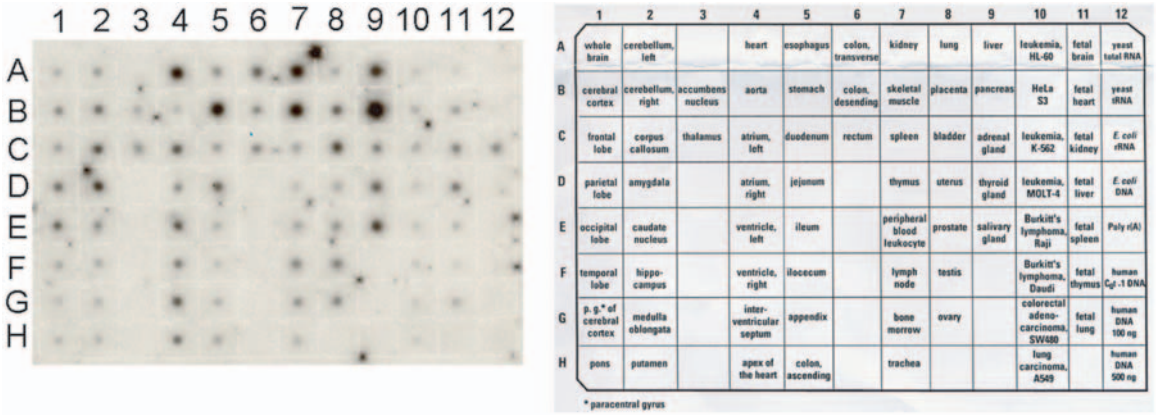
a.



b.

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                    Mitochondrial Localization Sequence / TM domain  ∇                  67
Human                     MGRLSWQVAA AAAVGLALTL EALPWVLRW- LRSRRRRPRR --EAPFFPSQ VTCTEALLRA PGAELAELPE
Mouse                     MGRSSWRLVF AAGAGLALAL EALPWLMRW- LLAGRR-PRR --EVLFFPSQ VTCTEALLQA PG--LPPGPS
Drosophila          MLITQIIMKQ IRDYPIVSTI SIAVSTVLAS EVIWKLVQC- SRSKREKASR VHEVIIFNEL GEICAAVHMR NSSMGSQKPQ
E. coli CLS         MVDPRYFKQD AGVGQWIDLM ARMEGPIATA MGIIYSCDWE IETGKRILPP PPDVNIMPFE QASGHTIHTI ASG------P
Salmonella Nuc                                                          MKKL ATWLLAAAFT TAALPAFAVE

                                                    RGV motif                          143
Human               GCPCGLPH-G ESALSRLLRA LLAARASLDL CLFAFSSPQL GHAVQLLHQR GVRVRVV--- TDCDYMALNG SQIGLLRKAG
Mouse               GCPCSLPH-S ESSLSRLLRA LLAARSSLEL CLFAFSSPQL GRAVQLLHQR GVRVRVI--- TDCDYMALNG SQIGLLRKAG
Drosophila          VSPCCNTHCS LRNVAKIVEQ IDRAVYSIDL AIYTFTSLFL ADSIKRALQR GVIIRII--- SDGEMVYSKG SQISMLAQLG
E. coli CLS         GFPEDLIH-- ---QALLTAA YSAREYLIMT TPYFVPSDDL LHAICTAAQR GVDVSIILPR KNDSMLVGWA SRAFFTELLA
Salmonella Nuc      PSVQVGYSPE GSARVLVLSA IDSAKTSIRM MAYSFTAPDI MKALVAAKKR GVDVKIVIDE RGNTGRASIA A-MNYIANSG

                  HKD motif: H K      D                                GS  N                 201
Human               IQVR-HDQDP GYMHHKFAIV DK-------- ---------- --RVLITGSL NWTTQAIQNN RENVLITED- DEYVRLFLEE
Mouse               IQVR-HDQDL GYMHHKFAIV DK-------- ---------- --KVLITGSL NWTTQAIQNN RENVLIMED- TEYVRLFLEE
Drosophila          VPVR-VPITT NLMHNKFCII DGFERVEEIR LLRKLKFMRP CYSIVISGSV NWTALGLGGN WENCIITAD- DKLTATFQAE
E. coli CLS         AGVKIYQFEG GLLHTKSVLV DG-------- ---------- --ELSLVGTV NLDMRSLWLN FEITLAIDD- KGFGADLAAV
Salmonella Nuc      IPLR-TDSNF PIQHDKVIIV DN-------- ---------- --VTVETGSF NFTKAAETKN SENAVVIWNM PKLAESFLEH

                                                                                       252
Human               FERIWEQFNP TKYTFFPPKK SHGSCAPPVS RAGGRLLSWH RTCGTSSESQ T
Mouse               FERIWEEFDP TKYSFFPQKH RGH
Drosophila          FQRMWRAFAK TEGSQIQLK
E. coli CLS         QDDYISRSRL LDARLWLKRP LWQRVAERLF YFFSPLL
Salmonella Nuc      WQDRWNQGRD YRSSY
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Figure S1 MitoPLD expression pattern and sequence comparisons. **(a)** A human multiple tissue expression array (Clontech) was hybridized with a [^{32}P]-labeled human MitoPLD probe spanning the open reading frame. Shown on the right are the sources of mRNAs used in the array. **(b)** Multiple sequence alignment of human (AAH31263), mouse (CAI24298), and *Drosophila melanogaster* (NP_609530) MitoPLDs, *Salmonella typhimurium* Nuc (AAL13395) and *E. coli* cardiolipin synthase (NP_753618). Only the C-terminal half of *E. coli* CLS (aa 242-481) was aligned. The box and open triangle respectively indicate the mitochondrial localization sequence (MLS) and cleavage site predicted for human MitoPLD protein by the MITOPROT program (http://ihg.gsf.de/ihg/mitoprot.html), which estimated a 78% probability that MitoPLD would localize to mitochondria. MLSs are generally amphipathic and positively charged; the MitoPLD MLS is amphipathic and contains 9 positively-charged residues and 1 negative charged residue. However, these guidelines refer primarily to imported proteins, which we show later does not appear to take place for MitoPLD, so the computer prediction is of less significance in this instance. Amino acid residues 5-27 (numbering according to the human sequence) were alternately predicted to function as a transmembrane helix by the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/). Other regions of interest are underlined. Red indicates highly conserved amino acid residues. Blue represents less highly conserved residues. TM, transmembrane; CLS, cardiolipin synthase. The RGV motif has not previously been described and its function is currently unknown; it could play a role in protein:protein or protein:lipid interactions since it lies at the back of the enzyme with respect to the active site (see Fig. 1h). The human MitoPLD gene localizes to human chromosome 17p11.2.

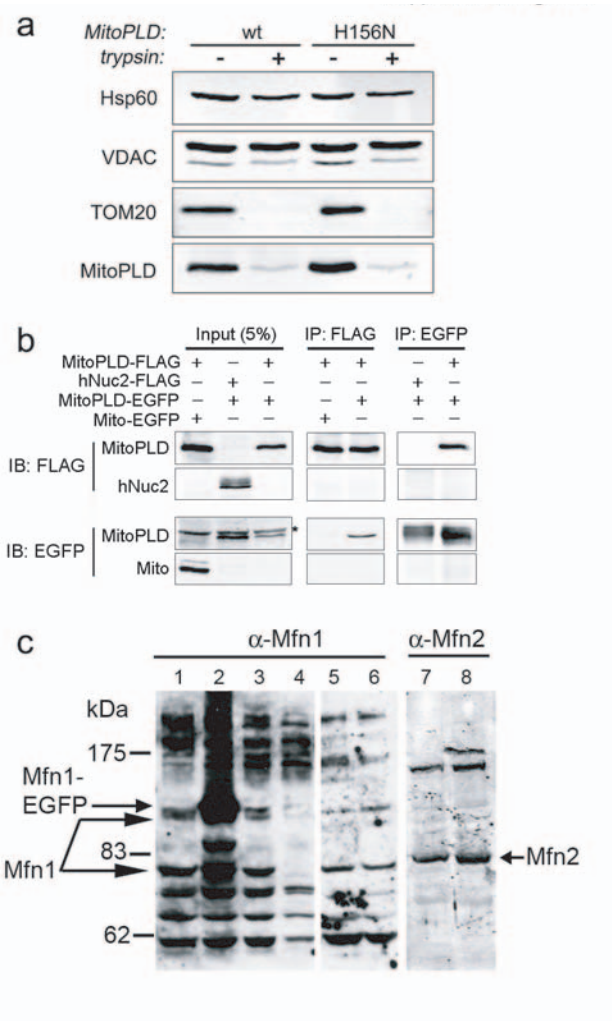

Figure S2 MitoPLD topology as determined by surface digestion using Trypsin, and lack of protein:protein interaction of MitoPLD with Mfn or effects of MitoPLD deficiency on Mfn expression. **(a)** Topology. Mitochondria purified from cells expressing FLAG-tagged wt or H156N MitoPLD were exposed to trypsin. Hsp60 (a mitochondrial matrix protein), VDAC (an integral outer membrane protein), and TOM20 (which projects from the outer membrane) were visualized using commercial antisera. **(b)** Co-IP. NIH3T3 cells were transiently co-transfected with plasmids for FLAG- and EGFP-tagged MitoPLD or control plasmids as indicated (hNuc2 is another presently uncharacterized HKD-containing gene that exhibits sequence similarity to bacterial Nuc but does not localize to mitochondria, unpublished data; Mito-EGFP is EGFP fused to the MLS of subunit VIII of human cytochrome C oxidase). Twenty-four hours post-transfection, the cells were lysed using 0.5% NP-40 in PBS, sonicated, and centrifuged at 13K x g for 5 min at 4ºC. The resulting supernatants were incubated with anti-EGFP (Abcam) or anti-FLAG (Sigma) antibodies in the presence of protein G-agarose (Sigma). The agarose was washed in lysis buffer, and the bound proteins were eluted using SDS loading buffer, subjected to 12% SDS-PAGE, transferred to nitrocellulose membranes and detected with an Odyssey infrared imaging system using the antibodies indicated. IP indicates antibody used for immunoprecipitation; IB indicates antibody used for western blot immunodetection. Asterisk indicates non-specific band detected by anti-EGFP antibody. Examination of the Input lanes indicates that all of the proteins were expressed at approximately equivalent levels. When FLAG-tagged MitoPLD was co-expressed with EGFP-tagged MitoPLD and immunoprecipated using either anti-FLAG or anti-EGFP antibody, the partner protein (EGFP-tagged MitoPLD or FLAG-tagged MitoPLD, respectively) was readily detected by western blot analysis. However, the partner proteins were not co-immunoprecipitated by Mito-EGFP or by hNuc2-FLAG, demonstrating specificity of the immunoprecipitation and detection reactions.**(c)** Mfn1 and 2 levels of expression are not altered in MitoPLD RNAi-targeted cells. Western blot analysis of parental cells, cells expressing tagged Mfn proteins, and control or MitoPLD RNAi-targeted cells were subjected to Western blot analysis using polyclonal antisera directed against Mfn1 and Mfn 2 with previously described protocols (4, 10). Left, Mfn1 analysis: In Lane 1, the parental NIH3T3 cells, many immunoreacting bands are observed. In Lane 2, a larger band is observed, indicating transiently-expressed EGFP-Mfn1 and demonstrating that the antibody does recognize Mfn1. Breakdown products, including one at approximately 80 kDa, which is close to the predicted size of Mfn1 (84 kDa), are also observed. In Lane 3, the antibody fails to recognize transiently EGFP-tagged Mfn2, demonstrating that it is isoform-specific. In lane 4, examination of mouse embryonic fibroblasts lacking Mfn1 indicates the disappearance of multiple immunoreactive bands – a major one migrating at approximately 80 kDa, a doublet almost as intense at approximately 110 kDa, and possibly several higher molecular weight bands (probably indicating incomplete denaturation and /or reassociation with other hydrophobic proteins during electrophoresis). Regardless, no difference in the level of Mfn1 is observed for the major band at 80 kDa between HeLa cells targeted using RNAi directed against Luciferase (Lane 5) versus ones targeted against MitoPLD (Lane 6). Similarly, no difference in the level of Mfn2 is observed between for the Luc (lane 7) or MitoPLD (Lane 8) RNAi targeted cell lines. Note – lanes 1-6 all derive from the same experiment and autorad, however, several non-relevant intervening lanes were excised from the figure between lanes 4 and 5. Lanes 7-8 derive from a separate western blot experiment.

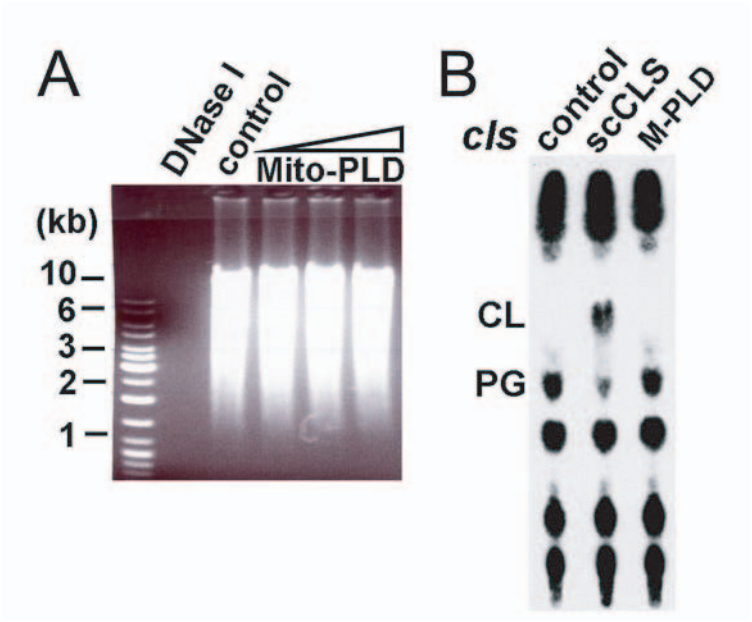

Figure S3 Neither nuclease nor CL synthetic activity can be detected for MitoPLD**. (a)** HeLa cell nuclear extract (200 μg) was incubated with either PBS (control), DNase I (10 unit) or MitoPLD overexpressing mitochondrial lysates (3 μg, 6 μg, 12 μg) for 2 hr at 37^°C. Nuclear DNA was precipitated by ethanol, resuspended, and electrophoresed on a 1% agarose gel. Shown on the left are DNA standards. (**b)** *S. cerevisiae* cells lacking cardiolipin synthase (YDL142C-3b, *crd1Δ*) were transformed with either a control vector or plasmids for the inducible expression of *S. cerevisiae* cardiolipin synthase (scCLS) or MitoPLD, labeled with [^{3}H]-palmitate, induced by galactose and processed for lipid extraction. Extracted lipids were separated by thin layer chromatography and visualized by autoradiography. CL, cardiolipin; PG, phosphatidylglycerol.

SUPPLEMENTAL INFORMATION, ADDITIONAL METHODS

SPLIT VENUS COMPLEMENTATION

The MitoPLD open reading frame was subcloned in frame and N-terminal to N- or C-terminal fragments of Venus fluorescent protein in a CMV-driven expression vector [1]. The resulting constructs were then co-transfected into NIH3T3 cells. The cells were incubated at 37°C for 24 hr, stained with MitoTracker Deep Red 633 for 30 min, fixed and visualized using confocal microscopy. Neither expression plasmid generated detectable yellow fluorescence when expressed alone or with control non-interacting proteins.

MITOCHONDRIAL FRACTIONATIONS

Mitochondria were prepared by sucrose density gradient as described previously [2,3]. In brief, cells (5×10^7) were washed in TD buffer (135 mM NaCl, 5 mM KCl, 25 mM Tris-Cl, pH 7.6), swollen for 10 min in ice-cold hypotonic CaRSB buffer (10 mM NaCl, 1.5 mM CaCl2, 10 mM Tris-Cl, pH 7.5, protease inhibitors), and then disrupted in a glass Dounce homogenizer with 7-12 strokes. Subsequently, MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 20 mM HEPES, pH 8.0) was added to stabilize mitochondria and nuclear contaminants removed by centrifugation (2 × 2,000 ***g*** for 5 min). The resulting supernatant was layered on top of a 1.0 M sucrose over 1.5 M sucrose step gradient (in 20 mM HEPES, pH 8.0, 2 mM EDTA, 2 mM dithiothreitol, protease inhibitors) and spun at 4 °C for 35 min at 85,000 ***g***. Mitochondria were collected at the 1-1.5 M interphase by lateral suction through the tube, washed (in 5 volumes of MS buffer), spun (for 20 min at 16,000 ***g***), resuspended in MS buffer, and used for all assays.

PROTEOLYTIC STRIPPING OF MITOCHONDRIA

Freshly isolated mitochondria (2 mg/ml in MS buffer) were incubated with Proteinase K (10 µg/ml, Roche) in the absence or presence of Triton X-100 (0.5%, Sigma) at 4 °C for 30 min, or with trypsin (10 µg/ml) at 37°C for 30 min. Digestions were stopped by the addition of PMSF (2 mM, Sigma) and SDS loading buffer and boiling at 95°C for 5 min.

MITOCHONDRIAL FUSION ASSAY

Mitochondrial fusion assays were carried out as described previously [4]. In brief, individual pools of cells respectively expressing mitochondrially-targeted EGFP and DsRed2 (Clontech) were mixed and plated on poly-L-lysine pretreated coverslips 12 h before cell fusion. Cycloheximide (20 µg/ml) was added 30 min before fusion and kept thereafter. The cells were incubated with 50% PEG (polyethylene glycol) 1500 (Roche) for 60 sec, washed four times with complete media, grown for 7 hrs and then fixed.

GENERATION OF HELA CELLS STABLY EXPRESSING shRNA.

A 19-nucleotide (nt) sequence, nt 2351-2369 (GCCAGCTTGGAAGTTAACT) matching human MitoPLD mRNA (GenBank accession number NM_178836) was chosen to perform MitoPLD RNAi targeting. Small hairpin (sh) RNAs were synthesized as complementary DNA oligonucleotides, annealed, and cloned into the *Bgl*II/*Acc65*I sites of a modified pSUPER vector [5] containing a blasticidin resistance gene. The resulting plasmids were transfected into HeLa cells using LipofectAMINE Plus and stably-transfected cells selected by blasticidin (10 µg/ml). An siRNA sequence corresponding to nt 1235–1253 (GATTTCGAGTCGTCTTAAT) of the *Photinus pyralis* (firefly) luciferase mRNA (GenBank accession number M15077) was used as a negative control.

PHOSPHOLIPID ANALYSIS

NIH3T3 cells were labeled with 2 µCi/ml [^{3}H] palmitic acid (American Radiolabeled Chemicals) for 24 h. The mitochondria were then isolated as described in the "Mitochondrial Fractionations" section. Mitochondrial pellets were homogenized in $CH_3OH/CHCl_3$/0.1 N HCl (10:5:4, v/v) and the lipids recovered by acid organic extraction (half-volumes each of $CHCl_3$, 0.1 N HCl, and 0.5 M NaCl) [6]. An organic fraction was dried down, dissolved in chloroform, and separated on a 10 x 10-cm silica gel 60 HPTLC plate (EMD Chemicals) either one-dimensionally using chloroform/methanol/water/ammonium hydroxide (120:75:6:2, v/v) as the solvent [7] or

two-dimensionally using chloroform/methanol/water (32.5:12.5:2, v/v) for the first dimension and chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, v/v) in the second direction [8]. The plate was sprayed with EN^{3}HANCE (PerkinElmer Life and Analytical Sciences) and exposed to x-ray film (BioMax MR, Kodak) for 2 days at -80 °C.

IN VITRO *DNASE ASSAY*

The DNA fragmentation was assayed by incubating HeLa cell nuclei with lysates of mitochondria overexpressing MitoPLD at 37°C for 2 hr using a method described previously [9].

IMMUNOFLUORESCENCE STAINING

Unless stated otherwise, cells were fixed with 3% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100, and blocked with 5% normal goat serum. The cells were then immunostained using primary antibodies against the specific proteins, followed by fluorescent dye-conjugated secondary antibodies. Stained cells were visualized using a Leica TCS SP2 confocal microscope. Images were processed using Adobe Photoshop 7.0, and quantitative analyses were performed using the ImageJ analysis software package (National Institutes of Health). All experiments were performed at least three times with similar results.

ESTIMATION OF MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi_m$).

NIH3T3 cells were transfected with MitoPLD-EGFP. Twenty-four hours post-transfection, cells were incubated with 100 nM TMRE (Molecular Probes) at 37 °C for 20 min, washed, and visualized by a Leica TCS SP2 confocal microscope equipped with a bipolar temperature controller (Harvard Apparatus).

ELECTRON MICROSCOPY

NIH3T3 cells were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde, stained sequentially in 2% OsO_4 and 1% uranyl acetate, dehydrated by a series of ethanol washes and embedded in Spurr resin for sectioning and analysis. Samples were analyzed with the use of a JEOL 1200EX transmission electron microscope at the Stony Brook University Microscopy Imaging Center.

Supplemental References.

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