# Science Advances

# Supplementary Materials for

## Structural insights into crista junction formation by the Mic60-Mic19 complex

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Movies S1 and S2



Fig. S1. Overview of mitochondrial architecture

#### Fig. S1. Overview of mitochondrial architecture

(A) Mitochondria are surrounded by two membrane systems. The outer mitochondrial membrane (OMM) envelopes the organelles and mediates communication and exchange of molecules with the cytosol and other membrane-bound intracellular compartments. The inner mitochondrial membrane (IMM) consists of two structurally and functionally distinct domains. The inner boundary membrane (IBM) is in close proximity to the OMM, comprising the aqueous intermembrane space (IMS) compartment. Large membrane areas, termed cristae, extend from the IMM and wrinkle into the matrix. These cristae membranes adopt the shape of branched tubules, sheets or discs and determine the characteristic ultrastructure of mitochondria. IBM and cristae membranes largely differ in their protein content. Whereas the IBM particularly contains metabolite carriers and translocation machineries for macromolecules, like polypeptides, cristae membranes are exceptionally protein-rich and harbor the respiratory chain complexes and the F<sub>1</sub>F<sub>0</sub>-ATP synthase for oxidative phosphorylation. The connections between cristae and IBM are termed crista junctions (CJs). These specialized membrane regions exhibit an exceedingly high curvature and are thought to act as diffusion barriers for metabolites and proteins. (B) The formation and stabilization of CJs is mediated by an evolutionary conserved hetero-oligomeric protein complex, the mitochondrial contact site and cristae organizing system (MICOS). The core complex consists of six subunits in yeast and seven in metazoa. MICOS is made of a Mic60 and a Mic10 module with distinct properties and functions. Mic60 (formerly known as mitofilin, IMMT, Aim28 or Fcj1) is the centerpiece of the membrane-bridging subcomplex that forms contact sites between IMM and OMM through interactions with partner protein complexes, like the sorting and assembly machinery (SAM). The Mic60 protein consists of an N-terminal transmembrane segment anchored in the IMM and a large hydrophilic domain in the IMS that exhibits an extended coiledcoil region and a C-terminal mitofilin signature domain. Mic60 is firmly associated with Mic19, a peripheral membrane protein that may act as a redox sensor through an intramolecular disulfide bond. In metazoa, the Mic60 module additionally contains Mic25 that belongs to the same protein family as Mic19. Studies in yeast and human mitochondria suggest that the N-terminal domain of Mic19 and parts of the Mic60 IMS domain differentially contribute to membrane contact site formation together with the polypeptide transport-associated (POTRA) domain of Sam50. Homooligomers of Mic10 form the backbone structure of the second MICOS subcomplex and contribute to the formation and stabilization of membrane curvature at CJs. The oligomeric state of Mic10 is regulated by Mic26 and Mic27 in an antagonistic manner and modulated by the IMM phospholipid cardiolipin. The two MICOS modules are connected by Mic12 in yeast or Mic13/QIL1 in metazoa. P: POTRA domain of Sam50, N-ter. Mic19: N-terminal region of Mic19, CC: coiled-coil domains, CHCH: coiled-coil-helix-coiled-coil-helix domain, Mito: mitofilin domain, CC dimer: Mic60 coiled-coil domain dimer. Ablation of MICOS leads to the collapse of CJs and the detachment of cristae from the IBM (A, bottom). Cristae accumulate as lamellar membrane stacks in the matrix. MICOS-deficient mitochondria show defects in oxidative phosphorylation and a variety of stress responses. (C) Representative EM micrograph of mitochondria from fixed mic60/1 S. cerevisiae cells reconstituted with plasmid-borne Mic60. Filamentous densities near the CJs are indicated by white arrows. (D) Same as in C, but reconstituted with the tetramerization mutant  $Mic60^{1274D/F280D}$ . Interference with Mic60 tetramerization results in a reduction of CJs. (E) and (F) Additional EM micrographs (as in C and D) and quantitative analysis of CJ diameter of mitochondria from fixed mic60 $\Delta$  S. cerevisiae reconstituted with Mic60 (n = 74, mean diameter 13.8 nm) and with the tetramerization variant  $Mic60^{1274D/F280D}$  (n = 50, mean diameter 10.4 nm).



Fig. S2. Oligomerization and interaction of Mic60 and Mic19

#### Fig. S2. Oligomerization and interaction of Mic60 and Mic19

(A) BN-PAGE analysis of purified  $tMic60_{CC}$  and the tetramerization variant M291D/F297D. (B) SDS-PAGE (left) and BN-PAGE (right) analysis of the  $ctMic60_{sol}$  and  $ctMic60_{sol}^{R525C}$  under reducing and non-reducing conditions. 'WT' represents  $ctMic60_{sol}$  under non-oxidized conditions, whereas the lanes 'WT ox' and 'R525C ox' show  $ctMic60_{sol}$  and the R525C variant, respectively, after oxidation using CuSO4. (C), (D) SDS-PAGE analysis and quantification of liposome co-sedimentation assay using different  $ctMic60_{sol}/ctMic19$  single amino acid substitution variants (C) and their respective complexes (D). S, supernatant; P, pellet. The light grey bars in (D) represent ctMic19 and its variants and the dark grey bars  $ctMic60_{sol}$  and its variants. Measurements were done in triplicate and error bars indicate the s.d. of each data set. SDS-PAGE data shown in the main Figs. 3C and 4E are not included here. (E) BN-PAGE analysis of  $ctMic60_{sol}$  variants and their complexes with ctMic19 variants. Proteins and protein complexes in all figures were visualized with Coomassie Brilliant Blue.



#### Fig. S3. Structural details of Mic60 and Mic19.

(A) Tetrameric structure of  $ltMic60_{CC}$ . Tetrameric helical assemblies are often found in membraneremodeling proteins, such as in SNARE complexes (59) or the stalks of dynamin proteins (60), although the detailed topologies differ. (B) Surface conservation plot of the tetramer interface (magnified area) and the monomer of  $ltMic60_{CC}$ . The other monomers are shown in cartoon representation. Conserved residues are colored dark magenta, variable residues dark cyan. (C) Localization of K366, which was exchanged to cysteine to stabilize the tetramer via a disulfide bridge. K366<sup>1</sup> refers to monomer 1 and K366<sup>3</sup> to monomer 3. (D) Structure prediction of the C- terminal region of Mic60 from co-evolution analysis. The mitofilin domain is colored in magenta and LBS 1/LBS 2 in orange. (E), (F) Surface conservation plot of one monomer of Mito2\_CHCH. The second monomer is shown in cartoon representation. The GS-linker is colored in yellow. Both magnifications in (E) show the conservation of the mitofilin-CHCH domain interface. The CHCH domain or helix  $\alpha 3^{M}$  from the mitofilin domain are displayed as cartoon representation for better visualization. The magnifications in (F) represent the conservation of the mitofilin-dimer interface.

ctMic60 ltMic60 scMic60 hsMic60 drMic60 drMic60 cdmMic60 ceMic60	1 1 1 1 1 1 1	MLRTSLP MLRACQLSGV MLRACQLSGV MLRVCWKG-A MLRACRKAQA MYRLAVRDQC MFRIASKRSV	SVRALGSRPA TAAAQSCLCG TVAAQSCLCG HAAARQCVCG AVLXECLCG KCALQRTLQQ SQTLKRARQQ	AVTAAG KFVIRP KFVIRP KVKAHP RISLRP T'ANNRQFGG SNQAA	SSSGGREQ	ReWolaa - LRPCRRYST - LRPCRRYST - LeHCRRYTT - LRTSRSYST GRROCEEOGO - PPKPFVOPP	RRAQLNVAQ GSSGLTTGK SSSGLTAGK AGNSGSSAGK TRSSGSSAAK QGDQGYQGYQ KKSGGCKGLA	LVSGATIVSL IAGAGTLFVG IAGAGTLFVG IVASTLTVG IIGASVLLTG SLPPHMREAG LVGATVVGAG	G G G FGKVVLFV&P G		RFYTDDA PA R	70 5 74 74 73 74 100 70
ctMic60 ltMic60 scMic60 hsMic60 drMic60 drMic60 ceMic60 ceMic60	71 6 75 75 74 75 101 71	SPETTSTT ATINCVKSGR ASRK-IVIR VEKTIPYSDK VEKTIPYSDK VEKTVPYSDQ VEKTVPYSDK VEKNVPGAGS VESTVPPVKQ	APP AGRA G LPEMVIG-PA LPEMVIG-PA LPONUGAPP LPELAIGAPA VIXVALOEP VEDAVIG	AYNVPLPKKS PYTVPLPKKS SPVPIOKKP PASLPVPKKP PFKG-ITKN 	Lorg Lag IQSGPLKISS VQSGPLKISS ETVKPLQISS LKSGPLQISS VNDQIDKVKS LOKTKQQIGD	TTPLPPPKK MATFEGOPAA NTGTTVASKK VSEVMKESKQ VSEVMKDSKL LSEATKDSKQ MSEVMKESKM GIETVTSTVD LKDAVWAVP	K P-ASQLQKQK P-VAQSKTK P-VAQSKTK P-KARAKSD PKKEKVEAPL SVTSKVTGLF K	GDTPASATAP GDTPASAAS- PAPPPSVEEA SPPPPPFTE GGGSGDDKS-	PAPAPALQSL SLNL		IRNYILULTS SKLLVRVGT RNTLWTIAT SAAGDTLSV SAAGDTLSV SATGEAVSV VKATPAEEKR ELAPLPFVT	119 47 44 158 157 172 167 184 128
ctMic60 ltMic60 scMic60 mmMic60 drMic60 cdmMic60 ceMic60	120 48 45 159 158 173 168 185 129	LSALAFGGV ATVGFYVGGV STAFYAGI PAPAVOPES PAPAVHEDT PAPGT-SDS PAPAVHKES PSKPSEVSKT PREPTHVDPV	WY RVNDNFH TL3 LX LX LX LX 	DFFTTYVPYG IGEGKPTPAL TNEGKSTSET ARECOKECT VTEEHCKECD	EQAVLYIEEL SEEASSS9- TEEAFSS8- EPEPAVKE- HEAVHKSVHK KDVTP	DLKKRFPNTA LMMDOFG OKMDKFG IRERPPEEVA VRERPPEEVA RPAEEVT LKERPAEEVS VOKPAAA MIPKPSP	DRUGS SRRSD ELECDN-VPL DFESNN-VPF ARUAQEKQE ARUAQEKQE ARUAQEKAE ARUYQEKAE ARUYQEKAE AAPAPAAKPK DVUFAKNQQL	LGDSVKVAPH AESIVEMYEE AEDLLETYEH QVKTESLAKS QVEMESLAKS LDAUAALTAG GEKTKFIAAS DNPLPRDVVE EEKTKIATHS	SGASWRVADG FRDEKMOASR HDRPTLFIE IEDAUROTAS IEDAURTSS IEDAUSSAK IEDAUSSAK IEDAUSSAK IEDAUSSAK AEGKVRLATE	SETSAROSSS MSID DSWD VIDOAIAAON VIDOAIGAOE VIDOAIGTOE LAVKEYN AKIKTIN	IQAVESAKKE GIKAKS AAVQAVNAHS AAVQAVNAHS AALTAIAAHT AAVQAVNTHA VAIGVUKGFN AINEHA	219 100 253 252 262 267 252 190
ctMic60 ltMic60 scMic60 mmMic60 drMic60 xlMic60 ceMic60	220 107 104 254 253 263 268 253 197	AKUTRAKPAV GELGTKVDRI NDILSGLTGS NIIKAAMDNS NIIKTAMDNS DKUREAMD-S OKUREAMD-S OKUREAMD-S SIIKQTVDA	IEEAKKKEEE PNRGADP SQ'IRSN EIAGEKKSAQ ETAGEKKSAQ ETPPDEKSTQ (ISADKKSLQ VENGENSLWT KHANWENVTS	KEEQTPKEAA WRTVEGALKE WRTVEGALKE ALNEALNE WRDLEDALKV TLKNR ALQR	ASV CEKKTV LETS GAV RENIEV RRKAVDEAAD RRKAVDEAAD RARAVDEAAD ASARDTAVAT AEAEARVDSG	PKPEPPKSP AAJPASKE KRILSL3P ALJKAKSE ALJKAKSE ALPKAKSE SLJKAKSE AERAARSA QEVDGRNY	S SPAAAIVA RLEDES INIETEN EKMKSVIEN EKMKTIIED EKLRSVIDK IERMOSIIGD OEKIVACEIA	AALVEKKEE AKKKEVAGAK AKKREIAGAT AKQSKIDSAR SKKSLINGAK ISAAATAQNA GKRDSTTATN	PHI DAAPGRI PHI DAAPGRI PHI DAAPGRI PO LAAPGRI LI ISABENI KKYEA VRDKI PLI LNAQETA	PEUDEPSRWP PEU	PASPIDE       IV         EQLG       -         IVDIDIVVK       -	319 158 144 343 342 357 357 337 280
ctMic60 ltMic60 scMic60 mmMic60 drMic60 xlMic60 cdMic60 ceMic60	320 158 144 344 350 358 338 281	PDAAEPVVOE SOKRATP OLKE KVOAAOSEAK KVOAOSEAK KVEAAOSEAK KVEAAOSEAK KVEAAOSEAK LVNKSROESA	LVRMINDIII LV2SVNAAVA IISINDLIN VVSQTHELVV VVSQTHELVV IVSQTELVV VAQTELVS VEDKYWRNVE VINQTKDLIE	VINHDG AVNEQS GADDSN GADDFKREL GADDFRKEL LARKEFQEL LARKEFQEL KARNYFIDEI KSRQGFALEM	DSITPEVLPG DSITPDITPG ANITPEIQAN DSVTPEVQSG ESIFFGLSLA KSILPNVDIH	WKGMSVSDLA WKGMSISDLA WKGLS WKGLT DKKLN AKD	AMEKYGA bipped DKLSTDLNS GKLSTDLNS GKLSADDLNS GKLSPDLNS GKLSPDLNS -iskeDLDL KNLNEDELNA domain	T GKAKEKIA TYAAVHDAPT ENSIKKSNO LIAHAHRRID LIAHAHRRID LIAHAHRRID LIGHAHRRID LIGHAYTHVL LIAHAHLKVD	KVC KTRDMK KUKSAL OAIN JLINIS LN OUNRE DAE K OUNRE DAE K	AAROCEAAOO EDIRTINVAES ETIREAISINY ATEROHITIA ATEROHITIA VREQIHIEVA AREQOHIEAA UREDIHISKA	VKQKIDE VAVOYG-QAS KIQKTS-EVI IBKOKL-BEK IBKOKL-BEK IBCOKL-BEK IBCOKL-BEK IBSVRGDIS IBEORL-ALE	390 220 209 442 441 443 451 430 372
ctMic60 ltMic60 scMic60 hsMic60 drMic60 xlMic60 dmMic60 ceMic60	391 227 210 443 442 444 452 431 373	DKTANELVSR KDTHESFEIR TEDTYCENS RADSAVAKA RTDSAVAKA KACERAVIS KACERAVISKA EATRACTEYH RTASEKISIE	ARSREVEITO ARSREVEITO REFERITO IDHIRSEIOA IDHIRSEIOA IDHIREITA IDHIREITA IDHIREITH IDAERKIAV MSRVGRONEL	AFR BEEEM OFLUE FNAFK DORKIEEVR DORKIEEVR DORKVEEVR DEKKVEEVR BEEKKEEVR BIERAUVESR	ARVKASYDAK ACIEPKISEE ENEKKIKSKO DATEVEMRTQ DATEVEMRTQ EVIJEVEMRTQ ASDKLIRLQ SSTEGELENQ	VOLIORERO LASALKANEO LEERIKANEO LERCAAAHTD LRCAAAHTD LRCAAAHTD LRCAAAHTD LRCAAAHTD LRCAAAHTD LRCAAAHTD LRCAAAHTD LRCABAHAD LKCABAHAD	LAE RINGL ANDAK SNOV HIRDVIRVOE HIRDVIRVOE HIRDVIRVOE HIRDVIRVOE HIRDVIRVOE HIRDVIRVOE HIRDVIRVOE HIRDVIRVOE HIRDVIRVOE	IE AVEL RH AIDSMK VE GLUSIT VKE EKSPEQ EKSPE EKSPEQ EKSPEQ EKSPE	FAREVEQVE FKILSEKLD FKILKD KIE ISEKLSEQEL ISEKLSEQEL ISEKLSEQEL ISEKLSEQEL FLORTATEKA	RERDGRLGRI BROGRLSRI FRRLSE FRRLSE FRRLSE FRRLSE MYRL FRRLGE LHSK VGAA	CELSAAVADI EALNGSVODI EBINSEVNDI DNFILDINIA DSFILDINIA DGFILDINIA GGTILDINIA SRIEGIEEAI	490 326 309 542 541 543 551 530 472
ctMic60 ltMic60 scMic60 hsMic60 drMic60 xlMic60 dmMic60 ceMic60	491 327 310 543 542 544 552 531 473	ERLTADWN SV AEAVD QVDTL SK TDRS SKI YARTRGIE GA YARTRGIE GA YARTKGIE GA YSRTKGIE GA AERADAERTA GSRVALDNEN	TOTNLRT OL VMKSEVISOL ISKNEALVOL VOSHAVASDE VOSHAVASDE VOSHAVASDE VOSHAVASDE VSCHAAASDE NOAQALWAAC RRAKQEWIAC	02" HVAUPAVRAS SLLTTLIKNK TFODETKSR ARKSHOLWLS ARKSHOLWLS ARKSHOLWLS ARKSHUHWLS (ALWASVRAA HNLLDTLKHG LBS	DDAR HAGDES SVK INNNLPDVN VEALKYS-MK VEALKYS-MK VEALKYS-MK VEALKFT-MK TPGVHY NKAGN 1	- C CC IDSELARLKI IDSELARLKI IDSELSRLKI TSSAETPTIP TSSAETPTIP TAVGDOPTEP KDRLRP N-IDERRLP	FIREIVALKE ICDIIPGRPS ISSLISTENK IGSAVEAIKA ISSAVEAIKE IEGAVRAIKE ITTAVGIRS IKNEINAIAK INESINLIKE LBS2	IAAG KCCS NCS SCA VAK VNP	SKKGNKNEG	KEGKISCKCK		548 397 409 610 611 619 591 534
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ctMic60 ltMic60 scMic60 hsMic60 drMic60 xlMic60 ceMic60	641 482 495 703 702 704 712 689 628	C C2 <sup>M</sup> IDNAAREMAA IDNAVEVVA IDNAVEVVA IDNAVEVVA IELAAREVNO IELAAREVNO IELAAREVNO IELAAREVNO IELAAREVNO I	LI GNS KILSR LK GYP SVL D LK GYP SVL D LK GES RVAD LK GES RVAD	DWLAEVRYL Swie Darkti Swie Darkti Dwla Armi Dwla Armi Dwla Armi Dwla Arlii Swig Arlii Dwly Drr Syl	C3 <sup>M</sup> EVROATEVIO EIE DI DATO EVORIVE III EIROUISTI	ABARLOSIRL CEIRSS RANASAVGIGT AYASAVGIGT AYASAVGIGT AYASAVGIGT AYASAVGIGT AHAAASGILY ABAAVSSIRS	EQQ 693 527 TOPE 758 TOVOE 757 TOP 757 TOVO 765 L 739 TY 679		<ul> <li>Tetramer</li> <li>Interaction</li> <li>Membrar</li> <li>Mitofilinini</li> <li>Introduce</li> </ul>	interface on (Mic60-Mid e binding Dimer interfa ed disulfid br	:19) ce variant idge	

Fig. S4. Sequence alignment of Mic60.

#### Fig. S4. Sequence alignment of Mic60.

The following sequences are aligned: *Chaetomium thermophilum* (ctMic60, Uniprot accession code G0SHY5), *Lachancea thermotolerans* (ltMic60, C5E325), *Saccharomyces cerevisiae* (scMic60, P36112), *Homo sapiens* (hsMic60, Q16891), *Mus musculus* (mmMic60, Q8CAQ8), *Danio rerio* (drMic60, Q6PFS4), *Xenopus laevis* (xlMic60, A0A1L8HKP3), *Drosophila melanogaster* (dmMic60, P91928) *Caenorhabditis elegans* (ceMic60, Q22505). Amino acids are colored according to their chemical and physical properties (positive charge: blue, negative charge: red, hydrophobic: green, proline and glycine: brown, all others: grey). For sequence conservation greater than 70%, the background is highlighted. Residues involved in tetramerization are labeled with  $\bigcirc$ , in interaction with Mic19 with  $\bigcirc$ , in membrane binding with  $\bigcirc$ , in mitofilin dimerization with  $\bigcirc$  and the residue chosen for cysteine replacement with  $\bigstar$ .



Fig. S5. ITC data of Mic60 and Mic19.

#### Fig. S5. ITC data for the interaction of Mic60 and Mic19.

(A-K) ITC experiments with different ctMic60<sub>sol</sub> variants and ctMic19 variants. At 10 °C, a concentrated solution of Mic19 in the syringe was titrated into a Mic60 solution present in the sample cell and the resulting heat change was monitored. Mic60 concentrations in the sample cell varied between 44-81  $\mu$ M and Mic19 concentrations between 390-810  $\mu$ M. (L-M) Overview of K<sub>DS</sub> ( $\mu$ M) and binding numbers derived from the ITC experiments shown in (A-K). The deviation represents the root-mean-square error of the fit. (L) includes the ctMic60<sub>sol</sub> variants, which have been titrated with ctMic19 and (M) the ctMic19 variants, which have been titrated into ctMic60<sub>sol</sub> solutions.



Fig. S6. S. cerevisiae in vivo analysis.

#### Fig. S6. S. cerevisiae in vivo analysis.

(A) Mitochondria harboring a chromosomally encoded Mic60-Protein A fusion construct together with plasmid-borne copies of either scMic60<sup>WT</sup> or scMic60<sup>I274D/F280D</sup> were solubilized with digitonin and subjected to immunoprecipitation. After elution with glycine buffer, samples were analyzed by SDS-PAGE and Western blot (left panel). Quantification (right panel) of Mic60 antibody (for scMic60<sup>WT</sup> and scMic60<sup>I274D/F280D</sup>) and PAP (for Mic60-Protein A) signals demonstrates the reduced homotypic interaction propensity of scMic60<sup>I274D/F280D</sup> (n=4, data represent mean +- standard deviation). (B) Representative electron micrographs of S. cerevisiae mitochondria in ultrathin sections of cells expressing the indicated scMic60 and scMic19 variants. 'scMic19' represents the mic19A strain complemented with plasmid pRS416-scMic19 (WT) (see also Figs. 2E-G). Bottom right panel: number of crista junctions per mitochondrial section. 100 mitochondrial cross sections were counted with maximally two mitochondria from the same cell. Each data point represents one mitochondrial cross section, and mean and standard deviation are shown. Statistically significant differences in the mean values are indicated. (C) Comparison of steady state protein levels in isolated mitochondria of S. cerevisiae strains lacking the chromosomal MIC60 gene (mic60 $\Delta$ ) transformed with an empty vector (e.v.), or plasmids encoding either wild-type (WT) scMic60 or the scMic60 variants I532D and T539D, respectively. Mitochondrial samples were subjected to SDS-PAGE and immunoblotting with the indicated antisera. Note that the I532D variant was expressed at lower levels compared to WT. (D) Protein steady state level analysis as in (C) using mitochondria of S. cerevisiae strains lacking Mic19 (*mic19* $\Delta$ ) or expressing WT scMic19 or the scMic19 L143D or L147D variants, respectively. Note that the L147D variant was expressed at lower levels compared to WT. Accordingly, also lower levels of the MICOS components Mic60 and Mic26 were observed. (E) Experiment as described in (C); analyzed scMic60 variants were V530D and I274D/F280D/V530D. The Mic60 variant with three amino acid substitutions was expressed at slightly lower levels compared to WT. (F) Mitochondria as described in (C) additionally containing a Protein A-tagged variant of Mic19, were solubilized in digitonin-containing buffer and subjected to IgG affinity chromatography. Elution fractions were analyzed by blue native PAGE und immunodetection with antibodies raised against scMic60. (G) SDS-PAGE and Western blot analysis of immunoprecipitations with mitochondria lacking Mic60 (mic60A) or expressing Mic60 variants I532D and T539D in a scMic19-Protein A containing background. Quantification of PAP and scMic60 antibody signals shows different binding capacities of scMic60 variants to scMic19 compared to Mic60-WT (n=4, data represent mean +- standard deviation). (H) Mitochondria of yeast cells expressing scMic60-Protein A that either lack Mic19 ( $\Delta mic19$ ) or express scMic19<sup>L143D</sup> or Mic19<sup>L147D</sup> from plasmid pRS416 were subjected to immunoprecipitation. Samples were analyzed by SDS-PAGE and Western blot.





(A) Maximum projections of Z-stacks of mitochondrial stainings using 175 nM DiOC6 in wildtype (WT) *S. cerevisiae* and *MIC60* knockout cells containing either an empty plasmid or plasmids for the re-expression of WT-Mic60 ('rescue') or the indicated Mic60 variants, respectively. The bar diagram shows a quantitative analysis of mitochondrial networks from 3 independent experiments (black circles). About 300 cells were counted for each sample (p-values were derived from an unpaired t test). The white scale bar indicates 2  $\mu$ m. (B) Same comparative analysis as in (A), but with cells harboring scMic19 variants.



Fig. S8. Sequence alignment of Mic19.

The following sequences are aligned: *Chaetomium thermophilum* (ctMic19, Uniprot accession code G0S140), *Lachancea thermotolerans* (ltMic19, C5E3G4), *Saccharomyces cerevisiae* (scMic19, P43594), *Homo sapiens* (hsMic19, Q9NX63), *Mus musculus* (mmMic19, Q9CRB9), *Danio rerio* (drMic19, Q502T3), *Xenopus laevis* (xlMic19, Q7ZYP1), *Drosophila melanogaster* (dmMic19, Q9VA18), *Caenorhabditis elegans* (ceMic19, Q21551). Amino acids are colored according to their chemical and physical properties (positive charge: blue, negative charge: red, hydrophobic: green, proline and glycine: brown, all others: grey). For sequence conservation greater than 70%, the background is highlighted. Residues involved in interaction with Mic60 are labelled with  $\bullet$ .

	ltMic60 <sub>CC</sub>	Mito1_CHCH	Mito2_CHCH
Data collection			
Space group	P42 <sub>1</sub> 2	P1	P2 <sub>1</sub>
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	54.2, 54.2, 134	43.82, 48.52, 70.74	58.20, 85.37, 61.50
α, β, γ (°)	90, 90, 90	82.70, 79.14, 72.41	90.00, 101.72, 90.00
Resolution (Å)	45-2.84	46.12-2.15	49.21-2.50
	(3.01-2.84) *	(2.28-2.15) *	(2.65-2.50) *
$R_{\rm meas}$ (%)	16.8 (264)	9.0 (111.5)	13.0 (191.1)
Ι/σΙ	13.1 (0.99)	8.28 (0.92)	9.16 (0.75)
Completeness (%)	99.7 (99)	91.8 (92.3)	98.8 (97.7)
Redundancy	13.5 (12.4)	2.5 (2.5)	4.0 (4.0)
CC(1/2) (%)	99.9 (76.0)	99.8 (52.9)	99.8 (39.4)
Refinement			
Resolution (Å)	44-2.84	46.12-2.15	40.74-2.50
No. reflections	5,157 (487)	27.211 (2.766)	20.337 (1.994)
$R_{\rm work} / R_{\rm free}$ (%)	24.4 / 28.9	25.0 / 27.8	23.2 / 25.9
No. atoms			
Protein	1,127	3,693	4,394
Ligand/ion	0	36	39
Water	0	19	8
<i>B</i> -factors (Å <sup>2</sup> )			
Protein	94.4	65.4	79.5
Ligand/ion	0	92.0	95.4
Water	0	44.3	58.9
R.m.s. deviations			
Bond lengths (Å)	0.003	0.003	0.003
Bond angles (°)	0.58	0.50	0.58
PDB accession code	7PUZ	7PV0	7PV1

## Table S1. Crystallographic data collection and refinement statistics

\* Data in highest resolution shell are indicated in parenthesis.

### Table S2. Overview of Mic60 and Mic19 constructs.

Summary of all constructs used in this study. Lt: Lachancea thermotolerans, ct: Chaetomium thermophilum, sc: Saccharomyces cerevisiae.

ltMic60		ctMic60				scMic60	Comments	
Boundaries Construct name		Boundaries	Mutations	Construct name	Boundaries	Mutations	Construct name	
207-382	ltMic60cc							Crystallized coiled-coil domain
		208-691		ctMic60 <sub>sol</sub>	1-540		scMic60	Wild type protein
		208-691	V455D/F461D	ctMic60 <sub>sol</sub> <sup>455D/F461D</sup>	1-540	1274D/F280D	scMic60 <sup>1274D/F280D</sup>	Broken tetramer interface
		208-691	L676D	ctMic60 <sub>sol</sub> L676D	1-540	V530D	scMic60 <sup>V530D</sup>	Broken mitofilin dimer interface
		208-691	V455D/F461D/L676D	ctMic60_v455D/F461D/L676D	1-540	I274D/F280D/V530D	scMic60 <sup>1274D/F280D/V530D</sup>	Broken tetramer and mitofilin dimer interface
		208-691	Q674A	ctMic60 sol Q674A				Disturbed Mic60-Mic19 interaction
		208-691	V678D	ctMic60 <sub>sol</sub> <sup>V679D</sup>	1-540	1532D	scMic60 <sup>I532D</sup>	Disturbed Mic60-Mic19 interaction
		208-691	L685D	ctMic60 <sub>sol</sub> L685D	1-540	T539D	scMic60 <sup>T539D</sup>	Disturbed Mic60-Mic19 interaction
		208-691	R574D/R575D	ctMic60_sol				Reduced membrane binding
		208-691	R581D/K582D	ctMic60_sol				Reduced membrane binding
		208-691	R631D	ctMic60 <sub>sol</sub> <sup>R631D</sup>				Reduced membrane binding
			ctMic19			scMic19		Comments
		Boundaries	Mutations	Construct name	Boundaries	Mutations	Construct name	
		1-164		ctMic19	1-170		scMic19	Wild type protein
		1-164	R126A	ctMic19 <sup>R126A</sup>				Disturbed Mic60-Mic19 interaction
		1-164	V129D	ctMic19 <sup>V129D</sup>	1-170	L143D	scMic19 <sup>L143D</sup>	Disturbed Mic60-Mic19 interaction
		1-164	L133D	ctMic19 <sup>L133D</sup>	1-170	L147D	scMic19 <sup>L147D</sup>	Disturbed Mic60-Mic19 interaction
		1-164	F150D	ctMic19 <sup>F150D</sup>				Disturbed Mic60-Mic19 interaction
Fusion	proteins	ctMic60	ctMic19	Mutation		Fusion proteir	1	Comments
Mito1_	снсн	624-691	116-164		(	ctMic60 <sup>624-691</sup> -GSGS-ctM	1ic19 <sup>116-164</sup>	Crystallized mitofilin and CHCH domain
Mito2_	СНСН	565-586- GS-622-691	116-164		ctMic60 <sup>565-586-GS-622-691</sup> -GSGS-ctMic19 <sup>116-164</sup>		-ctMic19 <sup>116-164</sup>	Crystallized mitofilin-dimer and CHCH domain
Mito2_C	HCHL676D	565-586- GS-622-691	116-164	L676D	ctMi	c60565-586-GS-622-691-GSGS	-ctMic19 <sup>116-164</sup>	Broken mitofilin domain dimer and CHCH domain

## Table S3. Plasmids used in this study.

Plasmid	Description	Reference	
pRS416	CEN, empty vector	Stratagene	
pRS416-Mic60	CEN, Mic60	This study	
pRS416-Mic60 <sup>I274D/F280D</sup>	CEN, Mic60 <sup>I274D/F280D</sup>	This study	
pRS416-Mic60 <sup>V530D</sup>	CEN, Mic60 <sup>V530D</sup>	This study	
pRS416-Mic60 <sup>I274D/F280D/V530D</sup>	CEN, Mic60 <sup>I274D/F280D/V530D</sup>	This study	
pRS416-Mic60 <sup>I532D</sup>	CEN, Mic60 <sup>I532D</sup>	This study	
pRS416-Mic60 <sup>T539D</sup>	CEN, Mic60 <sup>T539D</sup>	This study	
pRS416-Mic19	CEN, Mic19	This study	
pRS416-Mic19 <sup>L143D</sup>	CEN, Mic19 <sup>L143D</sup>	This study	
pRS416-Mic19 <sup>L147D</sup>	CEN, Mic19 <sup>L147D</sup>	This study	

CEN - Centromer

## Table S4. S. cerevisiae strains used in this study.

Strain	Genotype	Reference
YPH499	WTMATa ura3 lys2 ade2 trp1 his3 leu2	Ref. 54
Δ <i>mic60</i> -pRS416	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	MIC60::KanMX4 pRS416	
$\Delta mic 60$ -Mic 60	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	MIC60::KanMX4 pRS416-Mic60	
$\Delta mic60$ -Mic60 <sup>I274D/F280D</sup>	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	<i>MIC60::KanMX4</i> pRS416-Mic60 <sup>I274D/F280D</sup>	
$\Delta mic 60$ -Mic $60^{V530D}$	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	<i>MIC60::KanMX4</i> pRS416-Mic60 <sup>V530D</sup>	
$\Delta mic60\text{-Mic60}^{I274\text{D}/\text{F}280\text{D}/\text{V}530\text{D}}$	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	<i>MIC60::KanMX4</i> pRS416-Mic60 <sup>I274D/F280D/V530D</sup>	
$\Delta mic 60$ -Mic $60^{1532D}$	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	<i>MIC60::KanMX4</i> pRS416-Mic60 <sup>1532D</sup>	
$\Delta mic 60$ -Mic $60^{\text{T539D}}$	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	<i>MIC60::KanMX4</i> pRS416-Mic60 <sup>T539D</sup>	
$\Delta mic19$ -pRS416	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	MIC19::KanMX4 pRS416	
$\Delta mic19$ -Mic19	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	MIC19::KanMX4 pRS416-Mic19	
$\Delta mic19$ -Mic19 <sup>L143D</sup>	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	<i>MIC19::KanMX4</i> pRS416-Mic19 <sup>L143D</sup>	
$\Delta mic19$ -Mic19 <sup>L147D</sup>	MATa ura3 lys2 ade2 trp1 his3 leu2	This study
	MIC19::KanMX4 pRS416-Mic19 <sup>L147D</sup>	

	Table	<b>S5</b> .	Antibodies	used	in	this	study.
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Antibodies	Source	Identifier
anti-Atp2	Pfanner lab/ van der Laan lab	GR861
anti-Mic60	This study; amino acids 140-295	n/a
anti-Mic60	This study; amino acids 168-295	n/a
anti-Mic26	Pfanner lab/ van der Laan lab	GR3335
anti-Mic19	Pfanner lab/ van der Laan lab	GR3358

#### Movie S1 and S2. EM tomograms of crista junctions in fixed S. cerevisiae.

EM tomograms of mitochondria from  $mic60\Delta$  S. cerevisiae reconstituted with Mic60 (Movie S1) or the Mic60 tetramerization mutant scMic60<sup>I274D/F280D</sup> (Movie S2). See also fig. S1C-F. Arrows point to the CJs.

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