Supplemental Data

ChChd3, an Inner Mitochondrial Membrane Protein, is Essential for Maintaining Cristae Integrity and Mitochondrial Function

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Supplemental Methods

Antibodies- The following monoclonal (mAb) and polyclonal antibodies (pAb) have been used throughout the study: The mouse mAb Mitofilin (Mitosciences), mouse mAb Sam50 (Santa Cruz), mouse mAb DLP1(BD Biosciences), rabbit pAb Opa1 (Affinity BioReagents), mouse mAb Flag (sigma), mouse mAb α-Tubulin (Sigma), mouse mAb HSP90 (BD Biosciences), mouse mAb HSP70 (BD Biosciences) mouse mAb Prohibitin (Abcam), mouse mAb AIF (Santa Cruz), rabbit pAb VDAC (Affinity Bio Reagents), rabbit pAb ANT (Santa cruz), rabbit pAb GRP75 (Santa Cruz), rabbit pAb LC3 (Cell signaling), rabbit mAb S6K (Cell signaling), rabbit pAb SMAC / Diablo (Calbiochem), mouse mAb NDUFS3 (Mito sciences), rabbit pAb Mitoneet (1) rabbit pAb phospho S6K (Cell signaling), mouse mAB PARP (Santa Cruz), Total Ox-Phos Human WB antibody cocktail-MS601 (Mitosciences), rabbit pAb Cox-IV (abcam), mouse mAb Mfn1 (abcam), ECL-HRP labeled sheep anti-mouse and donkey anti-rabbit IgG (Amersham).

Mass Spectrometry Analysis- Gel bands from the immunoprecipitated samples of ChChd3 were excised, reduced with DTT (5 mM) and alkylated with iodoacetamide 10 mM). Trypsin was added (250 ng) and incubated overnight at 37°C. Gel digests were acidified with 0.5% formic acid and purified using Zip Tip (Millipore, Billencia, MA). Samples were mixed 1:1 with cyano-4-hydroxycinnamic acid matrix in methanol:acetonitrile:water (36%:56%:8%) (Agilent, Santa Clara, CA) and spotted on the MALDI (Matrix-assisted laser desorption/ionization) target. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry of peptides was performed on a 4800 MALDI-TOF-TOF (Applied Biosystems/MDS-Sciex, Foster City, CA). Reflector-positive spectra were obtained by acquiring 500 shots from mass to charge ratio (m/z) 500–4000 amu (atomic mass units). MS/MS spectra were acquired in 2 kV-positive modes with collision-induced dissociation (CID) on at resolution 200 full width at half maximum; for 3000 shots. The MS/MS data was analyzed on Global Proteome server 3.0 (Applied Biosystems). Samples with no or minimum data from TOF/TOF experiments were also analyzed by LC-MS/MS (2) and data searched with Mascot.

Isolation and Fractionation of Mitochondria- Mitochondria from mouse liver were isolated and gradient purified as described previously (3). In brief, fresh mouse liver was minced, washed and homogenized in MSHE plus BSA [210 mM mannitol 70 mM sucrose, 5 mM HEPES, pH 7.4, with KOH, 2mM EGTA, 0.5% fatty acid free BSA and protease inhibitor cocktail (Roche)]. The homogenate was centrifuged for 10 minutes at 600g to remove unbroken cells and nuclei and the supernatant was spun at 15,000g for 10 minutes to pellet mitochondria. The mitochondrial pellet was washed twice in MSHE with BSA followed by one wash in BSA- free MSHE and layered over a gradient of 35% Histodenz (Sigma), 17.5% Histodenz, and 6% Percoll (Sigma) and was centrifuged at 45,000g for 45 minutes at 4 ºC. Mitochondria were collected from the 17.5-35% interface, centrifuged at 15,000g for 15 min, and resuspended in a small volume of MSHE buffer.

Mitochondrial subfractions were obtained by a swell-shrink procedure and purified by using a sucrose gradient ultracentrifugation method (3). Gradient-purified mitochondria were resuspended in hypotonic buffer (10 mM KCl, 2 mM HEPES, pH 7.2) at 5 mg/mL for 10 minutes on ice to swell mitochondria. One-third volume of hypertonic buffer (1.8 mM sucrose, 2 mM ATP, 2 MM MgSO4, 2 mM HEPES, pH 7.2) was then added and allowed to incubate for an additional 5 min. Mitochondria were then sonicated for 15 seconds and layered on a sucrose gradient containing 0.76, 1 and 1.32 M sucrose and spun at 75,000g for 3 hours at 4 ºC. The IMS-soluble fraction was collected from the uppermost supernatant. The OM fraction was collected from the 0.76 and 1 M sucrose interface, washed with MSHE, and pelleted by centrifugation at 120,000 g. Mitoplasts (MP) were collected from the pellet, washed with MSHE, and centrifuged at 15,000g. MP were sonicated 6-7 times for 15sec on ice with brief intervals. The solution was

spun at 15,000 g to remove intact MP, and the resulting supernatant was spun at 140,000g for 45 min at 4ºC to pellet the IM / SMP fraction. The soluble matrix fraction was collected from the supernatant.

Fluorescence Microscopy- For fluorescence microscopy experiments, HeLa cells grown to 50-60% confluency on glass cover slips were transfected using Fugene 6 (Roche) transfection reagent following manufacturers protocol. Approximately 20 hours after post transfection, cells were fixed with 4% paraformaldehyde in DPBS for 30 minutes at room temperature. Cells were washed for five times with DPBS and coverslips were mounted on glass slides using ProLong Gold antifade reagent (Invitrogen). Images were captured on Radiance 2000 laser scanning confocal microscope (BioRad).

RT-PCR- Total RNA was extracted simultaneously from control and ChChd3 knockdown HeLa cells maintained under similar conditions, using RNA aqueous for PCR kit (Ambion) by following the manufacturer's protocol. RNA was treated with DNAse I to remove DNA contamination and subjected to the reverse transcription, using Superscript III first strand synthesis system for RTPCR (Invitrogen), with 2 μg of total RNA, 5 μM oligo $(dT)_{20}$ and 200 units of Superscript III reverse transcriptase, at 50 $^{\circ}$C for 50 minutes. 2 μl of the cDNA from reverse transcription reaction was used in the PCR reaction using Platinum *Taq* DNA polymerase High Fidelity (Invitrogen). The following gene specific primers were used for the PCR amplification: ChChd3: gaaaagaatccaggcccttc (forward), cttcgaaccagatggagagg (reverse); Sam50: ggtgataagccgtcaagcat (forward), aatggggtgtagaggtgcag (reverse); Mitofilin: ggaacgcagaaaggcagtag (forward), tgagctgcttggacctttt; Actin: gctcgtcgtcgacaacggctc (forward), caaacatgatctgggtcatcttctc (reverse).

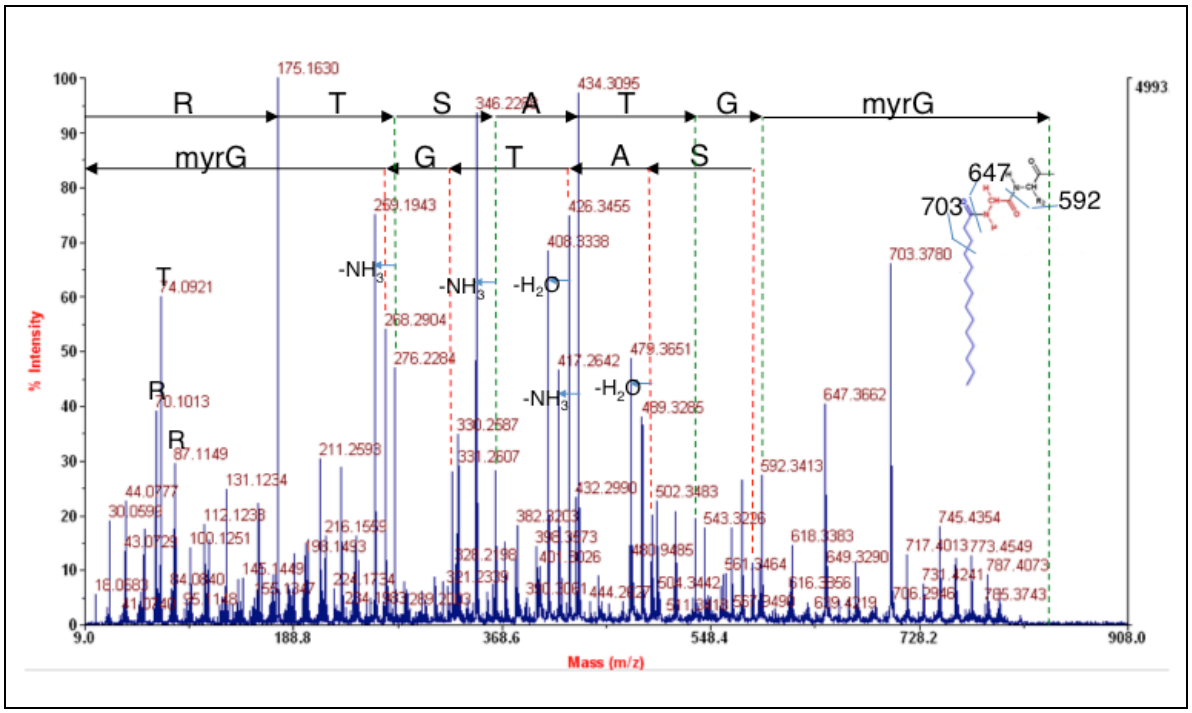

Fig. S1. MS/MS analysis identifies N-terminus of ChChd3 is myristylated.
Flag-tagged ChChd3 was transiently expressed in HEK 293 cells and immunoprecipitated with flag resin. Immunoprecipitated ChChd3 was extracted from SDS-PAGE and analyzed by mass spectrometry as described in experimental section.

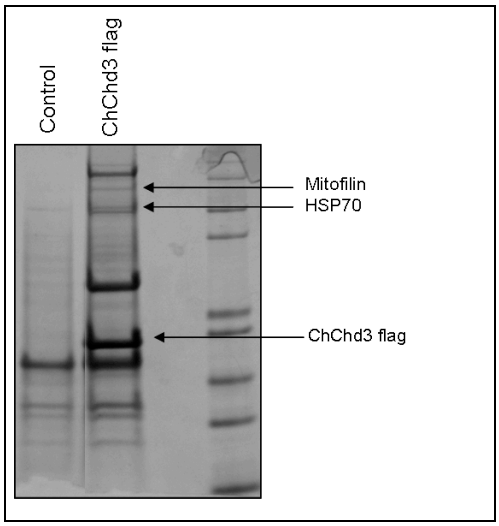

Fig. S2. MSMS analysis of immunoprecipitated samples of ChChd3 flag in HEK293 cells identifies Mitofilin and HSP70.
Flag-tagged ChChd3 and empty vector very transiently expressed in HEK293 cells and immunoprecipitated with flag resin. Samples were separated on SDS-PAGE and stained with Coomasie blue.

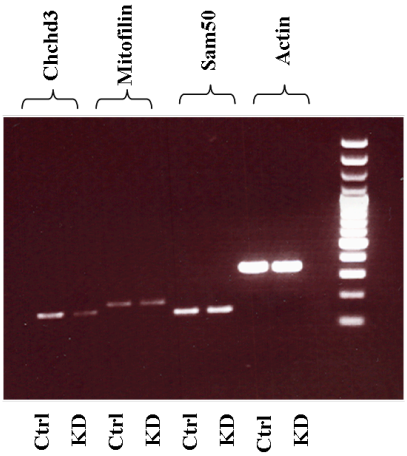

Fig. S3. RT-PCR analysis of control and ChChd3 knockdown cells showing no changes in Mitofilin and Sam50 mRNA levels.
Total RNA was isolated from the control and ChChd3 knockdown cells simultaneiously and reverse transcribed and used in PCR as described in the experimental section. Actin was used as a loading control.

Mitofilin:	IMMT_HUMAN Mass: 83626 Score: 582 Queries matched: 23									
	Mitochondrial inner membrane protein OS=Homo sapiens GN=IMMT PE=1 SV=1									
	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
	13	526.8226	1051.6307	1051.6026	0.0281	0	11	9.4	2	K.QHITLALEK.Q
	15	530.3108	1058.6070	1058.5720	0.0350	1	60	0.00016	1	K.KVQAAQSEAK.V
	20	558.3130	1114.6115	1114.5870	0.0245	0	34	0.051	1	K.AVDEAADALLK.A
	25	575.3152	1148.6158	1148.5826	0.0333	0	40	0.014	1	K.LSEQELQFR.R
	27	577.3102	1152.6059	1152.5887	0.0172	0	48	0.0018	1	R.ERPPEEVAAR.L
	34	621.8743	1241.7340	1242.6819	-0.9479	1	(6)	30	8	R.KAVDEAADALLK.A
	35	622.3577	1242.7008	1242.6819	0.0189	1	67	2.7e-05	1	R.KAVDEAADALLK.A
	36	623.3071	1244.5996	1244.5885	0.0111	0	71	1.1e-05	1	R.YSTSGSSGLTTGK.I
	48	493.6116	1477.8128	1477.7889	0.0240	0	44	0.0037	1	K.EVAGAKPHITAAEGK.L
	49	741.4309	1480.8473	1480.8249	0.0224	1	(27)	0.21	1	K.ATEKQHITLALEK.Q
	50	494.6352	1480.8838	1480.8249	0.0589	1	53	0.00042	1	K.ATEKQHITLALEK.Q
	51	755.4171	1508.8197	1508.8021	0.0176	0	26	0.28	1	K.LHNMIVDLDNVVK.K
	53	508.3168	1521.9285	1521.8667	0.0618	1	45	0.0028	1	R.KAHQLWLSVEALK.Y
	55	764.4248	1526.8351	1526.8205	0.0146	0	81	8.3e-07	1	K.VVSQYHELVVQAR.D
	56	509.9675	1526.8808	1526.8205	0.0603	0	(50)	0.001	1	K.VVSQYHELVVQAR.D
	60	792.4453	1582.8761	1582.8243	0.0518	0	62	6.2e-05	1	R.ELDSITPEVLPGWK.G
	73	912.4769	1822.9393	1822.8809	0.0584	0	76	1.8e-06	1	R.GIEQAVQSHAVAEEEAR.K
	74	608.6556	1822.9449	1822.8809	0.0640	0	(68)	1.3e-05	1	R.GIEQAVQSHAVAEEEAR.K
	85	689.0226	2064.0460	2064.0011	0.0449	1	25	0.18	1	R.VQEQELKSEFEQNLSEK.L
	87	698.3903	2092.1490	2092.0661	0.0829	1	60	6.5e-05	1	R.LRGIEQAVQSHAVAEEEAR.K
	107	785.4282	2353.2628	2353.1662	0.0966	1	96	1.3e-08	1	R.RLSQEQVDNFTLDINTAYAR.L
	113	889.4839	2665.4299	2665.3130	0.1170	1	41	0.0031	1	K.GMSVSDLADKLSTDDLNSLIAHAHR.R
	116	944.8725	2831.5958	2831.5253	0.0704	0	34	0.013	1	R.QTASVTLQAIAAQNAAVQAVNAHSNILK.A
HSP70:	gi|462325|sp|P08107|HSP71_HUMAN Mass: 70294 Score: 305 Queries matched: 9									
	Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2)									
	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
	1	1110.5488	1109.5415	1109.5505	-0.0090	0	18	49	1	K.LLQDFFNGR.D + Deamidation (NQ)
	2	1197.6741	1196.6668	1196.6876	-0.0208	0	44	0.12	1	K.DAGVIAGLNVLR.I
	3	1417.7356	1416.7283	1416.7513	-0.0230	1	27	8.4	1	R.LVNHFVEEFKR.K
	4	1465.7803	1464.7730	1464.8048	-0.0318	0	26	8.7	1	K.AQIHDLVLVGGSTR.I
	5	1487.6791	1486.6718	1486.6940	-0.0221	0	60	0.0046	1	R.TTPSYVAFTDTER.L
	6	1580.7894	1579.7821	1579.7994	-0.0173	1	11	3.7e+002	5	K.LLQDFFNGRDLNK.S + Deamidation (NQ)
	7	1687.8744	1686.8671	1686.8940	-0.0269	0	29	5.4	1	R.IINEPTAAAIAYGLDR.T
	8	1814.9218	1813.9145	1813.9434	-0.0289	1	39	0.54	1	K.NQVALNPQNTVFDAKR.L
	10	2786.3203	2785.3130	2785.3558	-0.0428	0	56	0.016	1	K.QTQIFTTYSDNQPGVLIQVYEGER.A

Table S1: LC-MS/MS data for mitofilin and HSP70 identified in immunofprecipitated samples of ChChd3 flag.

Supplemental References

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2. Navarro, L., Koller, A., Nordfelth, R., Wolf-Watz, H., Taylor, S., and Dixon, J. E. (2007) *Mol Cell* **26**, 465-477
3. Pagliarini, D. J., Wiley, S. E., Kimple, M. E., Dixon, J. R., Kelly, P., Worby, C. A., Casey, P. J., and Dixon, J. E. (2005) *Mol Cell* **19**, 197-207