ENTH$_{DDM+}$
C
Histodenz gradient
2%
5%
20%
30%
40%
Mic34
LUVs (-CL)
LUVs
LUVs
D
Histodenz
2%
40%
Mic34
Mic34 (-CL)
Mic19
ENTH$_{DDM-}$
ENTH$_{DDM+}$
E
Mic34
LUVs
LUVs (-CL)
LUVs
F
ENTH$_{DDM+}$
ENTH$_{DDM-}$


A
Peri
Sphero
Integral
Periph
KDa
76
82
42
Mic34
Mic40
MBP
B
Non-induced
MBP-induced
C
MBP-Mic34
Z = - 2.2 nm
Z = - 2.8 nm
Z = - 8.4 nm
Z = - 17.9 nm
Z = - 7.8 nm
Z = - 4.0 nm
Z = - 7.8 nm
500 nm
MBP-Mic40
Z = - 4.5 nm
Z = - 6.2 nm
Z = + 5 nm
200 nm
D
Vesicle number
60
50
40
30
20
10
0
Direct association
No association
61.1%
67.7%
Mic34
Mic40
ΔLBS1+2
WT
E
Mic34
ΔLBS1+2
76
60
MBP
MBP-ΔLBS1+2


A
Mic34 O/E
(ng/ml Dox)
Parental
0
100
10
1000
1000
100
10
0
34
V5
load
1 2 3 4 5
10^x cells/ml
Time (Days)
B
ΔLBS1+2
ΔLBS1
WT
Dox-
Dox+
WT
ΔLBS1+2
ΔLBS1
- + - + - +
V5
load
1 2 3 4 5 6
10^x cells/ml
Time (Days)
C
Parental
Mic34 WT O/E
K
1N1K
D
****
Mitochondria branches
Parental
WT O/E
E
Cell cycle
Mic34↑
1K1N
2K2N
2K1N
1K1N
1K2N
F
Mic34 WT O/E
Parental
%Reticulated mito.
1K1N
2K1N
2K2N
1K2N
G
R170D F174D
Dox-
Dox+
WT
R170D
F174D
- + - +
V5
load
1 2 3 4
10^x cells/ml
Time (Days)
H
*
WT
R170D174D