Supplemental Materials
Molecular Biology of the Cell
Fischer et al.

SUPPLEMENTAL INFORMATION

3 supplementary tables
5 supplementary figures

Protein import and oxidative folding in the mitochondrial intermembrane space of intact mammalian cells.

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Table S1 – *Identified potential human twin-CX_9C proteins*

Name	Alias	Isoforms	Accession number	Length (aa)	Motif 1 Motif 3	Motif 2 Motif 4
CHCHD1[a)]	C10orf34, **MRP10**		NP_976043.1	118	ERRREKGEATCITEMSVMMACWKQNEFRDDA	WKQNEFRDDACRKE**I**QG**F**LDCAARAQEARKM
CHCHD2 [a)]	**MIC17**		NP_057223.1	151	GTQPAQQQQPCLYE**I**KQ**F**LECAQNQGDIKLC	CAQNQGDIKLCEG**F**NE**VL**KQCRLANGLA*
Similar to CHCHD2 [a,b)]			XP_001718789	184	GTQPAQQQQPCFCE**I**KQ**F**LECAENQGDIKLC	CAENQGDIKLCEG**F**NE**VL**KQCRLANGLA*
CHCHD3 [a)]	MINOS3		NP_060282.1	227	KFKRYESHPVCADLQAKILQCYRENTHQTLK	YRENTHQTLKCSA**LA**TQ**Y**MHCVNHAKQSMLE
MIA40 [a)]	CHCHD4	CHCHD4.1 [a)]	NP_001091972.1	142	PCLGGMASGPCGEQ**F**KS**A**FSCFHYSTEEIKG	YSTEEIKGSDCVDQ**F**R**AM**QECMQK**Y**PD**L**YPQ
		CHCHD4.2 [a)]	NP_653237.1	155	PCLGGMASGPCGEQ**F**KS**A**FSCFHYSTEEIKG	YSTEEIKGSDCVDQ**F**RA**M**QECMQK**Y**PD**L**YPQ
CHCHD5 [a)]	MGC11, C2orf9, **MIC14**		NP_115685	110	QAALEVTARYCGRE**L**EQ**Y**GQCVAAKPESWQR HPIIRQIRQACAQ**PF**EA**F**EECLRQNEAAVGN	AAKPESWQRDCHYLKMSIAQCTSSHPIIRQI LRQNEAAVGNCAEH**M**RR**F**LQCAEQVQPPRSP
CHCHD6 [a)]	CHCM1		NP_115719.1	235	TIKPRRVEPVCSGLQAQILHCYRDRPHEVLL	YRDRPHEVLLCSD**LV**KA**Y**QRCVSAAHKG*
CHCHD7 [a)]	**COX23**	CHCHD7.B [a)]	NP_001011668	110	QRLRDPDINPCLSESDASTRCLDENNYDRER	LDENNYDRERCST**YF**LR**Y**KNCRR**FW**NS**I**VMQ
		CHCHD7.D [a)]	NP_077276	97	QRLRDPDINPCLSESDASTRCLDENNYDRER	LDENNYDRERCST**YF**LR**Y**KNCRR**FW**NS**I**VMQ
		CHCHD7.F [a)]	NP_001011671	85	QRLRDPDINPCLSESDASTRCLDENNYDRER	LDENNYDRERCST**YF**LR**Y**KNCRR**FW**NS**I**VMQ
CHCHD8 [a)]	E2IG2, **CMC3**		NP_057649.2	87	PLDQLISRSGCAASHFAVQECMAQHQDWRQC	CMAQHQDWRQCQPQ**V**QA**F**KDCMSEQQARRQE
CHCHD9	C9orf49		Q5T1J5	151	GTQPAQQQQPCFY**GI**KQ**F**LECAQNQGDIKLC	CAQNQGDIKLCED**F**SK**VL**KQCRLAKGLA*

Name	Alias	Isoforms	Accession number	Length (aa)	Motif 1 Motif 3	Motif 2 Motif 4
CHCHD10 [a)]	C22orf16		NP_998885.1	142	AAPQPLQMGPCAYEIRQFLDCSTTQSDLSLC	CSTTQSDLSLCEGFSEALKQCKYYHGLSSLP
NDUFA8 [a)]			NP_055037.1	172	KAAAHHYGAQCDKPNKEFMLCRWEEKDPRRC LDFFRQIKRHCAEPFTEYWTCIDYTGQQLFR	CRWEEKDPRRCLEEGKLVNKCALDFFRQIKR DYTGQQLFRHCRKQQAKFDECVLDKLGWVRP
NDUFB7 [a)]			NP_004137.2	137	AQLRLQLRDYCAHHLIRLLKCKRDSFPNFLA	KRDSFPNFLACKQERHDWDYCEHRDYVMRMK
NDUFS5 [a)]		Variant 1 Variant 2	NP_004543.1 [a)] NP_001171908.1	106 106	GEQPYKMAGRCHAFEKEWIECAHGIGYTRAE GEQPYKMAGRCHAFEKEWIECAHGIGYTRAE	GIGYTRAEKECKIEYDDFVECLLRQKTMRRA GIGYTRAEKECKIEYDDFVECLLRQKTMRRA
similar to NDUFS5 [a,b)]			XP_001720775.1	106	AEQPYKIAARCHAFEKEWIECAYGISVIRAE	GISVIRAEKECKIESDDFVECLLRQKTMRRA
NDUFS8			NP_002487	210	LRRYPSGEERCIACKLCEAICPAQAITIEAE	TTRYDIDMTKCIYCGFCQEACPVDAIVEGPN
COX6B1 [a)]			NP_001854.1	86	RFPNQNQTRNCWQNYLDFHRCQKAMTAKGGD	MTAKGGDISVCEWYQRVYQSLCPTSWVTDWDE
COX6B2 [a)]			NP_653214.2	88	RFPSQNQIRNCYQNFLDYHRCLKTRTRRGKS	RTRRGKSTQPCEYYFRVYHSLCPISWVESWNE
COX17 [a)]			NP_005685.1	63	EKKPLKPCCACPETKKARDACIIEKGEEHCG	ACIIEKGEEHCGHLIEAHKECMRALGFKI*
COX19 [a)]			NP_001026788.1	90	GSFPLDHLGECKSFKEKFMKCLHNNNFENAL	LHNNNFENALCRKESKEYLECRMERKLMLQE
COA5 [a)]	C2orf64, **PET191**		NP_001008216.1	74	YYEDKPQGGACAGLKEDLGACLLQSDCVVQE SPRQCLKEGYCNSLKYAFFECKRSVLDNRAR	DLGACLLQSDCVVQEGKSPRQCLKEGYCNSLK
TRIAP1 [a)]	**MDM35**		NP_057483	76	MNSVGEACTDMKREYDQCFNRWFAEKFL	FLKGDSSGDPCTDLFKRYQQCVQKAIKEKEI
EMI1 [a)]	C22orf39	Isoform 1 [a)] Isoform 2	NP_776154 NP_001159714	142 117	DGSGWQPPRPCEAYRAEWKLCRSARHFLHHY DGSGWQPPRPCEAYRAEWKLCRSARHFLHHY	HYYVHGERPACEQWQRDLASCRDWEERRNAE HYYVHGERPACEQWQRDLASCRDWEERRNAE

Name	Alias	Isoforms	Accession number	Length (aa)	Motif 1 Motif 3	Motif 2 Motif 4
UQCRH	QCR6		AAH93060	85	AAV**W**LCQ**L**AFCTDPLTTVREQCEQLEKCVKAR	VREQCEQLEKCVKARERLELCDERVSSRSHT
CMC1 [a)]	C3orf68		NP_872329.1	106	KIMREKAKERCSEQ**V**QD**F**TKCCKNSGVLMVV	KNSGVLMVVKCRKENSALKECLTAYYNDPAF
CMC2 [a)]	C16orf61		NP_064573.1	79	DLS**P**HL**H**TEECNVLINLLKECHKN**H**NI**L**KFF	NHN**I**LK**FF**GYCNDVDRELRKCLKNEYVENRT
p8 MTCP-1 [a)]	**CMC4**		NP_001018024.1	68	MPQKDPCQKQACEIQKCLQ**A**NSY**M**ESK	LQANSYMESKCQA**VI**QE**L**RKCCAQYPKGRSV
C1orf31 [a)]		Isoform 1 [a)]	NP_001013003.1	125	AAPSMKERQVCWG**A**RDE**Y**WKCLDENLEDASQ	LDENLEDASQCKK**L**RSS**F**ESSCPQQ**W**IK**Y**FDK
		Isoform 2	NP_001193570.1	79	AAPSMKERQVCWG**A**RDE**Y**WKCLDENLEDASQ	LDENLEDASQCKK**L**RSS**F**ESSCPQQ**W**IK**Y**FDK
C17orf89 [a)]			NP_001079990.1	74	LRA**F**PER**L**AACGAE**A**AA**Y**GRCVQASTAPGGR	APGGRLSKDFCARE**F**EA**L**RSCFAAAAKKTLE
C21orf129			Q96M42	142	LESSKCLTQGCCY**P**QA**WI**LACSAALLQALAS	TRKSGSTGNICSV**ML**N**VA**TGCVRIEK*
hypothetical protein XP_002342136 [a,b)]			XP_002342136.1	203	AEQPYKIPARHHP**F**EKE**W**RECAHGIGGIWGE	GIGGIWGEKECRIE**Y**D**YF**IECILWQKMMRCV
BAC85181			BAC85181	122	SQCQGAPQWICWD**V**DL**L**GRVCGQRDGALDRK DGALDRKQCICWD**V**DL**L**GRVCGHRDDALDRK	GALDRKKQCICWD**V**DL**L**GRVCGQRDGALDRK
lipoma HMGIC fusion partner-like 4 protein			NP_940962.1	247	VLLSMVLILGCITC**F**SL**F**FFCNTATVYKICA	FCNTATVYKICAW**M**QL**LA**ALCLVLGCMIFPD

a) Identified by Cavallaro et al
b) Listed with three different accession numbers by Cavallaro et al. and counted as three non-identical hits. However sequences are identical thus considered as one hit.

Bold: conserved as CX_9C from *Saccharomyces cerevisiae*

Bold and underlined: possible MISS/ITS-signal

Table S2 – *Plasmids and cell lines*

Cell line	Plasmid	Gene	Tag
HeLa	--	--	--
Mouse embryonic fibroblasts	--	--	--
Flp-In T-REx-293-Mock	pcDNA5/FRT/TO	--	--
Flp-In T-REx-293-Mia40	pcDNA5/FRT/TO	ORF *MIA40*	Strep
Flp-In T-REx-293-Mia40(SPS)	pcDNA5/FRT/TO	ORF *MIA40* (T_{157}; $T_{163} \rightarrow G$)	Strep
Flp-In T-REx-293-ALR	pcDNA5/FRT/TO	ORF *ALR*	HA
Flp-In T-REx-293-Smac	pcDNA5/FRT/TO	ORF *SMAC*	HA
Flp-In T-REx-293-NDUFA8	pcDNA5/FRT/TO	ORF *NDUFA8*	HA
Flp-In T-REx-293-Cmc1	pcDNA5/FRT/TO	ORF *CMC1*	HA
Flp-In T-REx-293-Cmc3	pcDNA5/FRT/TO	ORF *CMC3*	HA
Flp-In T-REx-293-Cox19	pcDNA5/FRT/TO	ORF *COX19*	HA
Flp-In T-REx293-Smac^{MTS}-Cox19	pcDNA5/FRT/TO	ORF *SMAC* (bp 1-177) ORF *COX19*	HA
Flp-In T-REx-293-Grx1-roGFP	pcDNA5/FRT/TO	ORF *GRX1*, ORF *ROGFP*	--
Flp-In T-REx-293-b2-Grx1-roGFP	pcDNA5/FRT/TO	ORF *CYTB2* (bp 1-258), ORF *GRX1*, ORF *ROGFP*	--
Flp-In T-REx-293-Su9-Grx1-roGFP	pcDNA5/FRT/TO	ORF *SU9* (bp 1-214), ORF *GRX1*, ORF *ROGFP*	--
--	pOG44	ORF *flp-recombinase*	--

Table S3 – *Primer*

Primer	Sequence (5' – 3')	Restric-tion site
Cox19 fwd. for pcDNA5/FRT/TO	CCCGGATCCGCCAACATGTCGACCGC	*BamHI*
Cox19 rev. for pcDNA5/FRT/TO	CCCGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTATTTT TTTGCCTCTGATTTTCCAC	*NotI*
Mia40 fwd. for pcDNA5/FRT/TO	CCAAGCTTGCCATGTCCTATTGCCGG	*HindIII*
Mia40 rev. for pcDNA5/FRT/TO	CCCTCGAGTTATTTCTCAAATTGTGGATGACTCCATCCTCCAGCAC TTGATCCCTCCTCTTCTTTG	*XhoI*
NDUFA8 fwd. for pcDNA5/FRT/TO	GGAAGCTTGCCATGCCGGGGATAGTGGAGC	*HindIII*
NDUFA8 rev. for pcDNA5/FRT/TO	GGGGTACCCTAAGCGTAATCTGGAACATCGTATGGGTATCCACTT CCCTTGGTCCAGAAATAAAAGCGGCTG	*KpnI*
Smac fwd. for pcDNA5/FRT/TO	CCAAGCTTACCATGGCGGCTCTGAAGAG	*HindIII*
Smac rev. for pcDNA5/FRT/TO	CCGGATCCTCATGCATAATCAGGTACATCATAAGGATAATCCTCA CGCAGGTAGG	*BamHI*
$Smac^{MTS}$ rev. for pcDNA5/FRT/TO	GGGGGATCCAATAGGAACCGCACACAGGGTTAC	*BamHI*
ALR fwd. for pcDNA5/FRT/TO	CCCAAGCTTGCCACCATGGCGGCGCCCG	*HindIII*
ALR rev. for pcDNA5/FRT/TO	CCCGGATCCCTAAGCGTAATCTGGAACATCGTATGGGTAGTCACA GGAGCCATCCTTCCAG	*BamHI*
Cmc3 fwd. for pcDNA5/FRT/TO	GGGGTACCGCCATGTCAACCTCAGTCCCTCAAGGC	*KpnI*
Cmc3 rev. for pcDNA5/FRT/TO	CCGGATCCCTAAGCGTAATCTGGAACATCGTATGGGTATCCACTT CCGTGGTGGGCACCGGCTTG	*BamHI*
Cmc1 fwd. for pcDNA5/FRT/TO	GGGGTACCGCCATGGCGCTCGACCCCG	*KpnI*
Cmc1 rev. for pcDNA5/FRT/TO	CCGGATCCCTAAGCGTAATCTGGAACATCGTATGGGTATCCACTT CCCATGCTTGTTGGAAGCTTCTGTAGC	*BamHI*
Mia40 fwd. $C_{157}\rightarrow A_{157}$	GGAAACATTAACTGGAACAGCCCATGCCTTGGGGG	--
Mia40 rev. $C_{157}\rightarrow A_{157}$	CCCCCAAGGCATGGGCTGTTCCAGTTAATGTTTCC	--
Mia40 fwd. $T_{163}\rightarrow A_{163}$	AACTGGAACTGCCCAAGCCTTGGGGGAATGGCC	--
Mia40 rev. $T_{163}\rightarrow A_{163}$	GGCCATTCCCCCAAGGCTTGGGCAGTTCCAGTT	--

Supplemental Material and Methods

siRNAs and antibodies – The following antibodies were used: anti-HA (Sigma), anti-HA (Roche), anti-ALR (Santa Cruz Biotechnology), anti-Smac (Sigma), anti-LDH (Santa Cruz Biotechnology), anti-PDH (Santa Cruz Biotechnology), anti-cytochrome *c* (BD Bioscience), anti-NDUFA8 (Abcam). Secondary antibodies were directed against mouse or rabbit (BioRad). The antibodies directed against Cox19 and Mia40 were raised in rabbits injected with the respective purified full-length protein. The following siRNAs were used Hs_GFER_1, Hs_GFER_6, Hs_CHCHD4_5, Hs_CHCHD4_6, control siRNA (Quiagen).

Preparation of Mouse Embryonic Fibroblasts (MEFs) – Pregnant C57Bl/6N females were sacrificed by cervical dislocation. The mouse embryos at embryonic day 14.5 were aseptically dissected from the mother and the embryo´s limbs and internal organs were removed. The carcasses were minced and incubated in a trypsin/EDTA solution to obtain MEFs. The reaction was stopped using DMEM medium supplemented with 10 % fetal bovine serum FBS, 1 % GlutaMAX (Invitrogen). After centrifugation at 200 g for 5 min, the MEFs were resuspended in medium and cultured at 37 °C and 5 % CO_2 until confluency. MEFs were splitted 1:10 and allowed to grow to confluency again. Experiments were performed using MEFs up to passage 5.

In silico screen – The SwissProt Homo sapiens genome was used to screen for potential candidates using a script written in the Python programming language (www.python.org) with Biopython extensions (Cock *et al.*, 2009). Protein sequences were recorded as a potential hit for twin-CX_9C proteins if they did not exceed a maximal length of 300 amino acids, contained at least two twin-CX_9C motifs that were at least five amino acids apart, and had a helix probability of larger than zero in at least one of the cysteine motifs. Hits that contained signal sequences for other compartments than mitochondria or transmembrane segments were discarded.

Immunofluorescence - Cells were cultured on poly-L-lysine covered cover slips for 24 hours. Fixation was performed with 4 % paraformaldehyde for 15 min. Then cell membranes were permeabilized with blocking buffer [20 mM HEPES pH 7.4, 3 % BSA, 0.3 % Triton X-100] for 1 hour. Cells were washed and incubated with primary (anti-HA, anti-Strep, anti cytochrome *c*) and secondary antibodies (anti-rat, ALEXA488; anti-mouse, ALEXA594) for 1 hour, respectively. Cells were stained for 15 min with 2 ng/ml DAPI in PBS. Then, cells were washed, cover slips transferred to microscope slides (cover medium: 30 % glycerol, 12 % polyvinyl alcohol, 60 mM TRIS, 2.5 % 1,4-diazabicyclo-2,2,2-octan) and analyzed by immunofluorescence microscopy (Microscope: Zeiss Axioskop2, Objective lense: Zeiss Plan-Neofluar 100x/ 1.30/ oil, Camera: F-Fiew Cool Snap, Software: CellF imaging software) . Pictures were processed for brightness and contrast using Photoshop CS.

Assay to assess protein oxidation in yeast cells (oxidation assay) – Yeast cells (strain YPH 499) were grown at 30 °C in S-Gal medium without methionine and cysteine. Synthesized proteins were pulse-labeled for 5 min at 30 °C in S-Gal medium without methionine and cysteine containing [^{35}S]-methionine at a concentration of 300 µCi / ml (Perkin Elmer). Pulse-labeling was stopped by adding cold methionine and cysteine in a final concentration of 20 mM. The chase was performed for variable times at 30 °C and was stopped by centrifugation for 15 sec at 16,000 g at 4 °C. The supernatant was removed, the pellet resuspended in ice cold 12 % TCA and the cells were lysed by sonification. TCA precipitation was performed by centrifugation at 13,000 g for 15 min and washing with 5 % ice cold TCA. Protein precipitates were dissolved in modification buffer [0.2 M Tris pH 7.5, 6 M Urea, 10 mM EDTA, 2 % SDS]. Samples were modified with a final AMS concentration of 15 mM for 1 hour at room temperature. Reduced controls were treated with 2 mM TCEP (final concentration) for 5 min at 96 °C prior to AMS modification, oxidized controls remained untreated. After modification samples were filled to 250 µl using lysis buffer A [30 mM TrisCl pH 8, 100 mM NaCl, 5 mM EDTA, 2 % SDS] and incubated for 5 min at 96 °C. Then, 750 µl of lysis buffer B [30 mM TrisCl pH 8, 100 mM NaCl, 5 mM EDTA, 2.5 % Triton X-100] was added and the mixture incubated at 4 °C for 1 hour. Samples were cleared by centrifugation at 25,000 g for 1 hour. The supernatant was subjected to immunoprecipitations with antibodies conjugated to protein A beads at 4 °C over night under gentle shaking. The samples were washed 4 times using lysis buffer C [30 mM TrisCl pH 8, 100 mM NaCl, 5 mM EDTA, 1 % Triton X-100] and once using lysis buffer D [30 mM TrisCl pH 8, 100 mM NaCl, 5 mM EDTA]. Proteins were eluted by adding Laemmli buffer [2 % SDS, 60 mM Tris pH 6.8, 10 % Glycerol, 0.0025 % Bromphenolblue] to the dried beads and subsequent boiling for 5 min at 95 °C. Samples were analyzed by Tris-Tricine PAGE and autoradiography.

Supplemental Figures

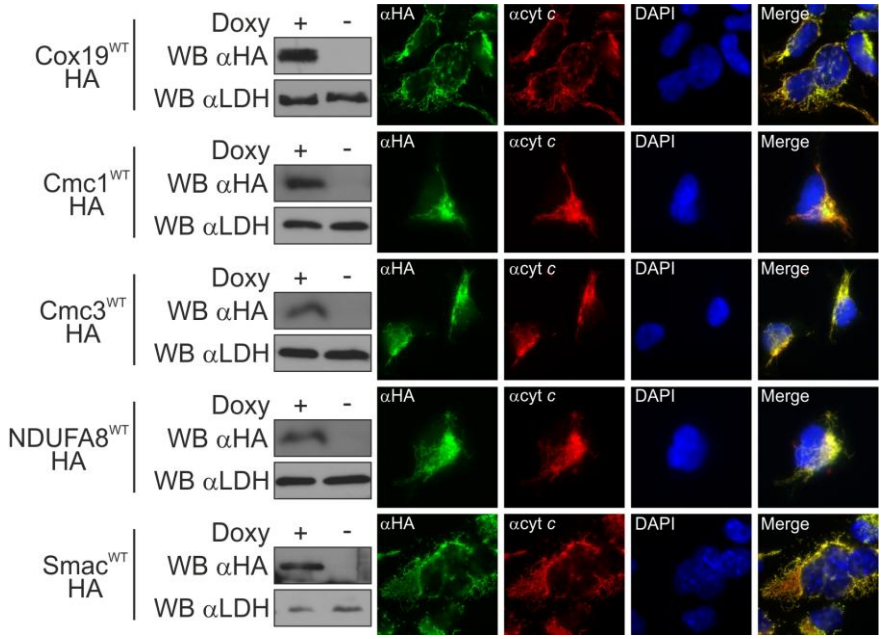

Fig. S1. Cox19-HA, Cmc1-HA, Cmc3-HA, NDUFA8-HA or Smac-HA localize to mitochondria. HEK293 cells stably expressing either Cox19-HA, Cmc1-HA, Cmc3-HA, NDUFA8-HA or Smac-HA were incubated for 24 hours with 1 µg/ml doxycyclin to induce expression. Cells were fixated, permeabilized and stained using primary antibodies against the HA epitope (αHA; green) and cytochrome *c* (αcyt *c*; red). Nuclei were stained with DAPI (blue). Cells were analyzed by fluorescence microscopy. In addition, some cells were lysed and analyzed by SDS-PAGE and immunoblotting against the HA epitope (αHA) and against lactate dehydrogenase (αLDH) that served as a loading control.

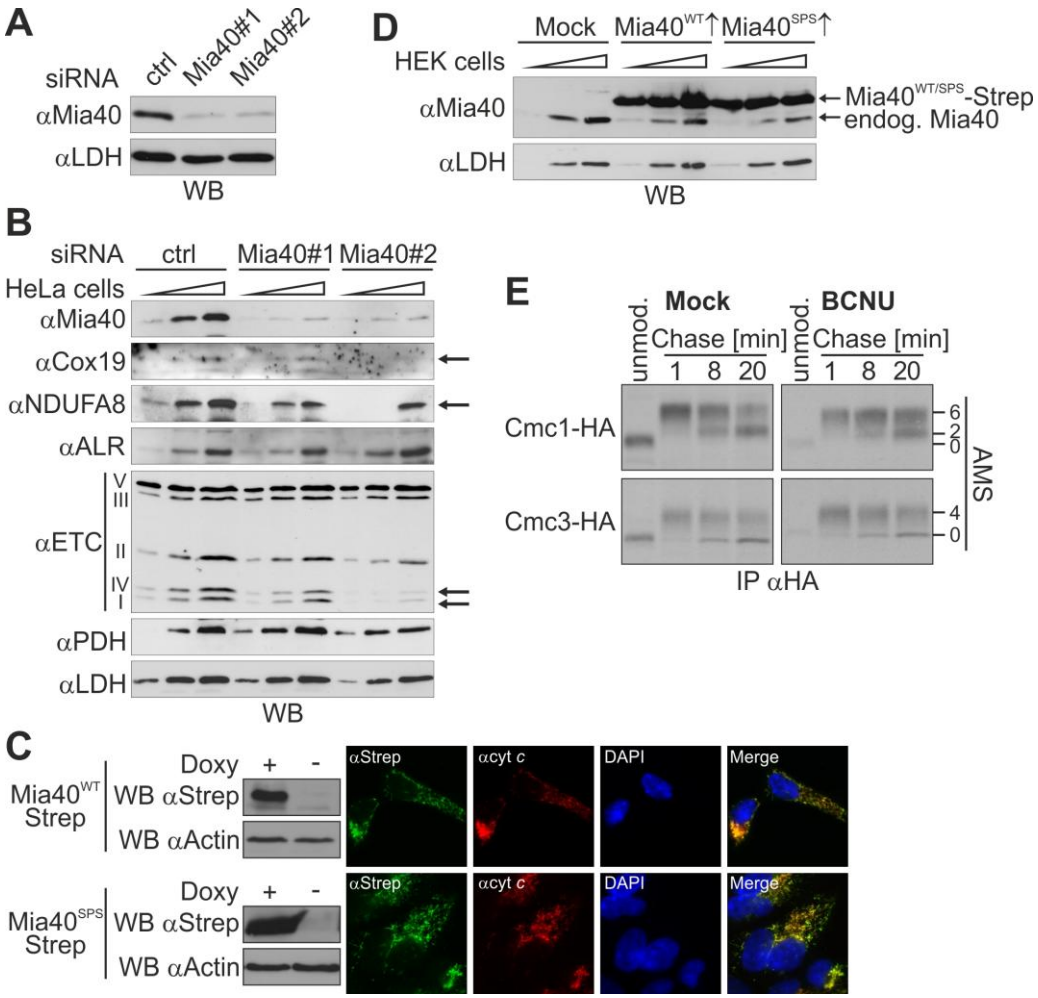

Fig. S2. Characterization of Mia40 depletion and overexpression. (A) Levels of Mia40 can be depleted to 10 % using siRNA. HeLa cells were transfected with control siRNA (Ctrl) or with two different siRNAs against Mia40 (#1, #2). 72 hours after transfection cells were lysed and analyzed by SDS-PAGE and immunoblotting using antibodies against Mia40 (αMia40) and lactate dehydrogenase (αLDH). **(B)** Depletion of Mia40 reduces the amounts of Mia40 substrates and complexes of the respiratory chain. As (A), except that immunoblotting was performed using antibodies against Mia40 (αMia40), Cox19 (αCox19), NDUFA8 (αNDUFA8), ALR (αALR), pyruvate dehydrogenase (αPDH), lactate dehydrogenase (αLDH) and against different proteins of the electron transport chain (αETC; complex *I*: NDUFB8; *II*: iron-sulfur subunit of complex II; *III*: ubiquinol-cytochrome *c* reductase core protein II; *IV*: cytochrome *c* oxidase subunit II; *V*: ATP5A). Proteins that are reduced in their amounts upon silencing of Mia40 are indicated by an arrow. **(C)** $Mia40^{WT}$-Strep and $Mia40^{SPS}$-Strep localize to mitochondria. HEK293 cells stably expressing $Mia40^{WT}$-Strep and $Mia40^{SPS}$-Strep were incubated 24 hours before the fixation for 4 hours with 1 µg/ml doxycyclin. Cells were fixated, permeabilized and stained using primary antibodies against the Strep epitope (αStrep; green) and cytochrome *c* (αcyt *c*; red). Nuclei were stained with DAPI (blue). Cells were analyzed by fluorescence microscopy. In addition, some cells were lysed and analyzed by SDS-PAGE and immunoblotting against the Strep epitope (αStrep) and against actin (αActin) that served as loading control. **(D)** In stable inducible cell lines Mia40 can be overexpressed up to 20 fold. HEK293 cells expressing either an empty plasmid (Mock), $Mia40^{WT}$-Strep or $Mia40^{SPS}$-Strep were incubated 24 hours before the lysis for 4 hours with 1 µg/ml doxycyclin. Samples were analyzed by SDS-PAGE and immunoblotting using antibodies against Mia40 (αMia40) and lactate dehydrogenase (αLDH). Both, endogenous and overexpressed Mia40 are indicated. **(E)** Inhibition of glutathione reductase decreases the efficient oxidation of the Mia40 substrates Cmc1 and Cmc3. Experiments were performed as described in Figure 3E using an antibody against the HA eptitope tag. Cmc1-HA and Cmc3-HA expression was induced in stable cell lines one hour before the experiment. The experiment was performed twice.

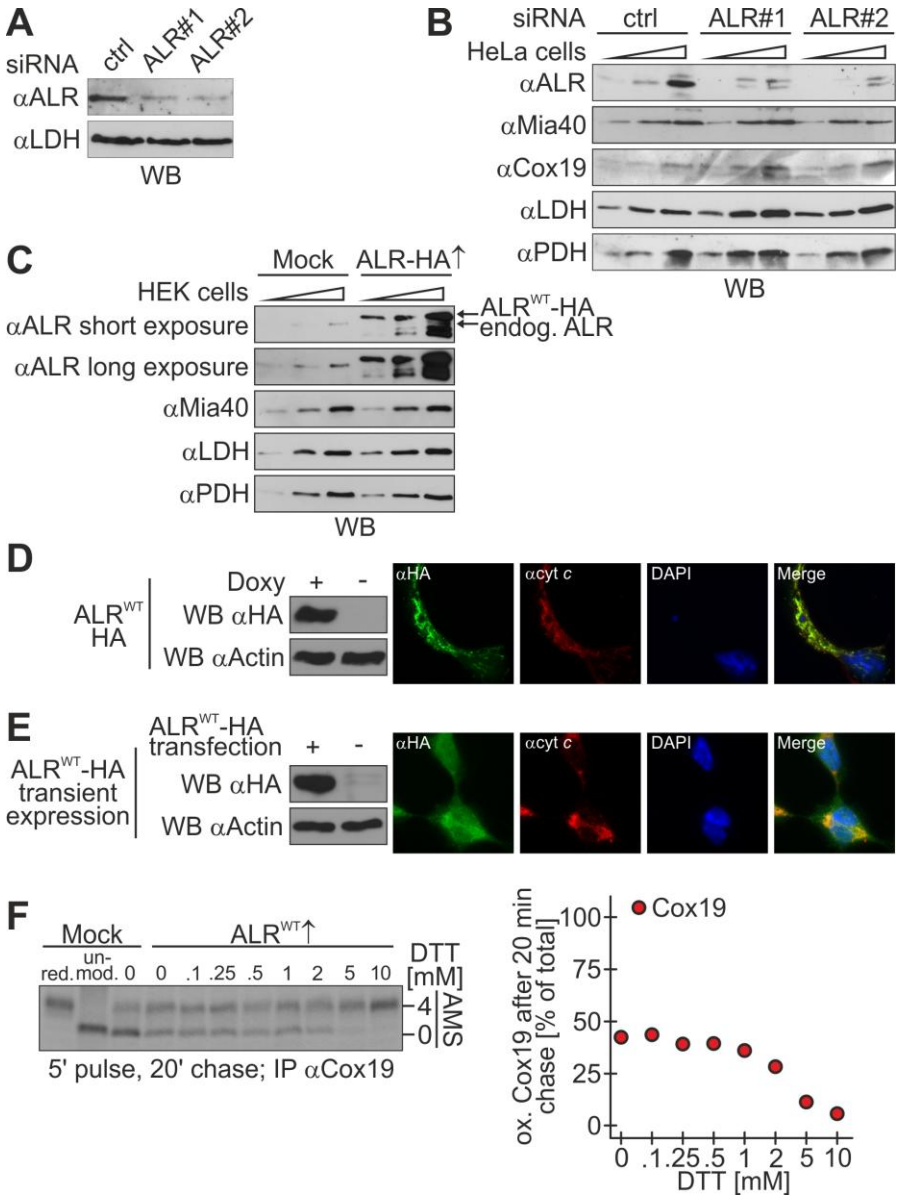

Fig. S3. Characterization of ALR depletion and overexpression. (A) Levels of ALR can be depleted to 10 % using siRNA. Hela cells were transfected with control siRNA (Ctrl) or with two different siRNA against ALR (#1, #2). 72 hours after transfection the cells were lysed and analyzed by SDS-PAGE and immunoblotting using antibodies against ALR (αALR) and lactate dehydrogenase (αLDH). **(B)** Depletion of ALR does not influence the amounts of Mia40 substrates. Experiment was performed as in (A), except that samples were analyzed by immunoblotting using antibodies against ALR (αALR), Cox19 (αCox19), pyruvate dehydrogenase (αPDH), lactate dehydrogenase (αLDH) and Mia40 (αMia40). **(C)** In stable inducible cell lines ALR can be overexpressed up to 10 fold. HEK293 cells stably and inducibly expressing ALR-HA or an empty plasmid were incubated 48 hours before lysis for 24 hours with 0.1 µg/ml doxycyclin or left untreated. The cells were lysed and analyzed by SDS-PAGE and immunoblotting using antibodies against ALR (αALR), Mia40 (αMia40), lactate dehydrogenase (αLDH) and pyruvate dehydrogenase (αPDH). Both, endogenous and overexpressed ALR are indicated. **(D)** ALR-HA localizes to mitochondria when expressed in stable inducible cell lines. HEK293 cells stably expressing ALR^{WT}-HA under the control of a Tet repressor were incubated 24 hours before the fixation for 4 hours with 1 µg/ml doxycyclin. Cells were fixated, permeabilized and stained using primary antibodies against the HA epitope (αHA; green) and cytochrome *c* (αcyt *c*; red*)*. Nuclei were stained with DAPI (blue). Cells were analyzed by fluorescence microscopy. In addition, some cells were lysed and analyzed by SDS-PAGE and immunoblotting against the HA epitope (αHA) and against actin (αActin). **(E)** Transiently overexpressed ALR-HA localizes to cytosol and mitochondria. HEK293 cells were transfected with 1 µg pcDNA3/ALR^{WT}-HA. 12 hours after transfection cells were fixated, permeabilized and stained using primary antibodies against the HA epitope (αHA; green) and cytochrome *c* (αcyt *c*; red*)*. Nuclei were stained with DAPI (blue). Cells were analyzed by fluorescence microscopy. In addition, some cells were lysed and analyzed by SDS-PAGE and immunoblotting against the HA epitope (αHA) and against actin (αActin). We conclude that overexpressed ALR only localizes to mitochondria if induction of expression with doxycyclin is stopped and the newly synthesized ALR is allowed to translocate

to mitochondria by incubation of cells with doxycyclin-free media overnight (compare to D). Conversely, continuous synthesis of ALR upon transient transfection results in cytosolic accumulation of ALR. **(F)** Titrations with DTT do not result in accelerated Cox19 oxidation in ALR-HA overexpressing cells. ALR overexpression delays oxidative folding of Cox19 (compare lanes 3 and 4). One explanation for this finding might be that high amounts of ALR lead to overoxidation of Mia40. This in turn might result in a decreased oxidation rate because a potential isomerase function of Mia40 could be impaired. For oxidative folding in the ER exposure to low amounts of reductants corrects defects of sulfhydryloxidase overexpression (Chakravarthi and Bulleid, 2004). We therefore applied increasing amounts of the reductant dithiothreitol (DTT) to cells overexpressing ALR and analyzed the oxidation state of Cox19 after a chase time of twenty minutes according to the experimental protocol applied to obtain Figure 4B. While we observed a clear impairment of oxidative folding at high concentrations of DTT, we did not observe an increase in oxidation rates at low DTT concentrations.

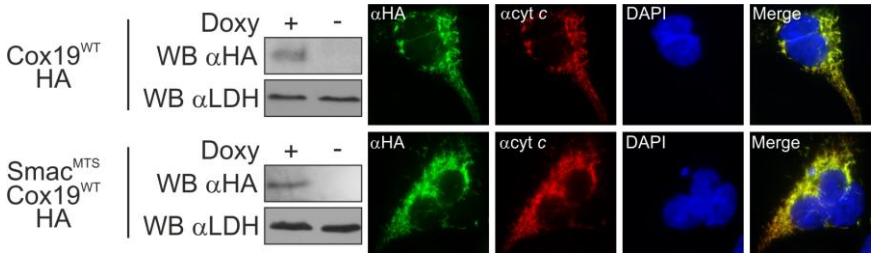

Fig. S4. Cox19-HA and Smac^{MTS}-Cox19-HA localize to mitochondria. HEK293 cells stably overexpressing Cox19^{WT}-HA, and Smac^{MTS}-Cox19^{WT}-HA were incubated for 24 hours with 1 µg/ml doxycyclin to induce expression. Cells were fixated, permeabilized and stained using primary antibodys against the HA epitope (αHA; green) and against cytochrome *c* (αcyt *c*; red*).* Nuclei were stained with DAPI (blue). Cells were analyzed by fluorescence microscopy. In addition, some cells were lysed and analyzed by SDS-PAGE and immunoblotting against the HA epitope (αHA) and against lactate dehydrogenase (αLDH).

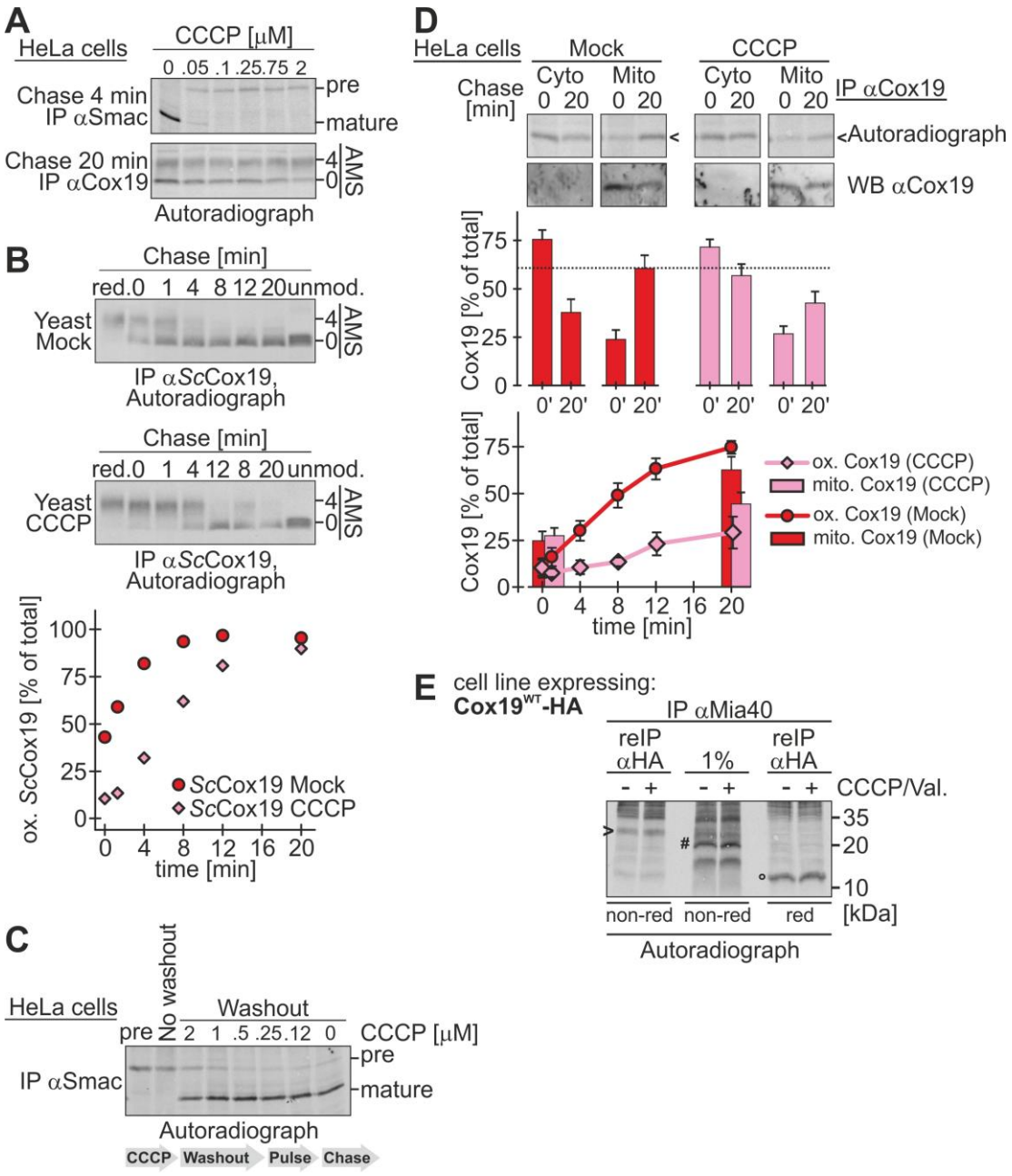

Fig. S5. Depletion of the membrane potential impairs import into mitochondria in a reversible manner. (A) Import of Smac and oxidation of Cox19 are sensitive to CCCP. HeLa cells were starved for 15 min, pulse labeled for 5 min with [^{35}S]-methionine and chased for either 4 min (IP αSmac) or 20 min (IP αCox19) with cold methionine. During all incubation steps the indicated amounts of CCCP were present. The reaction was stopped by adding ice cold 8 % TCA. TCA precipitation was performed and Cox19 samples were modified using AMS. Immunoprecipitation was performed against Smac (IP αSmac) or against Cox19 (IP αCox19) and the samples were analyzed by SDS-PAGE and autoradiography. Import of non-imported Smac (pre) was observed by cleavage of the presequence (mature). Oxidation of Cox19 was analyzed by comparing the amounts of reduced Cox19 (4 AMS bound) to oxidized Cox19 (0 AMS bound). **(B)** Oxidation of yeast Cox19 (*Sc*Cox19) is slowed down in *Saccharomyces cerevisiae* after depleting the membrane potential. YPH499 cells were pulse labeled for 5 min with [^{35}S]-methionine in SGal media. The labeling was stopped by adding cold methionine and the cells were incubated for the indicated times at 30 °C. 15 min before the experiment and during the whole pulse chase procedure the cells were treated with 20 μM CCCP or remained untreated (Mock). To stop the reaction cells were precipitated by brief centrifugation at 4 °C, and resuspended in ice cold 12 % TCA and lysed by sonification. TCA-precipitation, AMS-modification of free thiols, immunoprecipitation (IP) using an antibody directed against endogenous *Sc*Cox19 (α*Sc*Cox19) and SDS-PAGE were performed as for human cells. The reduced and the oxidized band of *Sc*Cox19 were quantified. The relative amounts of oxidized *Sc*Cox19 are shown in the graph for untreated cells (red circles) and cells treated with CCCP (pink rhomboid). **(C)** Mitochondria can import Smac after removing CCCP. HeLa cells were starved for 15 min in the presence of different concentrations of CCCP. After starvation the cells were washed with media without CCCP (CCCP washout) or washed with media containing 2 μM CCCP (no washout). Cells were pulsed for 5 min with [^{35}S]-methionine in the absence of CCCP (CCCP washout) or the presence of 2 μM CCCP (no washout). Then, cells were chased for 4 min in the absence of CCCP. Reactions were stopped by adding ice cold 8 % TCA. Immunoprecipitation was performed against Smac (IP αSmac) and the

samples were analyzed by SDS-PAGE and autoradiography. Import of non imported Smac (pre) was observed by cleavage of the presequence (mature). **(D)** Mitochondrial import of Cox19 is impaired by CCCP. HeLa cells were starved for 15 minutes, pulse-labeled for five minutes with [^{35}S]-methionine, and then chased with cold methionine for 0 or 20 minutes, respectively. All incubation steps were either performed in the absence (Mock) or presence of CCCP (CCCP). At the end of the chase the cells were transferred to ice and incubated with digitonin for 30 minutes to selectively permeabilize the plasma membrane. To separate the mitochondrial from the cytosolic fraction lysates were centrifuged. The supernatant and the pellet fraction were separated and ice cold TCA was added to a final concentration of 8 %. TCA-precipitation was performed and Cox19 was immunoprecipitated using an antibody against Cox19 (IP αCox19). Samples were analyzed by SDS-PAGE and autoradiography. Cox19 is labeled by an arrowhead. To assess steady state localization of Cox19, samples were also analyzed by immunoblotting against Cox19 (WB αCox19). Quantifications showing the relative amounts of Cox19 in the respective compartment are shown in the middle panel. The bottom panel shows the amount of oxidized Cox19 (ox. Cox19) in comparison to mitochondria-localized Cox19. Untreated cells (Mock) colored in red; cells treated with 2 μM CCCP colored in pink **(E)** Interaction of Mia40 and Cox19 is not influenced by CCCP. Cells stably expressing Cox19-HA or an empty plasmid (Mock) were induced for one hour and then radioactively pulse labeled for four hours. Then, thiol-disulfide exchange was inhibited by treatment with NEM, cells were lysed using an SDS-containing buffer, and Mia40 was immunoprecipitated (IP αMia40). 1 % of this immunoprecipitation was loaded as control. Then, a second IP with an antibody directed against the HA epitope was performed (reIP αHA) and analyzed by reducing and non-reducing SDS-PAGE and autoradiography. Arrowhead, disulfide-linked Mia40-Cox19 dimer; hash key, Mia40; circle, Cox19; red, reducing SDS-PAGE; non-red, non-reducing SDS-PAGE.

Supplemental References

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